Jameel M. Al-Khayri · Shri Mohan Jain
Dennis V. Johnson *Editors*

Advances in Plant Breeding Strategies: Industrial and Food Crops

Volume 6

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Preface

Contemporary plant breeders no longer need to rely solely on traditional methodologies in their work of assuring a sustainable and elastic level of world food production. However, human population is increasing at an alarming rate in developing countries, and food availability could gradually become a serious problem. Agriculture production is severely affected because of environmental pollution, rapid industrialization, water scarcity and quality, erosion of fertile topsoil, limited remaining arable land to expand production area, lack of improvement of local plant types, erosion of genetic diversity, and dependence on only few crop species for food supply worldwide. According to the FAO, 70% more food must be produced over the next four decades to feed a projected population of 9 billion people by the year 2050. Currently, only 30 plant species are used to meet 95% of the world's food requirements, which are considered as the *major crops.* The breeding programs of these crops have been very much dependent on the ready availability of genetic variation, either spontaneous or induced. Plant breeders and geneticists are under constant pressure to sustain and increase food production by using innovative breeding strategies and introducing minor crops that are well adapted to marginal lands and can provide source of nutrition through tolerance of abiotic and biotic stresses. In traditional breeding, introgression of one or a few genes into a cultivar is carried out via backcrossing over several plant life cycles.

With the development of new molecular tools, molecular marker-assisted backcrossing has facilitated rapid introgression of a transgene into a plant and reduced linkage drag. Continued development and adaptation of plant biotechnology, molecular markers, and genomics have established ingenious new tools for the creation, analysis, and manipulation of genetic variation for the development of improved cultivars. For example, molecular breeding has great potential to become the standard practice in the improvement of several fruit crops. Adopting a multidisciplinary approach comprised of traditional plant breeding, mutation breeding, plant biotechnology, and molecular biology would be strategically ideal for developing new improved crop varieties. This book highlights the recent progress in the development of plant biotechnology, associated molecular tools, and their usage in plant breeding.

The basic concept of this book is to examine the best use of both innovative and traditional methods of plant breeding to develop new crop varieties suited to different environmental conditions to achieve sustainable food production and enhanced food security in a changing global climate, in addition to the development of crops for enhanced production of pharmaceuticals and innovative industrial uses. Three volumes of this book series were published in 2015, 2016, and 2018, respectively: Volume 1, *Breeding, Biotechnology and Molecular Tools*; Volume 2, *Agronomic, Abiotic and Biotic Stress Traits*; and Volume 3, *Fruits*. In 2019, the following four volumes are concurrently being published: Volume 4, *Nut and Beverage Crops*; Volume 5, *Cereals*; Volume 6, *Industrial and Food Crops*; and Volume 7, *Legumes*.

This Volume 6, subtitled *Industrial and Food Crops*, focuses on advances in breeding strategies using both traditional and modern approaches for the improvement of individual crops. Included in Part I are 11 industrial plant species utilized as sources of raw materials for the production of industrial products including pulp and wood crops (acacia), fiber (cotton, jute, and ramie), rubber (guayule and rubber tree), and oil (jojoba and flax). In addition to agave which has industrial uses as a source of raw materials for hard fiber, biofuels, and pharmaceutical products as well as uses in food industries supplying raw materials for alcoholic beverages such as tequila, the main table sugar source (sugarcane) is included. Part II of this volume covers seven food plants selected for their utilization in food industries for the production of chocolate (cacao), cooking oil (oil palm, safflower, sesame, and sunflower), and natural flavors (saffron and vanilla).

Chapters are written by internationally reputable scientists and subjected to a review process to assure quality presentation and scientific accuracy. Each chapter begins with an introduction covering related backgrounds and provides in-depth discussion of the subject supported with high-quality color photos, illustrations, and relevant data. This volume contains a total of 138 figures and 78 tables to illustrate presented concepts. The chapter concludes with an overview of the current status of breeding and recommendations for future research directions as well as appendixes listing research institutes and genetic resources relevant to the topic crop. A comprehensive list of pertinent references is provided to facilitate further reading.

The book is an excellent reference source for plant breeders and geneticists engaged in breeding programs involving biotechnology and molecular tools together with traditional breeding. It is suitable for both advanced undergraduate and postgraduate students specializing in agriculture, biotechnology, and molecular breeding as well as for seed companies and policy-makers.

We are greatly appreciative of all the chapter authors for their contributions towards the success and quality of this book. We are proud of this diverse collaborative undertaking, especially since this volume represents the efforts of 60 scientists from 14 countries. We are also grateful to Springer for giving us an opportunity to compile this book.

Al-Hassa, Saudi Arabia — Jameel M. Al-Khayri
Helsinki, Finland — Shri Mohan Jain
Cincinnati, OH, USA — Dennis V. Johnson

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About the Editors

Prof. Jameel M. Al-Khayri is a Professor of Plant Biotechnology affiliated with the Department of Agricultural Biotechnology, King Faisal University, Saudi Arabia. He received his B.S. in Biology in 1984 from the University of Toledo and his M.S. in Agronomy in 1988 and Ph.D. in Plant Science in 1991 both from the University of Arkansas. He is a Member of the International Society for Horticultural Science and Society for In Vitro Biology as well as the National Correspondent of the International Association of Plant Tissue Culture and Biotechnology. His graduate work resulted in the establishment of in vitro regeneration protocols for spinach and zoysiagrass. For the last two decades, he dedicated his research efforts to date palm. He has authored over 60 research articles in refereed international journals and 25 review chapters and edited 7 journal special issues. In addition, he edited five reference books on date palm biotechnology and utilization of genetic resources and 7 volumes of the book series Advances in Plant Breeding Strategies. He has been involved in organizing international scientific conferences and contributed numerous research presentations. In addition to teaching, student advising, and research, he held administrative responsibilities as the Assistant Director of Date Palm Research Center, Head of the Department of Plant Biotechnology, and Vice Dean for the Development and Quality Assurance. He served as a Member of Majlis-ash-Shura (Saudi

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Prof. Shri Mohan Jain is a Consultant and Plant Biotechnologist, Department of Agricultural Sciences, University of Helsinki, Helsinki, Finland; he received his M.Phil. in 1973 and Ph.D. in 1978 at Jawaharlal Nehru University, New Delhi, India. He was a Postdoctoral Fellow in Israel and the USA; a Visiting Scientist/Professor in Japan, Malaysia, Germany, and Italy; and a Technical Officer, Plant Breeding and Genetics, International Atomic Energy Agency (IAEA), Vienna, Austria, 1999–2005. He is a Member of the International Association for Plant Tissue Culture and Biotechnology and Editorial Board Member of *Euphytica*, *In Vivo*, *Propagation of Ornamental Plants*, *Emirates J. Food and Agriculture*, and the series Forest Health and Biotechnology. His publications are more than 160 in peer-reviewed journals, book chapters, and conference proceedings. He edited 55 books and acted as a Chair Person in several international conferences worldwide and has been an Invited Speaker. He was awarded Nobel Peace Prize in 2005 in commemoration of the awarding to the IAEA of the Nobel Peace Prize for 2005. In addition, he was Former Consultant to the IAEA, the European Union, the Government of Grenada, Iranian Private Company, and the Egyptian Government. Currently, his research interests are on somatic embryogenesis, organogenesis, haploidy, somatic cell hybridization, somaclonal variation, and mutagenesis mainly in medicinal plants, date palm, and banana genetic improvement, genetic diversity, erosion, conservation, and utilization in the context of climate change and food and nutritional security.

Prof. Dennis V. Johnson is a Consultant and Former University Professor. He is a graduate of the University of California, Los Angeles, where he completed his B.A. (1966), M.A. (1970), and Ph.D. (1972) degrees in Geography, with specialization in agriculture and biogeography. He has taught at several colleges and universities, including the University of Houston, and was a visiting professor for 2 years at the University of Ceará, Fortaleza, Brazil. He also has worked extensively with international development agencies providing technical assistance to agriculture and forestry on projects and programs in Africa, Asia, Europe, and Latin America. He has published numerous articles on palm utilization and conservation and has edited or written books for the FAO, IUCN, and UNEP. He has also translated into English plant science books from Portuguese and Spanish. A decade ago, he began to focus his research on date palm, in particular its introduction to nontraditional areas, such as Spain, North and South America, and Australia. Moreover, he coauthored a book on date growing in the USA, has made presentations at five international date palm conferences, and coedited books on date palm, sago palm, and plant breeding.

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Part I
Industrial Crops

Chapter 1
Genetics and Breeding of Tropical Acacias for Forest Products: *Acacia mangium*, *A. auriculiformis* and *A. crassicarpa*

Arif Nirsatmanto and Sri Sunarti

Abstract Industrial forest plantations are playing an important role in the forestry sector for both economic and environmental reasons. Some fast- growing species of tropical acacias, such as *Acacia mangium*, *A. auriculiformis*, *A. crassicarpa* and *Acacia* hybrids are among the most dominant trees in forest industrial plantations in Southeast Asia countries: Indonesia, Malaysia, Vietnam, Thailand and Papua New Guinea. These trees are mainly planted to supply raw materials for pulp and paper, sawn lumber, the plywood industry and wood energy. Improvement through optimizing of silvicultural practices, exploring genetics and breeding of acacias has been gradually taking place. Although progress is still slow, as compared to agricultural crops, due to the age at rotation, larger tree size and extensive site management, some achievements in genetics and breeding of acacias have provided a significant benefit and impact in the operational scale of forest industries. Genetic diversity of acacias is low and most breeding is practiced conventionally under a recurrent selection strategy practiced successively from the first generation to advanced generation breeding cycles. Recently, breeding of acacias is also being developed through hybridization, application of biotechnology and molecular breeding.

Keywords Conventional breeding · Tree improvement · Molecular breeding · hybridization · Biotechnology · Forest industry

1.1 Introduction

The genus *Acacia* consists of trees and shrubs and is in the family Leguminosae, subfamily Mimosoideae. The genus includes more than 1000 species which are naturally distributed in Africa, the Americas, Asia and Australia (Anonymous 1982). Mangium (*Acacia mangium)* auriculiformis (*A. auriculiformis)* and crassicarpa

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(*A. crassicarpa*) are among the most dominant tropical acacias cultivated for the forest industries in Southeast Asia countries. These three acacias have a natural distribution in Australia, Papua New Guinea and Papua Province, Indonesia (Gunn and Midgley 1991; Thomson 1994), usually occurring in overlapping populations. Natural interspecific hybridization is potentially taking place among the acacias, especially between *A. mangium* × *A. auriculiformis* (Griffin et al. 2015a, b; Kha 2001; Sunarti et al. 2013).

Mangium is a major species for industrial forest plantation growth in the Southeast Asian countries of Indonesia, Malaysia, Vietnam, Thailand and Papua New Guinea (Harwood and Nambiar 2013; Turnbull et al. 1997). It was introduced as an exotic fast-growing tree for forest plantations beginning in the 1960s in Sabah, followed by Malaysia, Indonesia, Vietnam and Thailand (Pinyopusarerk et al. 1993; Turnbull et al. 1997). The main purpose of wood production is to supply raw material for pulp and paper, plywood, particleboard and sawn lumber. This species is also widely planted for other forestry purposes, such as reforestation and soil rehabilitation, and categorized as a multipurpose species. Recently it was reported that the total area of mangium plantations is around 1,350,000 ha, mostly in Indonesia, Malaysia and Vietnam, with the main purpose to supply raw material for the pulp and paper industry (Harwood and Nambiar 2014). The wood can also be combined into pulp mixtures including *Eucalyptus globulus* to obtain enhanced pulp properties (Wu et al. 2018).

Auriculiformis was introduced earlier than mangium, in the 1930s, particularly for land rehabilitation (Turnbull et al. 1997). Later it was cultivated for industrial purposes, mostly to supply raw material for sawn lumber. Due to higher levels of lignin, wood density and extractive contents in the wood of this species, there is a reduction in pulp yield (Jahan et al. 2008), making it less desirable for the pulp and paper industry. However, it has potential for hybridization with *A. mangium* to produce the hybrid, *A. mangium* × *A. auriculiformis*, which is suitable for pulp and paper needs (Kha 2001; Sunarti et al. 2013). Most auriculiformis plantations are managed by small growers using fewer silvicultural practices as compared to mangium (Hai et al. 2008; Harwood and Nambiar 2014; Huong et al. 2015). Like mangium, auriculiformis is also categorized as a multipurpose species and planted for reforestation and soil rehabilitation.

Although auriculiformis has had a long history since its introduction, the precise area of plantations is unclear because this species is mostly planted by small local growers with uncertain silvicultural practices and harvesting rotation. In Indonesia, auriculiformis is planted along with other acacia species or mixed with other tree species and crops; the time of harvest depends on the farmers' needs without consideration of the fixed age of the trees. Natural regeneration is practiced following the selective successive harvesting rotation. Unlike in Indonesia, auriculiformis plantations in Vietnam are better organized and managed in terms of silvicultural practices and harvesting (Hai et al. 2008; Harwood and Nambiar 2014; Huong et al. 2015).

Crassicarpa was first introduced as an alternative species in the pulp and paper industry in the 1990s (Turnbull et al. 1997). In drylands, the growth of crassicarpa is suboptimal as compared to mangium. However, following the enlargement of plantations into wetlands, crassicarpa was found to be one of the acacias adaptable to peat soils. It was reported that the area of plantations of this species is around 700,000 ha, mostly in Indonesia (Harwood and Nambiar 2013; Turnbull et al. 1997).

Most acacia plantation areas were established beyond the natural distribution of the tree species, and thus there were introduced as exotics. Seeds for plantations were first collected from natural populations, mainly in eastern Indonesia, Papua New Guinea and northern Australia (Gunn and Midgley 1991). At the time of initial plantation establishment, stand productivity was low due to improper silvicultural practices and unselected planting stock. Following the particular importance of high-productivity acacia plantations, efforts were made to adopt sound silvicultural practices; breeding was then carried out and has resulted in efficient and effective plantation management (Griffin et al. 2015a, b; Harwood et al. 2015). Mangium is the most advanced in terms of silvicultural practices among the three *Acacia* species, followed by crassicarpa and auriculiformis. Intensive and comprehensive research studies on nursery growth and silvicultural practices, such as cultivation, plantation management, fertilizing, weeding, pest and disease control, harvesting, wood properties and manufacturing processes in acacias have been conducted for 20–40 years with varied progress among the three *Acacia* species.

Breeding is one of the important factors in improving acacia plantations. After initial introduction and recognition that the acacias have potential for industry, early improvement through breeding was also pursued. Considering the need to supply large quantities of planting stock, genetic materials were sought and collected from a wide range of the natural distribution areas for establishing initial genetic trials, such as seed stands, provenance stands and first-generation seed orchards, in many sites representative of plantation regions. Although some advanced generation breeding programs were recently achieved, application of biotechnology and molecular breeding remains challenging. The role of certain research institutions and research projects in developing genetic and breeding is recognized for the four tropical acacias; such as the Center for Forest Biotechnology and Tree Improvement (CFBTI) in Indonesia, the Forest Research Institute Malaysia (FRIM), the Institute of Forest Tree Improvement and Biotechnology (FTIBRI) in Vietnam and the ACIAR Project on pest and disease of tropical acacias in Indonesia and Vietnam (ACIAR-FST/2014/068) (see Appendix I).

In this chapter, information on the genetics, achievements and challenges of breeding *Acacia mangium*, *A. auriculiformis* and *A. crassicarpa*) and the hybrid (*A. mangium* × *A. auriculiformis*) is presented. The chapter provides a history of the introduced species, cultivation status and breeding, biodiversity, future challenges in the involvement of biotechnology and molecular breeding.

1.2 Silviculture and Conventional Breeding

1.2.1 Current Cultivation Practices

The four acacias mentioned above are highly adaptable to a wide range of tropical regions, from sites with adverse conditions of dry and degraded soil, soil with a low pH and low rainfall, up to sites with good fertile soil and high rainfall (Mackey 1996; Pinyopusarerk et al. 1993; Turnbull et al. 1997). Although the main objective is to develop forest industries, most acacia plantations were initially established in degraded soils as part of reforestation and soil rehabilitation programs. Concerning the importance of high productivity of plantations, considerable efforts were undertaken to improve cultivation practices by assessing the suitability of site conditions. Due to the variability of site conditions, cultivation techniques in practice differ from site to site according to company preferences.

The main objectives typical of industrial acacia plantation in drylands are that they are planted mostly on degraded lands such as grasslands (*Imperata cylindrica*) and to some degree in logged-over areas (secondary forest). In the first rotation, fully mechanized site preparation is practiced in grassland areas by ploughing, followed by harrowing (Otsamo et al. 1996; Siregar et al. 1999). In the case of scrubland and logged-over areas, site preparation is through slashing, felling and chopping. Debris is then piled at the sides of the planting block. For subsequent rotations, mechanical site preparation is not done. After timber harvest, weeds and naturally-regenerated acacia seedling are slashed, followed by herbicide application (Hardiyanto and Nambiar 2014). Unlike in drylands, plantation areas for acacias in wetlands mostly take place in peat land where the only adaptable species is crassicarpa. Peat land drainage is implemented by controlling water levels at around 50–80 cm below the peat surface through a canal system (Tropenbos, APRIL and Ministry of Forestry 2010).

Planting stock for acacia plantations is mostly provided by raising seedlings from seed. In mangium, large-scale multiplication using stem cuttings from young seedlings is also practiced by some companies (Wong and Yuliarto 2014). Genetically-improved seed for seedling preparation is mostly collected from first- and second-generation breeding cycles (Harwood et al. 2015; Kurinobu and Rimbawanto 2002; Nirsatmanto et al. 2014, 2015; Werren 1991). In the *A. mangium* × *A. auriculiformis* hybrid, planting stock is prepared only through the large-scale multiplication of shoot and stem cuttings from selected clones. Ageing effects commonly occur in the multiplication of shoot and cutting for acacias in which the rooting ability of a cutting decreases as hedge plants or stool plants get older (Ahmad 1991; Monteuuis et al. 1995). Therefore, maintenance of juvenility is essential to the success of cuttings in acacias.

Stand density is one of the important factors in the silvicultural practices of acacia plantations. It is determined at the beginning of planting by the spacing arrangement. Considering crown development, growth and stand productivity, the initial plant spacing for acacias is 3 × 3 m, or a stand density of 1100 trees ha^{-1}, mainly for

the pulp and paper industry (Hardiyanto 2004). Further silvicultural treatments such as thinning and singling in dryland plantation areas are necessary to produce fine, straight logs if the wood is destined for sawn lumber (Beadle et al. 2007), plywood and particleboard.

Some acacias, such as mangium, are nitrogen-fixers (Galiana et al. 1990). This character will affect the fertilizer application in plantations, especially the levels of N. The first application of fertilizer when planting acacias is at planting time, commonly using TSP (triple superphosphate) or SP-36 (Hardiyanto 2004). Fertilizer is put into the planting hole and then the surface is covered by soil. This is done before planting the seedling to avoid direct contact between the seedling roots and the fertilizer. One month after planting, additional fertilizer is applied using NPK which is allocated in the dip around 10 cm from the planted seedling. In some companies, the additional fertilizer is also applied 2–3 times within the first 2 years. In the subsequent rotation, the levels of N application will be much reduced due to fixed nitrogen added to the soil from trees in previous rotations. Weeding should be practiced periodically to improve early seedling growth by reducing intense weed competition. Weeding can be manual or by the application of herbicides, and optimally done before fertilizer application.

In the past, the growth of the three *Acacia* species was commonly crooked with multi-stemmed leaders (occurrence of more than one main stem in one tree). However some recent breeding processes have succeeded in improving the stem form to be straighter and with a single stem (Harwood et al. 2015). For pulp and paper, multi-stemmed trees are not a matter of serious concern and thinning is not necessary.

The harvesting rotation for acacia depends on the purposes of the end-product. With fast growing tree species for biomass production, such as pulp paper and wood energy, the length of rotation is shorter, around 2–6 years. In the case of wood-based products, such as furniture, particle board and other sawn lumber products, the rotation is longer at more than 10 years. In some regions, especially when planted by the local people, a combination of the purposes of sawn lumber and wood energy is practiced, in which the main stem is used to furnish logs for sawn lumber while the trimmings are used for wood energy. Wood processing technology is important for producing good lumber from acacias, especially with respect to wood drying. This is because the water content of the wood is high and the lumber will easily split and crack if improperly dried, especially for younger harvested timber (Tenorio et al. 2012; Yamamoto et al. 2003).

1.2.2 Current Problems and Challenges

There are at least two main problems in plantation development of the four acacias. The first is the low stand productivity due to substandard silvicultural practices, particularly under adverse condition either in drylands or wetlands. The second is the increase of plantation tree damage due to pests and diseases.

Under current silvicultural practices, the productivity of acacia plantations has been increased in some regions. In addition, breeding practices have also succeeded in improving plantation productivity (Hardiyanto 2014; Harwood and Nambiar 2014; Nirsatmanto et al. 2015). However, there is still a large gap between supply and the total demand. In this case, expansion of plantations is necessary. Very large variations of planting-site conditions means the deployment of high-productivity acacia plantations is limited, although the key species have been reported to be highly adaptive to varied soil types. Growth will be more pronounced in a site with favorable soil conditions and climate, such as well-drained soil properties and higher annual rainfall (>1800 mm/year) (Mackey 1996). For peat areas, given the current issues regarding carbon emissions and peat fires, water levels in peatland drainage should be reassessed for sustainable peatland management. For instance, Indonesian Government Regulation number 71/2014 on the Protection and Management of Peat Ecosystems, declares that the water levels can be as high as 40 cm below the peat surface. However, that water level will suppress the growth of crassicarpa, the main acacia commonly planted in peat areas. This situation has become one of the most serious problems for crassicarpa plantations. It is necessary to have the appropriate silvicultural technique to encourage high-productivity acacia plantations in adverse environments. Plant exploration to identify adaptive genotypes is also an important challenge in acacia breeding.

In the past, there were no serious and significant impacts of pest and disease on acacias. However, recently acacia plantations are facing very serious threats both from pests and diseases resulting in very significant damage to plantations, mostly in mangium (Brawner et al. 2015; Harwood and Nambiar 2014; Harwood et al. 2015; Tarigan et al. 2011). Squirrels and monkeys are pests that seriously threaten mangium plantations. A large proportion of the plantations in Sumatra Island, Indonesia are attacked by these pests (Hardiyanto 2014). Diseases are another very serious threat, mainly in mangium. Root rot associated with *Ganoderma* sp. and wilt disease with *Ceratocystis* sp., cause high mortality in mangium plantations. For example, Suryantini and Wulandari (2018) studied the virulence level of three *Ganoderma* isolates to infect mangium seedlings, which revealed that *G. lucidum* isolated from mangium is of highest virulence, compared to *G. boninense* isolated from the oil palm and *G. applanatum* isolated from the rubber tree. Pathogen infections are commonly associated with tree wounds due to silvicultural practices, such as branch pruning and singling of multi-stemmed trees, as well as wounds from pests and wind damage. Exploring for resistant genotypes to such diseases is another important challenge in acacia breeding.

Acacia plantations for pulp and paper industries are usually monocultural with limited genetic diversity that is probably more susceptible to the consequences of climate change (Pawson et al. 2013). Climate change may impact directly on forest diversity through changes in temperature, rainfall, forest fires, and pest and disease attacks. Indirectly, climate change will strongly affect plantation forests requiring changes in forest management practices to mitigate the influences of climate change on productivity. Furthermore, Pawson et al. (2013) suggested that the management of forest plantations in future should take into account the plant rotation, weed

control and silvicultural practices and also established multi-species plantations for climate change mitigation and adaptation. Mixed plantations of clones, which are structured on a mosaic with variation in age classes, will be more productive, healthier and increase the biodiversity (Lindgren 1993), while increasing resilience to climate change (Pawson et al. 2013). Booth et al. (2014) examined the challenge on how climate change may affect locations in Southeast Asia suitable for growing mangium, auriculiformis and crassicarpa by considering a short rotation, with the relatively frequent opportunity to change planting stock and provenance resources. Covering a large area, an acacia plantations plays an important role in biological carbon mitigation through increased concentration of CO_2 by sequestering considerable amounts of carbon in biomass and soil (Kalita et al. 2016).

1.2.3 Improvement Strategy

Some improvement strategies are underway to overcome current problems of acacia plantations. Modified silvicultural practices related to successive second and third rotation plantations are being applied, particularly to improve conditions through reducing soil compaction from mechanical harvesting, appropriate fertilizer application and land preparation for the next planting. To increase the quantity of wood production, plantations are being enlarged through the expansion of plantation areas to include minimally-suited areas of soil fertility, soil drainage and rainfall.

The effort to manipulate genetics through breeding is another strategy to overcome problems. Although most breeding is still taking place conventionally through selection in successive advanced generations and hybridization, some involvement of biotechnology such as polyploid breeding and genomic selection has also been initiated (Baurens et al. 2004; Griffin et al. 2015a, b; Wang and Cui 2000). All breeding strategies will be focused on improving genotypes which show higher productivity both in growth and wood properties, resistance to pests and diseases and tolerance to adverse conditions.

1.2.4 Traditional Breeding Methodologies and Limitations

Breeding strategies using a recurrent selection system is regularly practiced in acacia and includes selection, mating and propagation, and repeated in successive generations of breeding (Namkoong et al. 1988). In each generation, the process is implemented by establishing a seedling seed orchard for three simultaneous objectives: (1) progeny testing, (2) seed production and (3) a genetic base for successive generations. The breeding strategy employs open-pollinated progeny from plus trees. This strategy is adopted due to the early flowering habit at 2–3 years of age, and the difficulty in vegetative propagation, as well as the limitation of available breeding techniques using controlled pollination (Wright 1976).

Due to the difficulty of full pedigree control, breeding populations of the four acacias are managed under multiple populations. In this strategy, a large quantity of genetic material for breeding is divided into several subpopulations of sublines, instead of one single large population (Barnes 1984; McKeand and Beineke 1980; van Buijitenen and Lowe 1979). Sublines are then created by establishing several small breeding groups, and mating among the parent trees with some degree of inbreeding is only allowed within the respective subline (McKeand and Beineke 1980). On the other hand, maintaining outbred offsprings will be directed in a seed production orchards consisting of a combination of a few selected superior seed parents from a respective subline. To avoid relatedness among sublines and to adjust the timing of flowering, a sublining system of tested families by provenance is recommended (Nikles 1989).

Selection for genetic gain in breeding population of acacias can be based on either phenotypic or genotypic values (Falconer 1981). Regarding the seedling seed orchards with multiple-tree plots, the genetic gain will be realized within-plot as well as by family selection (Wright 1976). In spite of the low potential of gain as compared with that by family selection, roguing the orchard by within-plot selection is a first step to improve genetic quality of the acacias.

A subsequent selection procedure conducted after the within-plot selection in seedling seed orchards of acacias is family selection. The primary purpose of this type of selection is to improve the genetic quality of seed produced in the orchards by culling trees in the poor families. Unlike the within-plot selections that were done largely dependent on breeder intuition, the family selection can be regarded as more controllable, because it is theoretically oriented and straightforward. However, in practice, due to the low genetic diversity in acacias, something short of modification in family selection is desirable to reduce some risk associated with selection intensity, relationship between selection intensity and genetic variability, and different outcrossing rates. After finishing the family selection, seedling seed orchards are used as orchards for operational seed production.

Beyond the strategy toward advance generation breeding populations using the sublining system in acacia breeding, selection of plus trees is conducted in each subline. Selected trees are considered as candidate before designation as plus trees, and they should be examined not only based on growth and form trait, but also on other essential traits such as flowering habit, wood property as well as pedigree. Candidate plus trees should be confirmed on their family origin to reduce the progress of inbreeding in the next generation, where a maximum of two plus trees per family are allowed to be tested in the subsequent generation. To maintain wide genetic diversity in the orchards and to increase genetic gain, new selected plus trees from other populations within the same provenance region of the acacias can be added into subsequent generation of the breeding population (Borralho and Dutkowski 1996). From two successive generational breeding cycles, improved

seed of mangium could result in a genetic gain of stand volume of 30–50% over the unimproved seed, and the second generation breeding showed 13% higher than the first generation breeding (Nirsatmanto et al. 2015).

Early selection is often practiced in seedling seed orchard establishments to increase the genetic gain per unit of time and thus the economic return on tree improvement activities (Lambeth 1980; McKeand 1988). To evaluate the efficiency of early selection in acacia seedling seed orchards, assessment of the genetic parameters is necessary. This is because genetic gain by this type of early selection is well known to be primarily dependent on the magnitude of heritability and the genetic correlation between selection age and rotation age (Cotteril and Dean 1988; McKeand 1988). Nevertheless, genetic parameters may change markedly as trees grow and develop (Balocchi et al. 1993; Namkoong and Conkle 1976). Likewise, for many breeding programs which are concerned with volume improvement, tree height was found to be good early predictor and selection trait for volume yield at rotation age of the acacias due to its typically higher heritability and stronger juvenile-mature correlation (Lambeth et al. 1983). Nirsatmanto et al. (2012) reported that early selection of mangium based on tree height brought more gain per year than later direct selection on 8-year rotation with optimal age for early selection found at 2 years of age.

Besides shortening the breeding cycle, large additional gain in productivity in acacias also can be achieved through selection of the best provenance for a given site and purpose. Therefore, acacia seedling seed orchards should be made up using families of plus trees selected from good provenances which proved to be promising based on previous provenance trials. This is because the acacias are introduced exotically into plantation areas far from their natural distribution. Hence, available provenance information of the acacias will become an important factor prior in seedling seed orchard establishment. A study of projected increase in stand volume of introduced provenances of mangium showed that those from Papua New Guinea and Queensland increased the genetic gain by 17–26% over the local land race (Nirsatmanto et al. 2014).

Although it has come a long way and made significant achievements in traditional breeding, work remains in acacia breeding due to their limitations. As described in preceding paragraphs, traditional breeding in acacias faces difficulty in controlled crosses among the parents and the ageing effect in propagation. In addition, breeding was explored mostly through the use of potential genetic variation which was reported at a low level. This low variation is the most current limitation in improving acacias through traditional breeding. This situation is aggravated by the decrease of acacia populations in natural areas due to illegal logging or other biological and physical threats. In this case, an infusion of genetic material from new collections into the advanced generation breeding population could not be successively practiced to maintain a large genetic variation. As a result, the potential for increasing productivity through the breeding will stop.

1.2.5 Role of Biotechnology

In tree breeding programs, biotechnology advances include new methods in vegetative propagation through in vitro, molecular biology through DNA markers and genomic DNA, polyploidy breeding and genetic transformation (Barrens et al. 2004; Burdon 1994; Griffin et al. 2015a, b). The role of biotechnology is expected to overcome some limitations and constraints in the breeding of forest tree species, such as shorter time for selection, maintain the juvenility of stock plants for vegetative propagation, and give more accurate means for modifying and combining of target genes (Burdon 1994).

Depending on the end-product uses, economic rotation of acacias can be harvested at around 6–10 years of age. Final selection in the breeding process is commonly practiced at the midpoint of rotation at around 4–5 years of age. Considering the genetic information from molecular studies such as quantitative trait loci (QTL), the selection procedure could be potentially started at early stages in the nursery (Burdon 1994). However, the time lag to proceed into the subsequent generation cycles based on the reproductive ability in the acacias is around 2–3 years (Nirsatmanto et al. 2012). Therefore, complete control of flowering using biotechnology, such as through induction of precocious flowering, is necessary to shorten the time lag. In another case, technology using species-specific SNP markers will also be useful to confirm acacia hybrid trees and for screening mass propagated ramets of selected acacia hybrid clones (Asif et al. 2017).

Other biological limitations of acacia breeding include the difficulty in making controlled cross-pollination due to the typical flowers, which are hermaphroditic with many tiny flowers in an inflorescence (Ibrahim 1993). An emasculation process is needed to make controlled crosses (Griffin et al. 2010). But then, due to the tiny flowers with polyad pollen, emasculation is difficult, although that does not mean it cannot be done. Therefore, other alternative methods for gene recombination are necessary, and hence biotechnology has great potential to be applied in acacia breeding, such as through genetic transformation, polyploid breeding, and genome and molecular breeding. In addition, the developed biotechnology could be used to optimize the low genetic diversity in the breeding of acacias.

Asexual propagation is commonly practiced to rapidly deploy genetically improved stock obtained from breeding programs. However the ageing effect influences low rates of success of clonal propagation in acacias (Ahmad 1990; Monteuuis et al. 1995). This is because material from mature stock plants usually results in a low level of rooting capability. In this case, maintaining the juvenility of stock plants for the mass scale clonal propagation is important. Developed technology through in vitro culture and somatic embryogenesis (SE) can help greatly not only to maintain the stock plants in juvenile stages, but also to accelerate the increased number of multiplied planting stock from vegetative propagation (Andrade et al. 2011; Hazubska-Przybyl and Bojarczu 2016).

The technique of tissue culture propagation of the four acacias has been developed well, and successfully produced plantlets for mass plantations (Ahmad 1991;

Galiana et al. 2003; Monteuuis 2004; Yang et al. 2006). Propagation by tissue culture of mangium was successfully practiced using nodal explants which were excised from 1-month old aseptically germinated seedlings and cultured on full strength MS basal medium (Murashige and Skoog 1962) supplemented with 6-benzylamino purine (BAP) or kinetin (Ahmad 1991). The types of auxin hormones such as indole butyric acid (IBA), alpha-naphthalene acetic acid (NAA) and Serradix 3 were used for root formation. Multiplication from juvenile material of mangium showed better rooting ability than the mature genotypes (Monteuuis 2004).

In vitro multiplication and rooting formation on acacia hybrids requires different medium as practiced on mangium, additional suitable regulators were necessary in the culture medium. The average of multiplication and rooting ability of the *Acacia* hybrid was higher than mangium but varied greatly among the clones (Galiana et al. 2003). The survival rate during acclimatization in the nursery under a misting system was also higher.

The first successful crassicarpa propagation from an excised 2-month-old phyllode in vitro seedling was achieved using MS basal media supplemented with 1-phenyl-3 (thiadiazol-5-yl), thidiazuron and α-naphthalene acetic acid (NAA) (Yang et al. 2006). The rooting medium from elongated adventitious shoots was MS media supplemented with 3-indolebutyric acid (IBA). The shoots produced 96.5% of rooting plantlets which were successfully acclimatized in a nursery bed on soil media with an 80% survival rate.

1.3 Germplasm Biodiversity and Conservation

1.3.1 Germplasm Diversity

The natural distribution of the three acacias is in northern Australia, southeastern Papua Province, Indonesia and southwestern Papua New Guinea (Gunn and Midgley 1991; Thomson 1994). The populations are separated into two larger groups on New Guinea Island and Australia, with some overlapping sites among the three acacias. The distinct differences in geographic distribution among the two islands is indicative of the differences in germplasm diversity for the three acacias. Genetic diversity among the three species is typically similar, and generally categorized as low, with mangium having the least genetic diversity among the three (Moran et al. 1988, 1989; Widyatmoko et al. 2010).

Among the two distinct natural distributions of mangium, Queensland, Australia and New Guinea Island (Papua Province and PNG), the Papua population is reported as having higher genetic diversity as compared to Australia (Butcher et al. 1998). In Australia, a decline in diversity occurs from populations in the north (Cape York) to populations farther south in Townsville and Daintree.

The natural distribution of auriculiformis is geographically wider than mangium. It covers not only populations in New Guinea Island and the northern part of Queensland, but also Northern Territory, Australia (Turnbull et al. 1997). It is reported that the populations from Queensland are closely related to the populations of New Guinea Island, rather than to the Northern Territory (Wickneswari and Norwati 1993). In addition, it was reported that the genetic diversity of auriculiformis is higher as compared to mangium and crassicarpa (Widyatmoko et al. 2010). The natural distribution of crassicarpa covers a similar ranges to mangium, which is New Guinea Island and northern Queensland. It was also reported that the genetic diversity of crassicarpa is low and comparable mangium.

1.3.2 Species Characterization and Phylogeny

In general, the three acacias are somewhat similar in morphology. However, the differences among the species can be recognized visually, mostly through the leaf, stem and bark pattern, and reproductive morphologies. According to Laksmi and Gopakumar (2009), morphological keys for A. *mangium* include rhytidome bark rectangular in shape; length of internode, short (~1.5 cm; leaves most spaciously arranged); phyllode, broad (~5.4 cm) and looking more like an *entire leaf*, rough textured due to the presence of four nerves on the dorsal side of the phyllode, which can be prominently perceived by touch; flower, creamy- white color; pod, highly coiled; funicle, orange color. In *A. auriculiformis* the rhytidome bark is rectangular in shape; phyllode, short (~12.6 cm), sickle-shaped; pod, highly coiled; funicle, curved and enclosing more than half of the seed, reddish-orange color. For *A. crassicarpa*: the rhytidome bark is square in shape; length of internode long (~3.3 cm) which means that leaves are arranged more compactly on the branchlets; phyllode, thick with a grayish tint and sickle-shaped; flower, greenish-yellow color; pod, flat and uncoiled; prominent protrusion in the middle of the seed; funicle, creamy-white color. However, dissimilar site and climate may show slight differences in morphology for the three species, especially for the bark color and rythidome shapes. Some morphology keys of the four tropical acacias grown in Central Java, Indonesia are presented in Figs. 1.1 and 1.2. Seed morphology of the three tropical acacias is presented in Fig. 1.3.

Rather than in morphological and biochemical markers, the revolution in biotechnology is in the molecular basis for DNA sequencing, which has become an important tool for accelerating an accurate understanding of phylogenetic and genetic diversity in a species. Random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism AFLP and restriction fragment length polymorphism (RFLP) are among the most common technologies employed in phylogenetic studies. In crops and horticultural species, phylogenetic studies using molecular markers have become a common tool and have resulted in the completion of several thousand phylogenetic analyses.

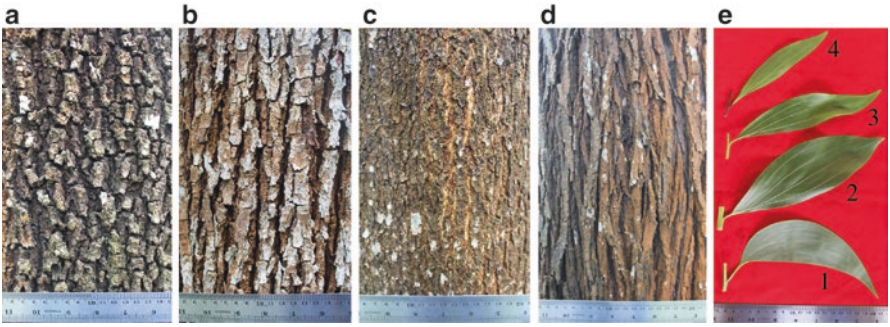

Fig. 1.1 Bark color and rhytidome shape from mature trees grown in Central Java, Indonesia for (**a**) *Acacia mangium*, (**b**) *A. auriculiformis* (**c**) *Acacia* hybrid *A. mangium* × *A. auriculiformis*, (**d**) *A. crassicarpa,* (**e**) the phyllode shapes from the respective species: (**1**) *A. crassicarpa*, (**2**) *A. mangium*, (**3**) *Acacia* hybrid, (**4**) *A. auriculiformis.* (Photographs by A. Nirsatmanto)

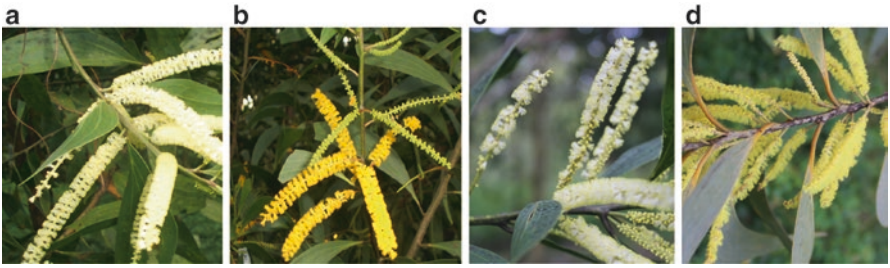

Fig. 1.2 Flower morphology of (**a**) *Acacia mangium*, (**b**) *A. auriculiformis*, (**c**) *Acacia* hybrid *A. mangium* × *A. auriculiformis*, (**d**) *A. crassicarpa.* (Photographs by A. Nirsatmanto)

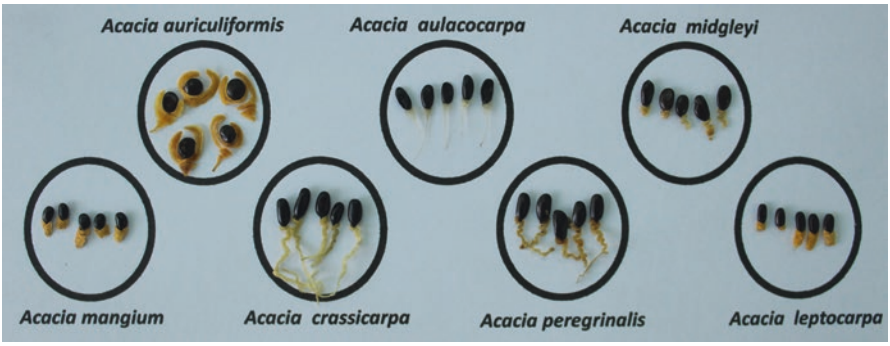

Fig. 1.3 Seed and funicle morphology of *Acacia mangium*, *A. auriculiformis* and *A. crassicarpa* compared with other Acacia species. (Photograph by A. Nirsatmanto)

Although still rare, phylogenetic studies of *Acacia* have been published. Brain and Maslin (1996) reported the classification of 48 species of the subgenus *Phyllodineae*, which is predominantly distributed in Australia. Harrier et al. (1997) studied the phylogenetic relationship of African acacias using RAPD analysis. Murphy et al. (2003) investigated the phylogeny of *Acacia* subgenus *Phyllodineae*, predominantly Australian species, to reassess the infrageneric classification. Alaklabi (2015) reported on the phylogenetics of *Acacia* species collected from various locations in Saudi Arabia using internal transcribed spacer sequence of nrDNA (nrDNA-ITS), and concluded that both pairs of *Acacia etbaica* with *A. johnwoodii* and *A. ehrenbergiana* with *A. tortilis* were close to each other. Widyatmoko et al. (2010) carried out a phylogenetic study of four tropical *Acacia* species based on the information derived from the RAPD analysis of nuclear DNA and SSCP (single strand conformation polymorphism) analysis of chloroplast DNA, and concluded that the four Acacias were classified into two groups, i.e. *A. aulacocarpa* and *A. crassicarpa* in one group, and *A. auriculiformis* and *A. mangium* in another.

1.3.3 Genetic Resources Conservation Approaches

Maintaining genetic resources of the acacias is very important for the sustainability improvement through breeding. This is because most of the breeding strategy in acacias has been through a recurrent selection system, in which the infusion of new genetic materials into successive advanced breeding populations is a prerequisite to maintaining long-term increases of genetic gain. Several approaches in genetic resource conservation of the acacias have taken place through in situ and ex situ conservation.

In Indonesia, for example, genetic resource conservation for mangium, auriculiformis and crassicarpa was commonly practiced in combination with seed sourcing. An in situ conservation strategy was implemented through the allocation of resources into identified seed stands and selected seed stands within their natural range. In this strategy, the structure of a stand is maintained in nature without human manipulation (Fig. 1.4). A selected seed stand is an area within or among the identified seed stand areas which generally has better stand growth and structure for seed production. In certain areas, overlapping populations among the three acacias are found within the same seed stand area. Although there is no exact minimum area, a seed stand is commonly allocated more than 10 ha. Higher genetic diversity could be maintained by this strategy.

Various models are practiced for ex situ conservation of the three acacias. These include establishment of seed sources in locations beyond the range of the natural distribution, seed banks and clone banks. The two latter are commonly practiced in combination with the seed sourcing and genetic testing. Provenance trials combining with provenance seed stands were established using bulk seed of several representative mother trees (>20 trees) within provenances collected from different natural populations. In this strategy, seed from some provenances, with a large

Fig. 1.4 Growth performances of *Acacia crassicarpa* in an in situ conservation area of Papua Province, Indonesia. (Photographs by A. Nirsatmanto)

single plot for each provenance (> = 1 ha), are planted together in the same site area. The provenances within the trial are isolated from each other using border space or a barrier to avoid pollen contamination. The best provenance performance suitable for the end-product is then selected as a provenance seed stand, while the unselected provenance stands are still maintained as genetic resources for other potential purposes of breeding. This kind of conservation strategy is commonly practiced by plantation companies in the beginning of plantation establishment.

In *Acacia* hybrid breeding programs (*A. mangium* × *A. auriculiformis*), clonal testing is necessary to select the best performer clones before large scale deployment into operational plantations. As multipurpose species with various end-product uses and a great variability among *Acacia* hybrids in terms of growth, wood properties and resistance to pests and diseases (Ibrahim 1993; Le and Ha 2016; Sunarti et al. 2013), combining clonal testing with clone bank purposes is another potential approach for genetic conservation of acacias. In this strategy, the clonal test is intended only to provide information of the clone performance without any further roguing (without removing poorer performance clone from the test). Therefore, genetic diversity in the clonal test could also be maintained for any other future potential use.

Acacia seed is orthodox with the length of storage time, under proper conditions, of 7 years or more (Hardiyanto 2004). With this character, therefore, seed banks are another potential approach for genetic conservation of the three tropical Acacias. This is increasingly important due to some problems in new collection of genetic material from the natural distribution populations. In this seed bank strategy, thou-

sands of individual and bulk-provenance genetic resource seedlots collected from the natural distribution population and several generation breeding cycles could be stored in dry cold storage for many years, anticipating future need.

1.4 Hybridization

In general terms, hybridization is the crossing of two genetically dissimilar individuals, either naturally or artificially (Chaudary 1984). Hybridization is a major method for creating new varieties, although nowadays the progress of biotechnology has promising achievements such as mutation, polyploidization and also gene transformation (Kha 2001). Requirements in conventional hybridization are simple and uncomplicated, but in some cases may be more difficult with forest trees than crop plants. The barriers to tree hybridization are usually related to characters of trees, such as due to size and reproductive biology cycles. Therefore, hybridizing of forest trees has made less progress as compared to agricultural plants. The potential of forest tree hybrids may show some special values for growth or desired products. There is also the possibility of the presence of hybrid vigor (heterosis) (Zobel and Talbert 1984). Hybrid vigor usually refers to a size superiority over both parents, such as diameter, height, stem volume, and any other measurable trait such as wood quality, yield, early maturity, resistance to pest and diseases, more adaptive to adverse conditions, and generally expresses itself differently (Kha 2001). The earliest hybrids in forest trees were *Pinus* (pine), *Quercus* (oak), *Picea* (spruce), *Juniperus* (juniper), *Populus* (poplar), *Salix* (willow) and *Eucalyptus* (eucalypt) (White et al. 2007). In the case of *Acacia* species, the *Acacia* hybrid was first found naturally in Malaysia in 1972, followed by other Asian countries such as Thailand, China, Vietnam and Indonesia (Ibrahim and Awang 1991; Luangviriyasaeng 2007; Sunarti et al. 2013). Comparison of the properties related to wood pulp quality among mangium, auriculiformis and the *Acacia* hybrid was recently reported; in general the wood properties of selected *Acacia* hybrid trees were superior to its parent species (Asif et al. 2018).

The most advanced breeding of *Acacia* hybrids is taking place in Vietnam where it has been in process since 1992, focusing on the natural hybrid *A. mangium* × *A. auriculiformis*, with trial plantings at Ba VI Enterprise (Ha Tay Province) and Trang Bom (Dong Nai Province) (Kha 2001). At that time, it was not directly used in wood production, but was in the process of careful selection and serial field testing. Suitable vegetative propagation methods are used to maintain good genetic characteristics of the selected hybrid clones. Clone selections were based on growth rate and wood properties such as density, pulp yield, chemical components, mechanical strength and physical properties. Only clones exhibiting better growth rates and improved wood quality characteristics than the parental species were further mass propagated for plantation growth.

The development of *Acacia* hybrids in Indonesia began in the 1999s by establishing a hybrid orchard to produce hybrid seeds in Wonogiri, Central Java; the breeding strategy was updated in 2009 (Sunarti et al. 2013). The breeding strategy was

established as an effort to obtain higher probability of *Acacia* hybrid vigor, whether naturally or artificially, through co-improvement methods applied in hybridizing seed orchards (HSO) and breeding gardens (Fig. 1.5).

The main purpose of the co-improvement method is to hybridize the selected superior parent trees to increase hybrid vigor, as well as to minimize hybrid breakdown. Some serial research involved in this strategy covered flower phenology,

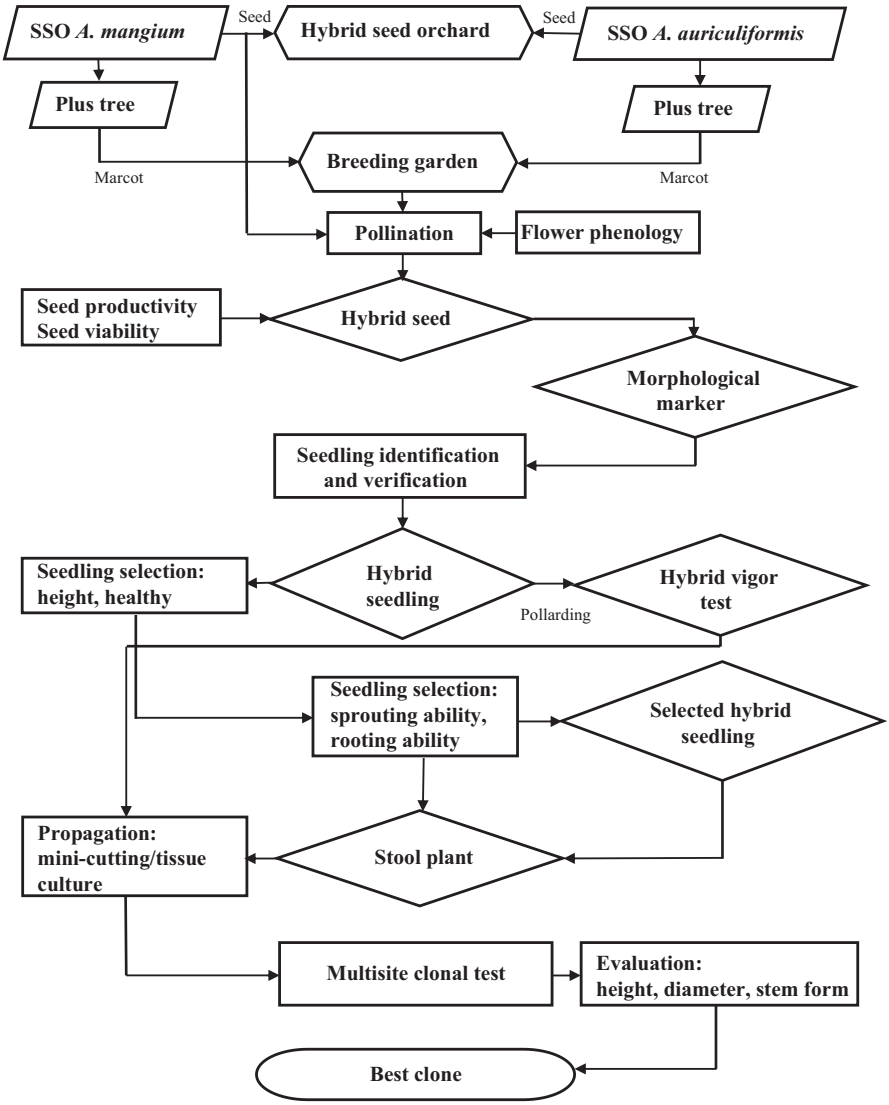

Fig. 1.5 Flow chart of breeding strategy of *Acacia* hybrid (*Acacia mangium* × *A. auriculiformis*). (Modified from Sunarti et al. 2013)

controlled pollination, hybrid seed quality, identification and verification of hybrids, clone multiplication and establishing multisite clonal tests.

Observations of flower phenology have focused on flower synchronization between *Acacia mangium* and *A. auriculiformis* parent trees. Weekly assessment of flowering was carried out using a scoring system (Ibrahim 1993). Controlled pollination was then practiced during the respective synchronized flowering periods using the direct controlled pollination method as proposed by Sedgley et al. (1992), modified in time of application (Sunarti et al. 2013). Obstacles to this crossing exercise are the limitation of receptive female and male flowers simultaneously due to internal factors of the genetics of the tree such as the hormonal system related to the physiological character of the trees (Opick and Rofle 2006). The unbalanced intensity of flowering between *A. mangium* and *A. auriculiformis* was another limiting factor affecting the success of the crosses, which may be due to particular phytohormones regulating the flowering system (Pallardy 2008).

Viable seeds produced from crosses were not as abundant as the number of flowers, and only a few of the florets developed into a mature pods, possibly caused by incompatibility between the stigma of the mother trees and pollen grain of the male trees (Chaudary 1984). These differences in receptivity between stigma and pollen is also suspected as the cause of empty seeds and inhibited the viable seed formation which commonly occurs in interspecific hybridization (Nghiem 2012).

In healthy hybrid seed, obtained from purposeful hybridization, the germination rate is low, as categorized by Wang (1991), indicating incompatibility between the stigma of *Acacia mangium* and the pollen grain of *A. auriculiformis*. This also causes the appearance of abnormal seedlings which are related to immature embryos and an imperfect fertilization process (Agrawal 1998). Seed with a normal embryo will germinate immediately under suitable conditions and produce vigorous seedlings.

Seedlings produced from such crosses using direct methods are putative seedlings because no emasculation is done of the pollinated female flowers, therefore inbreeding may occur within the flowers (Sedgley et al. 1992). Consequently, further identification and verification are required to select, and ensure, that the seedlings are true hybrids. Morphological markers developed by Rufelds (1988), simplified by Gan and Sim (1991), are efficient in identifying *Acacia* hybrid seedlings with an accuracy of 92.2%, confirmed with SCAR molecular markers (Sunarti et al. 2013). These morphological markers were identified by observing seedling leaf development from the appearance of the true leaves (pinnate) until the emergence of the phyllode which is slightly different between the *Acacia mangium* or *A. auriculiformis* parent species and their respective hybrids. The flow chart of identification is presented in Figs. 1.6 and 1.7.

Confirmed hybrid seedling were then tested to select superior clones based on growth performance, sprouting and rooting ability for further clonal multiplication by tissue culture and/or shoot cuttings. Hybrid multiplication by tissue culture using MS basal medium (Murashige and Skoog 1962) containing BAP of 0.5 mg/l for shoot multiplication and river sand for rooting has been achieved (Darus 1991;

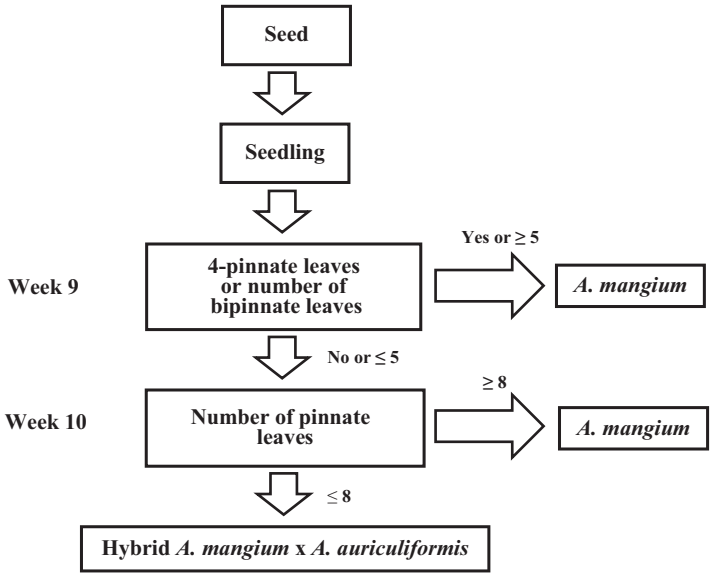

Fig. 1.6 Flow chart of morphological markers for *Acacia* hybrid (A*cacia mangium* × *A. auriculiformis*). (Modified from Gan and Sim 1991)

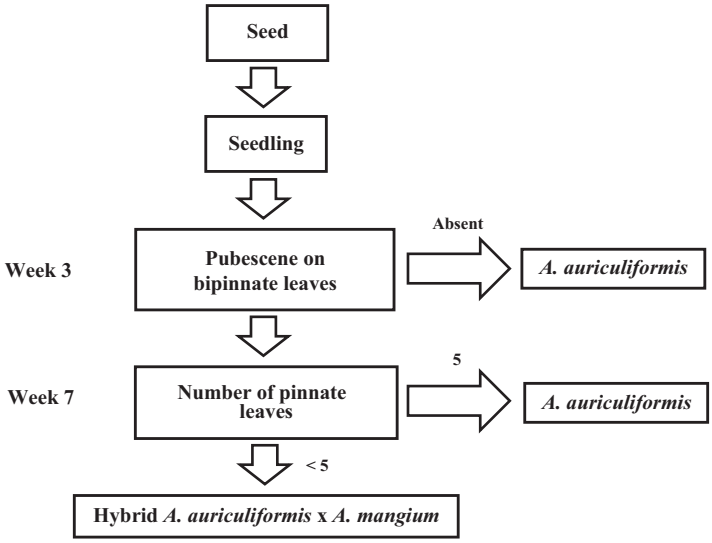

Fig. 1.7 Flow chart of morphological markers for *Acacia* hybrid (A*cacia auriculiformis* × *A. mangium*). (Modified from Gan and Sim 1991)

Galiana et al. 2003). Successful multiplication by shoot cuttings of the hybrid was first achieved in Vietnam in 1999 (Kha 2001). The most effective propagation method to mass-produce acacia hybrids is a combination of tissue culture technology and shoot cuttings, which is recommended to maintain the physiological vigor of the clones (Le and Ha 2016).

Before deploying selected clones into an operational plantation, a multi-site clonal test in several representative sites should be done to determine the appropriate clones which perform best within and across the sites (Libby and Ahuja 1993). Vietnam has established large-scale plantations (500,000 ha) using superior acacia hybrid clones which are the result of years of clonal testing, making it the most widely planted forest tree variety in that country (Le and Ha 2016). Meanwhile, in Indonesia, field trials of potential superior clones are being tested at multi-site locations with various site conditions to ensure their productivity within and across the sites (Sunarti et al. 2013). The clones are being tested not only for the growth productivity, but also for wood properties and resistance to pests and diseases.

1.5 Conclusion and Prospects

Acacia mangium, *A. auriculiformis* and *A. crassicarpa*, and an acacia hybrid, are among the major trees which are economically and ecologically feasible for forest industries, especially in the Southeast Asian region in Indonesia, Malaysia, Vietnam, Thailand and Papua New Guinea. The long history from the earliest introductions in 1930s, domestication and improvement in silviculture and breeding have taken place and resulted in significant increases in productivity in response to industrial demands. However, the increasing demand for wood from acacias for industries has resulted in a large gap between supply and demand. Recently, some serious problems have been encountered which reduced acacia plantation productivity, mostly due to biological factors, such as pests (monkeys, squirrels), root rot disease *Ganoderma* sp. and wilt disease *Ceratocystis* sp. In addition, developing new varieties which are environmentally adaptive to adverse condition, such as wetlands and to the impact of climate change, present challenges to the expansion of plantation areas.

Acacias have certain biological advantages, such as fast growth, adaptively to levels of adverse site conditions and nitrogen fixation, which provide an attractive opportunity for research in site management and breeding. However, there are some limitations in terms of reproductive morphological and genetics, such as flower morphology creating difficulty in controlled crosses, and a low level of genetic diversity. Taking these into consideration, traditional breeding through recurrent selection in successive generations should be incorporated into advanced breeding technology to employ molecular breeding and biotechnology to maximize potential and to ensure a continuous improvement to meet future demand and the challenges of acacia breeding.

Appendices

Appendix I: Research Institutes Relevant to Acacias

Institution	Area of specialization and research activities	Web-site
Center for Forest Biotechnology and Tree Improvement (CFBTI), Indonesia	Forest biotechnology, forest genetic conservation, forest tree improvement. Engaged in molecular genetics, genetic trials, seed production and hybridization	www.biotifor.or.id
Institute of Forest Tree Improvement and Biotechnology, Vietnam	Biotechnology and tree improvement. Engaged in Genetic trials, seed production, hybridization and polyploid Breeding	www.vafs.gov/en/
Forest Research Institute Malaysia (FRIM)	Forestry and environment, forest biotechnology, forest biodiversity, natural product, economic and strategic analysis, and social forestry. Engaged in molecular genetics, genetic trials and genomics	www.frim.gov.my/
ACIAR-FST/2014/068	Project on pest and disease of tropical acacias in Indonesia and Vietnam. Engaged in root rot disease, wilt disease and biological control agents (BCA)	http://aciar.gov.au/project/fst/2014/068

Appendix II: Genetic Resources of Acacias

Species	Important traits	Cultivation location
Acacia mangium	Good growth and wood properties suitable for pulp and paper, sawn lumber, wood energy industries, specifically on drylands	Indonesia, Vietnam, Malaysia
Acacia crassicarpa	Good growth and wood properties suitable for pulp and paper, sawn lumber, wood energy industries, specifically on wetlands	Indonesia
Acacia auriculiformis	Good growth and wood properties suitable sawn lumber and wood energy industries, specifically on degraded land and poor soil nutrients	Indonesia, Vietnam
Acacia hybrid	Good growth and wood properties suitable for pulp and paper, sawn lumber, wood energy industries specifically on drylands	Vietnam

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Chapter 2
Cotton (*Gossypium hirsutum* L.) Breeding Strategies

Saeed Rauf, Muhammad Shehzad, Jameel M. Al-Khayri, Hafiz Muhammad Imran, and Ijaz Rasool Noorka

Abstract This chapter is focused on the achievements and future prospects of cotton breeding and related biotechnology. Traditional plant breeding has been utilized for the development of pure-line selection for high yielding cotton genotypes in segregating generations through the pedigree method. Selection criteria include boll number plant^{-1}, boll mass, sympodial branches and ginning outturn percentage. Plant breeder efforts have been fruitful in releasing cotton cultivars with high yield potential and superior lint quality traits. Traditional breeding efforts resulted in the increase of seed cotton yield and fiber length. The calculated increase in the yield potential was 1.34 kg ha^{-1} year^{-1}. However, further genetic gains due to selection for high-yield potential reached a plateau in the last two decades and the recent increase in yield was due to better cotton husbandry techniques. Cotton ideotypes specifically for various agronomic and environmental conditions may be developed. Moreover, utilization of wild relatives for the introgression of disease resistance and abiotic stress tolerance is proposed through traditional plant breeding along with molecular markers to reduce linkage drags due to wild relatives. These high yielding cultivars with superior agronomic and adaptability traits may be further used for the development of transgenics. Genome editing technique such as CRISPR/Cas (clustered regularly interspaced short palindromic repeats: associated protein) is one of the emerging technologies to knock out genes or SNP (single nucleotide polymorphism) substitution at specific site with future prospects for the development of disease resistant crop cultivars.

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Keywords CRISPR · Genetic gains · Ideotype · Introgression · Transgenic · Wild relatives

2.1 Introduction

Cotton is an important cash crop, grown in more than 90 countries for its vegetable fiber which is processed by the textile industry. Globally cotton is cultivated on an area of 34.74 million ha which was about 3% of the total world arable land (FAO 2014). Among the countries, India, China, the USA and Pakistan are the largest producers of raw cotton. Cotton seed is used by the oilseed industry for vegetable fat and cooking oil production while seed cake is consumed by animal and poultry farming. The quality of cotton seed products is influenced by the fatty acid content and chemical composition, which is known to vary among cotton genotypes (Al-Bahrany and Al-Khayri 2000). One important health concern is the gossypol content and related sesquiterpene aldehydes in cotton seed meal, which function as natural phytoalexins against pathogens and pests (Tian et al. 2018).

Average global cotton production was 73.44 million mt during 2011–2016 which was 12% higher than the average world cotton production during 2001–2010, (65.23 million mt) and 36% higher than the average world cotton production during 1991–2000 (54 million mt). There was an increase of 21% in world cotton production when 1990–2000 and 2001–2010 are compared while an increase of 13% in global cotton production was seen in the present decade. World production grew by 1.5% year^{-1} in the last three decades. The global trade of cotton lint was only USD 21.30 million showing that most of the cotton was consumed and processed within cotton producing countries (FAO 2016). The cotton world map of production is shown in Fig. 2.1.

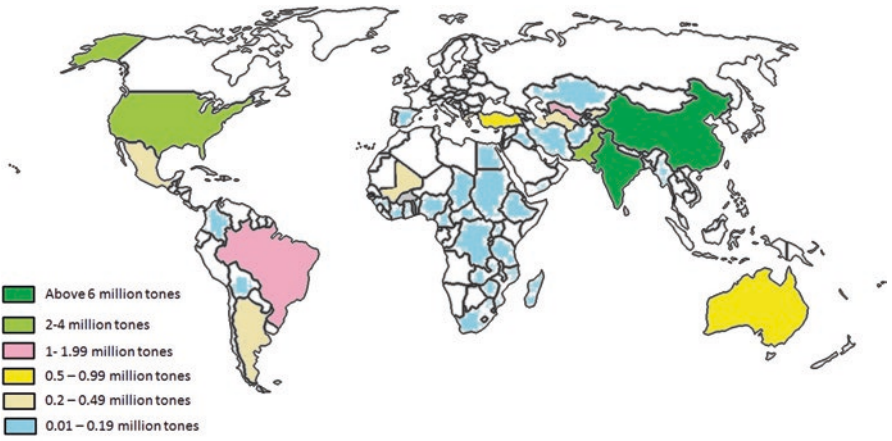

Fig. 2.1 The world map of cotton production. The map prepared as per food and agriculture statistics. (Source: FAO 2016)

Yield gains in cotton have been attributed to traditional plant breeding which developed cotton cultivars having high yield potential, superior lint qualities, tolerance to abiotic and biotic stresses, along with early maturity to convert this crop from a biannual to an annual growth habit. Cotton breeding is based on the exploitation of genetic variation within cotton germplasm and to select the transgressive plants with superior yield and lint quality. However, breeder selection efficiency is known to be dependent on the heritability of the traits. Since most economically-relevant traits are polygenic and affected by the environment, the variation among the traits was often masked by the environmental affect. The development of molecular marker systems and whole genome sequences analysis have led to the identification of genes and genomic regions associated with traits of interest by which selecting cotton cultivars is facilitated inbreeding programs (Fig. 2.2).

Cotton is one of the first crops in which transgenic cultivars have been successfully commercialized for large-scale cultivation during the mid-1990s (Zhang 2013). Transgenic cotton provided resistance against the boll worm complex which reduced the pesticide spray by nearly 50% (Traxler and Godoy-Avila 2004; Traxler et al. 2001). It is transformed with *Cry* genes encoding crystal protein δ-endotoxin (Bt toxin) obtained from *Bacillus thuringenesis* which is activated in the insect gut causing the death of the organism. Herbicide resistant genes reduced the yield losses due to weed competition for nutrients, light and soil moisture. The success of transgenic cotton in combating boll worms brought tremendous investment of the private sector for the research and development of transgenic cotton in various countries (Qiao 2015). Experimentation and evaluation of other transgenes have been carried out with a hope to commercialize them in future for sustainable cotton production. Details are presented in Sect. 2.5. Moreover, the cotton regeneration system has been optimized and several methods for development of transgenic cotton have been devised.

Establishment of functional genomic database in cotton is another milestone for cotton breeding and biotechnology that could help to develop molecular markers related to traits having importance in cotton breeding programs, mining of valuable

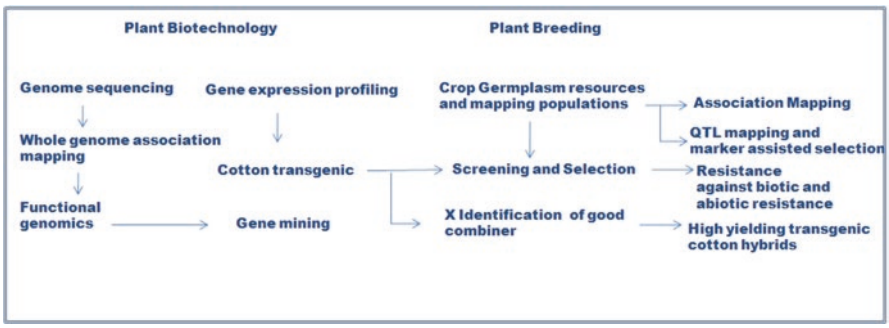

Fig. 2.2 Summary of integrated efforts for the improvement of cotton species through breeding and biotechnology

genes from genome analyses and help to understand the key metabolic pathways of commercial importance such as fiber development and oil content (Arpat et al. 2004).

This chapter reviews the achievements and future prospects of cotton breeding and biotechnology. It covers breeding and biotechnological aspects of cotton for high and sustainable production.

2.2 Cotton Sustainable Production

Cotton yield and production is an outcome of harvested bolls from a unit of population over multiple picks (Constable and Bange 2015). However, production is threatened by various social, economic, environmental, agronomic and biological factors (Gutierrez et al. 2015). Sustained cotton production strategies aim to improve these factors such as limiting degradation of the environment, loss of biodiversity, soil erosion, exhaustion and leaching of soil nutrients, evolution of new pathogens and biotype insects due to various practices of agriculture in cotton zones (Gutierrez et al. 2015). Precision agriculture may be implemented to accurately carry out various cultural activities from sowing to harvesting for continued success of the cotton crop with optimum use of farm resources, without affecting the environment and maximization of cotton farm income and profits (Roberts et al. 2002; Torbett et al. 2007).

The biological factors include the genotype performance, *the ability to respond successfully to a particular environment*. Cultivar performance depends upon its genetic potential, stability over the range of environment and resistance to various yield-limiting factors of a particular area. Cotton yield potential is defined as the harvested yield through current cultivars and crop husbandry practices under ideal conditions in the absence of yield limiting factors (Constable and Bange 2015). Conventional cotton breeding tools such as selection and hybridization have been used to widen the genetic potential of cotton and biotechnological tools such as recombinant DNA technology have been employed to induce resistance against various biological and environmental yield-limiting factors. Better crop stand also plays an important role in fully exploiting the benefits of the farm inputs and soil. Good crop stands depend on the optimum plant population per unit area, which is contributed by the percentage of seed germination, speed of germination (equals the sum of n1/d1 + n2/d2 + n3/d3 + …; where, n = number of germinated seeds and d = number of days) and seedling vigor (Gairola et al. 2011). Optimization of these parameters is improved through seed technologies.

2.3 Cotton Genetic Resources and Conservation

2.3.1 Cytogenetics and Evolution

Cotton has a narrow genetic base due to a bottleneck which occurred after evolving into allopolyploidy species, domestication of few high yielding genotypes and intense pure-line selection (pedigree selection method) by breeders (Kuraparthy and Bowman 2013; Rauf et al. 2010). The upland *Gossypium* species native to Africa *G. herbaceum* L.-like, carrying the A genome and *G. raimondii* Ulbr.-like species carrying the D genome which is native to South America (Paterson et al. 2012). Parental species intercrossed about 1–2 million year ago, when species carriers of the A genome spread into the Mexican region through transoceanic dispersal (Paterson et al. 2012). Mating between the species lead to the evolution of several tetraploid species, out of which *Gossypium hirsutum* and *G. barbadense* L. are widely cultivated throughout the tropical and subtropical regions of the world. A number of studies using molecular markers or phenotypic based studies have confirmed the presence of low genetic diversity in cotton elite germplasm (Rauf et al. 2010). Therefore, there was a need to broaden genetic diversity within cotton germplasm and to use wild germplasm resources for the improvement of cultivated species (Shaheen et al. 2012). The genus *Gossypium* comprises more than 50 species, out of which 4 are cultivated (Fig. 2.3). These cultivated species have 2 diploid and 2 tetraploid species. Among the 50 species, 7 species are allotetraploid and 43 are diploid (Paterson et al. 2012). The A genome is native to the Africa while A1 and A2 genomes are native to Afghanistan, China and Myanmar. The C and G genomes evolved in Australia, the D native to the Americas and the E genome in the Arabian Peninsula (Wendel 2000).

2.3.2 World Cotton Germplasm Collections

World collections of cotton germplasm exist in eight major sites: India, Brazil, France (CIRAD), the USA, Australia, Uzbekistan, Russia and China. Uzbekistan has the largest germplasm collection of *Gossypium hirsutum* (13,241 accessions) followed by China (7712), India (7633), the USA (6302), Russia (4503), France (2103), Brazil (1660) and Australia (1518 accessions) (Campbell et al. 2010). Uzbekistan (3019 accessions) also holds the world largest collection of *G. barbadense* followed by USA (1584 accessions), Brazil (1509), Russia (1057), China (633), India (534), France (483) and Australia (104 accessions) (Campbell et al. 2010).

Fig. 2.3 Ex situ wild germplasm collection at Central Cotton Research Institute, Multan, Pakistan. (**a**) *Gossypium somalense* (Gurke) J.B. Hutch., (**b**) *G. laxum* L.Ll Phillips, (**c**) *G. lobatum* Gentry, (**d**) *G. capitis-viridis* (Harv. & Sand.) Hochr., (**e**) *G. nelsonii* Fryxell, (**f**) *G. longicalyx* J.B. Hutch. & B.J.S. Lee, (**g**) *G. gossypioides* (Ulbr.) Standl., (**h**) *G. anomolum* Wawra & Peyr., (**i**) *G. harkensii* Brandegee, (**j**) *G. arboreum*L. red, (**k**) *G. arboretum.* (Photos by H.M. Imran)

Fig. 2.3 (continued)

Germplasm from various sources has been collected and maintained at the Central Cotton Research Institute, Multan, Pakistan (pers comm, Imran 2018). The collection includes 4243 accessions of *Gossypium hirsutum*, 1025 of *G. arboreum*, 556 of *G. herbaceum* L. and 109 accessions of *G. barbadense*. Several of the classified *Gossypium* species are not maintained in their native sites and cotton germplasm is vulnerable to diseases and insect infestation and many cotton species are at the verge of extinction (Campbell et al. 2010). A survey was carried out in Mexico (2002–2003) to determine the current status of the various diploid species of genus *Gossypium*. Increased population, rapid urbanization and pollution have threatened the local land races of diploid species of cotton. The populations of 7 diploid species were collected from door yards, garden plots and as feral plants (Ulloa et al. 2006). In situ preservation of all 11 diploid Mexican species was under threat. Therefore, collaborative efforts are required to preserve these cotton germplasm resources. The conventional method of in situ conservation was not applicable and ex situ conservation including seed bank for conservation of wild population should be considered (Almeida et al. 2009). In order to preserve cotton germplasm in situ techniques such as nodal cultures and cryopreservation are recommended (Altman et al. 1990).

2.3.3 Utilization of Wild Germplasm

Wild germplasm can be utilized for introgression of cytoplasmic male sterility, diseases and abiotic stress resistance, and to widen the genetic diversity of the cultivated species (Shaheen et al. 2012; Table 2.1). Synthetic species can be reconstituted from donor species i.e. *Gossypium herbaceum* and *G. raimondii.* Linkage drag from wild types i.e. introgression of undesirable alleles along with genes of interest is a major limitation for use of wild species in cotton improvement breeding. The deterioration of yield and quality, a divergent gene regulatory system, chromosome structural differences, hybrid break down and genome assortment without introgression are major limitations in the utilization of wild germplasm for cotton breeding (Diouf et al. 2014; Zhang et al. 2014). Molecular markers were used to reduce the linkage drags in backcross and transgressive breeding populations or development of substitution lines (Wang et al. 2011). Introgression of useful genes such as disease or insect resistance, especially from wild relatives, also introduced non-targeted or undesirable genes (such as photoperiod sensitivity, late maturity, poor fiber, yield traits) which reduce the overall performance of introgressed populations and thus these breeding lines were not directly commercialized. Use of molecular markers in foreground and background selection could help to minimize the introgression of non-targeted loci and maximize the genome of recipient species along with genes of interest. This could also help reduce the number of backcrosses required to achieve a degree of homozygosity.

Table 2.1 Development of breeding material from wild species of cotton (*Gossypium* spp.)

Interspecific crosses	Breeding material	References
*G. hirsutum*cv. 86-1× *G. armourianum* Kearney	Resistant to sucking pest such as jassid, white fly due to intense hairiness	Junqi et al. (1995)
G. hirsutum × *G. arboreum*	Sterile triploid hybrids which may be amphiploid to transfer cotton leaf curl virus resistance	Tahir and Noor (2011)
G.hirsutum × *G. arboreum*	Eight cultivars were developed after introgression with lint yield. High yielding cotton cv.Shiyuan 321 (Jimian 24) occupied the highest area in Yellow River Valley	Zhenglan et al. (2002)
G. longicalyx × *G. armourianum* ×*G. hirsutum* *G. longicalyx* × *G. herbaceum* × *G. hirsutum*	Trispecies hybrids were resistant to nematodes infestation	Bell and Robinson (2004)
hirsutum Acala 44 × *G. barbadense* Pima S-7	Three QTL CM12, STS1, 314–7 had large effect over *Verticillium* wilt resistance	Bolek et al. (2005)
G. barbadense, G. arboreum and *G. thurberi* Tod.	Developed breeding lines after introgression with *hirsutum* had superior fiber quality, expanding genetic diversity and resistant to *Fusarium* wilt and *Verticilium* wilt	Pang et al. (2006)
G. anamolum	Genes for fiber fineness and strength and resistance to insect and disease	Mehetre (2010)
G. hirsutum × *G. barbadense*	Salt tolerant back cross inbred lines surpassing both parents due to transgressive breeding	Tiwari et al. (2013)
G. *gossypioides*	Resistant to cotton leaf curl virus disease and may be exploited for introgression	Azhar et al. (2013)
G. hirsutum × G. *arboreum* (followed by backcrossing to *G. hirsutum*)	Interspecific F1 hybrid was completely resistant to cotton leaf curl virus, resistance break down with back crossing scheme due to lack of introgression from *G. arborem*	Nazeer et al. (2014)
Synthetic amphiploid *G. hirsutum* × *G. arboreum* (AADDAA)	Amphiploid was resistant to *Verticillium* and drought resistance	Chen et al. (2015a, b)
G. *capitis-viridis* × (*G. hirsutum* × G. *austral* F. Muell.)	Trispecies hybrid was intermediate in canopy characteristics and resistant to insect	Chen et al. (2015a, b)
G. herbaceum (A1) × *G. raimondii* (D5)	Sterile hybrid with characteristics intermediate between the species. Synthetic species was used to understand speciation, genome interaction and evolution of tetraploid species	Wu et al. (2017)

Chromosome substitution lines from wide crossing between *Gossypium barbadense* × *G. hirsutum* were developed with the objective to combine the high yield, lint percentage with better lint quality traits. The chromosome by chromosome approach was exploited to narrow the search for the genotypes with high yield and premium quality fiber (Saha et al. 2010). Breeding potential of introgression lines (*G. barbadense* × *G. hirsutum*) showed that some of the lines had a predominance of additive variance and were positive contributors of alleles related to yield and quality. Developed introgression lines may be used for the development of hybrids and transgressive lines (Zhang et al. 2016).

2.3.4 Colored Cotton

Cotton produces white lint color. However, there is great variability of lint color within tetraploid cottons. Lint is available from various shades of brown color to the light green (Fig. 2.4). Colored cottons provide great potential for the textile industry to produce cloth without use of synthetic dyes and chemicals which can causes allergic reactions to consumers and produce toxic effluent. It is also a major component of organic cotton, i.e. cotton produced with less damage to the environment without the use of synthetic chemicals. However, colored cotton fiber qualities (fiber length, strength, micronaire) are inferior to the white cotton and cotton color is affected by the environment upon maturity. Therefore, there is need for the selec-

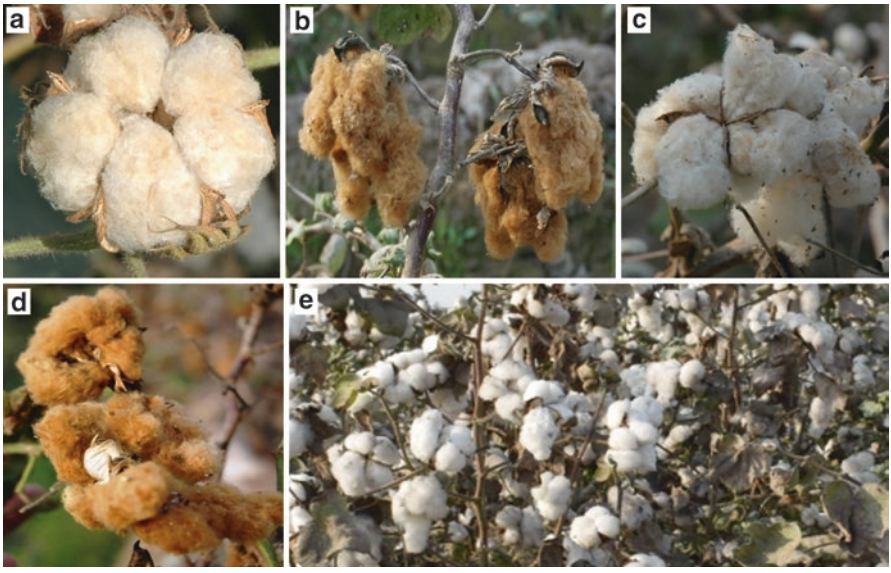

Fig. 2.4 Genetic diversity in cotton lint color. (**a**) Light brown, (**b**) Khaki, (**c**) Light green, (**d**) Dark brown, (**e**) White. (Photos by H.M. Imran)

tions of colored cotton with stabilized pigmentation and superior fiber quality for its acceptability in the textile industry.

Colored lint develops as a result of accumulation of natural pigmentation in the fiber after exposure to sunlight. All colors are genetically controlled and have monogenic inheritance. Green colored lint is controlled by the allele Lg (de Carvalho et al. 2014). There were about six alleles (Lc_1–Lc_6) for the brown color (Kohel 1985). Lc_1–Lc_2 produce brown color, Lc_3 produce dark brown color and Lc_4–Lc_6 produces light brown pigmentation in lint (Kohel 1985). Lc_1 was assigned to chromosome 7 and Lc_2 on chromosome 6 (Wang et al. 2014).

2.4 Traditional Cotton Breeding Programs

Traditional breeding based on basic principles and selection methods has been successfully employed in selecting cotton cultivars with greater yield potential, along with acceptable fiber quality traits. It involves developing crosses between good × good, or good × poor, which were selected through pedigree or recurrent selection in segregating population to establish pure lines. The selection pressure for early type cultivars resulted in improvement of harvest index per unit area, and switching the cultivars from perennial to annual growth habit and reduction in monopodial branches with subsequent increase in the direct fruiting branches. A positive impact of breeding has been noted over the fiber quality traits and genetic gains for fiber quality traits (Kuraparthy and Bowman 2013). However, it is known that cotton yield has reached a plateau and further genetic gain in lint yield was not possible due to drain of genetic variation within elite cotton germplasm (Rauf et al. 2010). Several types of cotton ideotypes have been proposed to develop cotton cultivars with high yield potential or sustainable yield under various agro-ecological conditions and biotic and abiotic stresses Table 2.2.

Rapid genetic gain in the seed cotton yield potential of Pakistani cultivars was noted until the 1980s (Fig. 2.5). Yield potential expanded from 600 to 3500 kg ha^{-1} from 1916 to 1988; afterward there was no change in the yield potential of the released cultivars. The increase in the yield potential was about 4.4% per year, about 1.34 kg ha^{-1} $year^{-1}$. There was also steady increase in the ginning outturn and staple length of the Pakistani cultivars; both traits increased by 0.5% and 0.6% per year, respectively (Figs. 2.5 and 2.6).

Correlation analyses showed that there was high significant ($P \leq 0.05$) and positive relationship between the actual and potential yield, showing that release of high-yielding cultivars had profound effects over actual cotton seed yield in Pakistan (Table 2.3). Correlation between the potential seed cotton yield of cultivars with ginning outturn and staple length was also positive and significant ($P \leq 0.05$) showing that high-yielding cultivars also had superior fiber quality and that the increase in yield potential was not at the expense of fiber quality (Table 2.3). There was no improvement in yield potential later in the 1990s–2005 period. However, there was some increase in actual yield which may be due to better crop management, early

Table 2.2 Various ideotypes of cotton to full fill ambitious breeding objectives of cotton

Breeding objective	Plant ideotypes	References
High yield potential	Compact canopy, high number of boll per meter^2, multiple bolls per fruiting point (boll borne in cluster), medium size boll, good boll opening, earlier anthesis, long reproductive duration, increase photosynthetic rate	Loison et al. (2017a, b) and Sekloka et al. (2008)
Mechanized farming	Uniform opening, small to medium compact canopy, direct fruiting branches, early maturing, good boll opening, normal broad shaped leaf at the base and okra leaf at the canopy	Karthikeyan et al. (2015)
Early maturity	Effective flowering time, plant height at harvest, length of fruiting branch, height to node ratio, average boll retention at first fruiting branch	Sekloka et al. (2008)
Insect resistant	Long frego bract, okra leaves, hairiness, small leaf area, red colored leaves, glandless leaves, nectariless	Taggar and Arora (2017)
Drought resistance	Small leaf area, early maturing, intense hairiness, high cuticular wax, longer root length, greater stem reserve mobilization	Rauf et al. (2016)
Heat resistance	Cell membrane stability, ability to bear flower and retain boll at lower nodes, leaf and stem angle, lower leaf senescence, canopy architecture, leaf thickness, leaf angle	Loison et al. (2017a, b) and Pauli et al. (2017)

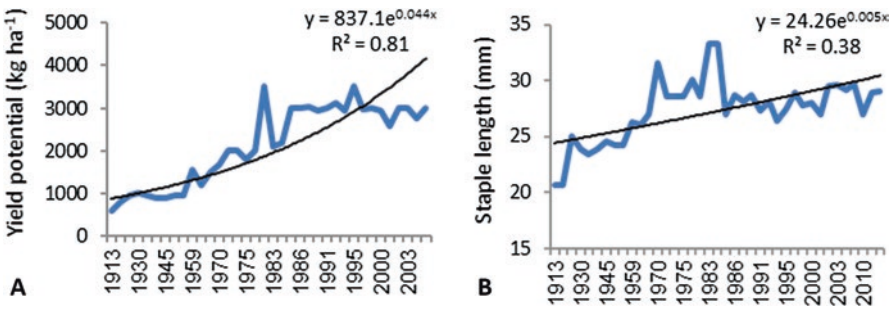

Fig. 2.5 Change in yield potential (**a**) and staple length (**b**) of cotton cultivars release in various periodicals. (Figures were produced by Saeed Rauf from public data published by Pakistan Central Cotton Committee)

sowing and control of insect infestations. Studies have also shown that an increase in lint yield occurred at the expense of seed yield. Estimated genetic gain in lint yield in Cameroon was about 3.3 kg ha^{-1} year^{-1} due to increased ginning outturn. However, no genetic gains were noted for physiological traits such as radiation-use efficiency, aerial biomass, harvest index, leaf-area index and seed-cotton yield (Loison et al. 2017a, b).

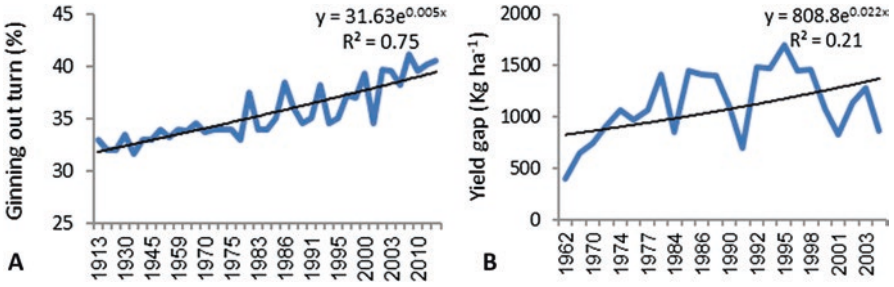

Fig. 2.6 Change in ginning out turn (**a**) and yield gaps (**b**) of cotton cultivars release in various periodicals. (Figures were produced by Saeed Rauf from public data published by Pakistan Central Cotton Committee)

Table 2.3 Correlation coefficients between yield and quality traits

Traits	Yield potential	Actual yield	Yield gap	Ginning out turn (G.O.T.)
Actual yield (kg ha^{-1})	0.72∗			
Yield gap (kg ha^{-1})	0.66∗	−0.06^{ns}		
Ginning out turn (%)	0.76∗	0.61∗	0.22^{ns}	
Staple length (mm)	0.62∗	−0.21^{ns}	−0.02^{ns}	0.47∗

∗Significant at $p \leq 0.05$; *ns* non significant $P \geq 0.05$

Plant breeders also developed hybrid cotton with significant economical heterosis for fiber yield and quality traits (Lian-gen 2011). It is principally grown in India, China and Vietnam, while India pioneered hybrid cotton. In India, 40% of cotton cultivation uses hybrid seed, while in Vietnam about 70% of the cotton area is planted by the hybrid seeds. Development of high-yielding hybrids could expand the yield potential of the cultivars. The yield advantage of hybrid cotton in China was more than 20% over the open- pollinated cultivars. Bt hybrid cotton was considered an example of successful integration of traditional cotton breeding with biotechnology, as hybrid Bt cotton was based on the manifestation of heterosis between non-Bt and Bt breeding lines (Dong et al. 2004). Recently several cotton hybrid cultivars with high yield, lint percentage, multiple resistance and wide adaptability have been released for general cultivation in various parts of the world (Table 2.4). New technologies such as molecular markers have been employed to study the genetic purity of hybrids (Dongre et al. 2011). Hybrid cultivars also exploit heterosis for the development of long or extra-longstaple cotton through interspecific hybridization between the *G. hirsutum* × *G. barbadense* species.

Table 2.4 Cotton (*Gossypium* spp.) hybrid performance under various agro-environment conditions

Crosses	Heterosis	References
Hybrid transplantation technology	Transplanting Bt hybrid could help to escape the chilling stress under controlled condition and provided additional 1 week growth period	Dong et al. (2005)
Hybrid performance over organic vs. conventional cultivation	37–71% more bolls under organic condition and superior fiber traits	Blaise (2006)
Hybrid Ji-FRH3018 CMS-3096 × Ref 866	High resistance to bollworm, *Fusarium* wilt and *Verticillium* wilt and high yield and lint quality	Guo et al. (2010)
Cytoplasmic male sterility source from *G.harknenssii*	Negative impact of CMS source over number of bolls per plant and fiber quality traits	Tuteja and Banga (2011)
Cotton hybrids CRI-28, CRI-29, XZM 2 and Jimian18.	Gene differential expression in hybrids when compared to parents that changed over various phonological stages	Zhu et al. (2011)
Commercial hybrid Xiangzamian 3	Hybrid had better canopy cooling ability. Maximum heterosis for net photo synthesis rate was observed during post noon when temperature exceeded 45 °C	Zeng et al. (2012)
G. *hirsutum* × *G. barbadense* chromosome segment introgression lines	Stable heterotic loci (hLP-A4-3) was detected in all 3 years	Guo et al. (2013)
Okra leaf hybrid F1 s, crossed 2 sterile near isolines and 3 restorer near isolines	Lint percent 7–12%, boll weight (6–11%), Canopy light intensity (2–147%), 1–10% in net photosynthetic rate (Pn), and −3 to 3% in lint yield over check hybrid (Zhongza 29)	Zhu et al. (2008)
Recombinant inbred lines	29 QTLs were linked to mid parent heterosis. Genetic basis of heterosis in cotton was due to dominance, partial dominance or epistasis	Shang et al. (2016)

2.5 Genomic Database

The presence of a large number of sequence data sets of cotton has led to creation of an integrated cotton functional data set (Zhu et al. 2017a, b). Cotton functional data set (CottonFGD, https://cottonfgd.org) has been established and allows the easy, quick and user-friendly access to the cotton functional genomic data set including all the *Gossypium* species genome data published. Cotton FGD was exploited to access genes for leaf shape and arginase (Zhu et al. 2017a, b). The comparison of data sets from various upland and sea island cottons identified various polymorphic sites for the development of molecular markers for these traits. These sequences were used for marker-assisted selection (MAS) within F2 populations to identify the genomic regions associated with these traits. Marker-assisted selection along with Cotton FGD tools narrowed down the genomic region associated with traits of interest resulting in the identification of 81 genes. Among the identified genes, *ATHB-51* (*GhD01G2042*), a homeobox-leucine zipper protein was a homologue to the leaf shape gene in *Arabidopsis thaliana*. The draft genome sequence of *G. raimondii* species has been published (Wang et al. 2012) which

showed that the *G.raimondii* genome contains more than 40,976 protein-coding genes. However, the species genome underwent tremendous recombination with more than 40% gene synteny (Wang et al. 2012). Some key genes related to fiber elongation were explored through expressed sequence tags (Arpat et al. 2004). More than 80 genes related to various developmental stages of fiber development were identified (Arpatet al. 2004). Several fiber related genes such as *GhTTG1-GhTTG4*, *GhGa20ox1-3*, *iaaM*, *GbPDF1* and *GhJAZ2* (fiber initiation); *GhSusA1* and *PHYA1* (fiber length); *GbPDF1, E6*, *GhExp1* and *RLK* (fiber strength); *GbTCP*, *WLIM1a*, *PAG1*, *GhCaM7*, *ACO*, *GhHOX3* and *PIP2s* (fiber elongation), have been reviewed and are considered primary genes expressed at various stages of fiber development (Ashraf et al. 2018). The genes were not known to regulate fiber development pathways. *Susy* gene was found to be positively involved while *GhPRP5* was negatively involved in the same fiber elongation pathways. Resistance genes to biotic and abiotic stress along with their putative functions are listed in Tables 2.5 and 2.6, respectively.

Table 2.5 Development of transgenic for protection against various types of biotic stresses

Transgenes	Resistance	References
Hen egg white lysozyme, GAFP4, *FreB* gene (VDAG_06616) *Gastrodia* antifungal proteins	Resistant to *Verticilium* wilt	Wang et al. (2016), Wenfang et al. (2017) and Rehman et al. (2018)
GR79 EPSPS and *N-acetyltransferase* (*GAT*) genes	Resistant to glyphosate	Liang et al. (2017)
cry1Ac	BT toxin induction in *Gossypium hirsutum*	Anayol et al. (2016)
Galanthus nivali agglutinin (GNA) and *Amaranthus caudatusagglutinin*	Genes with anti-aphid function having 75% control over the pest.	Yang et al. (2017)
Pyramiding RNAi and BT technology	Pyramiding RNAi and BT technology was effective to control resistant *Helicoverpa armigera* types	Ni et al. (2017)

Table 2.6 Characterization of various trans-genes under abiotic stresses

Transgenes	Tolerance	References
Isopentenyl transferase gene	Increased salt tolerance due to higher chlorophyll and cytokinin contents which delayed leaf senescence	Liu et al. (2012)
LOS5/ABA3 (LOS5) encodes a molybdenum co-factor	Drought resistance due to lower transpiration losses and over accumulation abscisic acid	Yu et al. (2012)
Annexin gene, *GhAnn1*	Enhance drought and salt tolerance	Zhang et al. (2015)
PeDREB2a and *HhERF2* from 86 AP2/ERF	Transgenic plants containing PeDREB2a and KcERF showed tolerance to salt and drought stresses	Bo Li et al. (2016)

2.6 Mutation Breeding

Mutation breeding programs were initiated to improve cotton in various parts of the world during the 1960s. High fruiting, diseases resistance, early maturity and lower monopodial growth habits were the main targets of these breeding programs (Muthusamy et al. 2005; Iqbal et al. 1994).The mutants were generated with exposure of plant tissues such as seed (30 kR), pollen (10 Gy gamma rays), shoot tip (5–50 Gy gamma rays), ovule (50 Gy gamma rays) or the chemical mutagen 1–5 mM of ethyl methane sulfonate (EMS) (Aslam et al. 2018; Muthusamy and Jayabalan 2011, 2014; Muthusamy et al. 2005; Saeed Iqbal et al. 1994). Lower doses and concentrations generated higher genetic variation for traits such as early flowering, plant height, number of bolls, yield of seed cotton, ginning percentage, seed index, harvest index and fiber traits (Muthusamy and Jayabalan 2011, 2014). Cell lines of cvs. (Acala SJ2, B1654) were developed by exposure of 2.13 μmol to sulfonyl urea. The embryogenic resistant cell had LD_{50} several hundred times greater than unselected lines (Rajeskaran et al. 1996). Regenerated plants were resistant to primsulfuron (0.06–0.21 μM), and were cross tolerant to two imidazolinone herbicides, imazethapyr and imazaquin (Rajeskaran et al. 1996). The Coker-2312 cell lines were exposed to the progressive higher doses of glyphosate (20 mM) (Tong et al. 2010). Regenerated calli line R1098 can tolerate about 1.48 kg acid ha^{-1} glyphosate (Tong et al. 2010).

A team at the Nuclear Institute of Agriculture Biology, Faisalabad, Pakistan, led by the late Rana Saeed Iqbal Khan, developed a revolutionary cv., NIAB-78, obtained by irradiating the F_1 cross of (Deltapine × AC-134). The Government of Pakistan awarded him the highest presidential award for the creation of this cultivar and for his services to the cotton community (pers comm, Hafiz M. Hassan Mumtaz 2019). The developed mutant was early maturing and with high yield potential which increased the overall cotton production of Pakistan several fold and was suitable for integration into the double cropping system (wheat-cotton) of Pakistan. It occupied about 90% of the area during early 1980s and total Pakistan cotton production increased from 3 to about 12.8 million bales (www.niab.org.pk). However, the cultivar was abandoned due to its susceptibility to CLCuVD during the mid-1990s (pers comm, Hafiz M. Hassan Mumtaz 2019). A high yielding mutant NIAB-92 was developed by irradiating seed of Stoneville231 with gamma rays at 30 kR from ^{60}CO. It has a semi-hairy, compact canopy with 0–2 monopodial branches as compared to its parental cultivar (Iqbal et al. 1994). Later on, two new mutants (NIAB-999, NIAB-111) were released for cultivation which were high yielding, heat tolerant and CLCuVD resistant. NIAB-777 was developed by crossing NIAB-78 with REBA-288. The pollen of REBA-288 were irradiated with 10 Gy of gamma rays before pollinating NIAB-78 (Aslam et al. 2018). Plants were selected for high yield, better fiber quality and disease resistance during segregating which resulted in the evolution of cv. NIAB-777 (Aslam et al. 2018). A mutant with high fiber quality (fiber strength = 40.5) was isolated from segregating populations generated after exposure of MD15 to 3.2% v/v ethylmethane sulfonate (Bechere et al. 2013).

2.7 In Vitro Applications

A prerequisite for genetic transformation is the development of a reliable in vitro regeneration system. Cotton is a recalcitrant species for tissue culture and regeneration via somatic embryogenesis is confined to only few cultivars i.e. Cocker lines. A Cocker line such as 312 had high regenerable response and a heart-shaped globular embryo cultured on semi-solid media which germinated into plantlets (Trolinder and Goodin 1987). Research efforts were carried out for the optimization of genotype independent protocols for regeneration and genetic transformation.

Cotton regeneration protocols are claimed to be optimized in several cultivars around the globe (Pathi and Tutega 2013). For instance, Chinese cultivar YZ-1 showed higher regeneration potential (81.9%) when compared with Coker lines 312 and 212 (Jin et al. 2006). A selection toward regeneration potential in elite cultivar was carried out for two cycles and selected cell lines within the elite cultivar were designated as Max-R (Mishra et al. 2003). The regeneration response was also known to be dependent over the explants, in addition to the growth media such as vitamins and plant growth regulators (Pathi and Tutega 2013). Explants such as embryo axes showed higher regeneration response due to direct regeneration in contrast to the leaf disc segments. Shoot-tip explants have been used for transformation and transgenic meristematic cell of Indian cotton cultivars were regenerated into plantlets (Satyavathi et al. 2002). In vitro regeneration in cotton was shown to have genetically controlled traits and showed moderate heritability when local genotypes were crossed with Cocker lines (Rauf and Rahman 2005). Overdominance to additive type of gene action was involved in the expression of traits such as callus induction, embryogenic callus and germinating embryo Factors affecting cotton response to in vitro culture were reviewed by Ahsan et al. (2014).

Various in vitro techniques may be exploited to widen the genetic diversity within cotton species. Techniques such as in vitro fertilization or embryo rescue could be used to facilitate the wide cross among the cultivated and wild species. Interspecific crosses among species were attempted in the field and fruit boll abscission was prevented by repeated application of plant growth regulators (BAP, NAA) and embryos were rescued and cultured over growth medium for 15 days after pollination (Gill and Bajaj 1984). Cotton embryo culture was improved by the manipulation of components of the culture medium, including nutrients and plant growth regulators (Fuller et al. 2011).

Protoplast fusion of diverse species and genera provide an innovative tool to attempt wide crosses and to transfer the genes of interest from cyto-morphologically incompatible species. Moreover, protoplast fusion could also introduce cytoplasmic genes from donor species (Wang et al. 2007). Theoretically protoplast fusion could be attempted between any species, where protoplast may be successfully isolated and plants may be regenerated from cell culture. Although cotton is a recalcitrant species for in vitro regeneration, reports on the protoplast fusion between various species have been presented in the literature and viable embryos containing asymmetrical protoplast of different species were regenerated. Protoplast fusion starts with the production of in vitro callus culture. The callus of highly regenerable cot-

ton cultivars such as Coker201 is obtained by culturing on MS media (Murashige and Skoog 1962) supplemented with suitable plant growth regulators. The regenerable calli are subcultured on a regular interval. Cell suspension culture is done in growth media devoid of agar and with suitable growth regulator such as BAP 0.1 mg L^{-1} and naphthalene acetic acid 1 mg L^{-1}. Isolation medium for protoplast has been reported as MS salt (Murashige and Skoog 1962), 5 rnM MES (2-(N-morpholino) ethanesulfonic acid), 0.7 M mannitol, 5% (w/v) Cellulysin cellulose and 1% (w/v) Macerase pectinase at a pH of 5.7) (Renfroe et al. 2001). The cells were plasmolyzed before incubation in the isolation medium. They were incubated in isolation media for 5 h at 28 °C and purified by filtration with nylon mesh having 100 mm pores and centrifuged at 125 RCF for 6 min and layered over 20% (w/v) sucrose solution. The protoplast of recipient and donor species is mixed in 2:1. Pigmented lines may be used as markers for identification of hybridity. The protoplast of the species is fused following Evans and Bravo (1983). The fusion is promoted by using 50% polyethylene glycol solution (MW = 6000). Polyethylene glycol fusing solution was eluted with either a glycine buffer followed by a wash with culture media, or by a Tris buffer (Renfroe et al. 2001). The details of the various reports are presented in Table 2.7.

2.8 Transgenic Cotton

Transgenic cotton is cultivated on more than 25 million ha, which is about 70% of the total world cotton area (Anderson and Rajasekaran 2016). Genetic transformation of cotton is categorized as *Agrobacterium*-mediated transformation; biolistic transformation, in planta pollen tube pathway or pollen tube transformation are involved. *Agrobacterium*-mediated transformation is a widely used and reliable method of transformation and is achieved by co-cultivation of explants with *Agrobacterium* cultures. Recombinant DNA plasmid containing genes of interest along with reporter genes are first inserted in T-DNA or other plasmids. The recombinant plasmid is then inserted in *Agrobacterium* through various methods including electroporation. *Agrobacterium tumefaciens* have the capacity to transform the plant cells with recombinant T-DNA in cotton cells. Triple genes carrying *Cry1AC*, *Cry2Ab* and *EPSPS*are used to transform T-DNA to produce recombinant plasmid (Naqvi et al. 2017). The sequences are retrieved from the NCBI. The gene sequences are optimized according to upland cotton (Naqvi et al. 2017). The 2X 35S promoters and terminator sequences are used to induce expression and termination for *Cry1Ac* gene. The 2x 35S promoter was first cloned in pBlue Script SK-zero using *SwaI* and *BamHI* restriction site while *Cry1Ac* was cloned in pBlue SK-35S using *BaHI* and *HindIII* restriction sites. The terminator is cloned using HindIII and SalI restriction sites.*Cry2Ab* cassette comprise figwort mosaic virus promoter, chloroplast signal peptide, partial *Cry2Ab* (270 bp) and G7 terminator (Naqvi et al. 2017). The cassava mosaic virus promoter (700 bp) is used along *EPSPS* (1.9 kb) and E9 terminator to induce the expression of the herbicide resistant gene.

Table 2.7 Protoplast fusion between various species of cotton (*Gossypium* spp.)

Species	Growth condition	Plant morphology	Chromosome no.	References
G. hirsutum (Coker 201) ×*G.klotzschianum* Andersson	Electrofusion Fused protoplast was cultured on KM8P medium supplemented with 2.685 µ*M* α-naphthaleneacetic acid and 0.465 µ*M* kinetic	Plants were different from their parents	71–81	Sun et al. (2004)
G. hirsutum ×*G. bickii* Prokh. and *G. hirsutum* × *G. stockii* Masters	Electrofusion RAPD markers confirmed the hybridity	Hybrids were intermediate and produced viable seed	78	Sun et al. (2005)
G.hirsutum (Coker201) × *G. davidsonii* Kellogg	RAPD (random amplified polymorphic DNA) and SSR were used to confirm hybrids	Regenerated were difficult to transfer into soil and therefore grafted on the root stock	74–84	Sun et al. (2006)
G.hirsutum (YZ-1) × *G. davidsonii*	Asymmetric hybrids were obtained by electrofusion. Both protoplast were treated with chemical and radioactive to retard growth before fusion	Plants were intermediate for many characters and showed recipient like morphology. Simple sequence repeats and amplified polymorphic DNA markers were used to identify hybrids	40–73	Fu et al. (2009)
G .hirsutum L. (cv. Coker 312) × *G. trilobum* (de Candolle) Skovsted	Symmetric electro fusion Molecular markers were used to identify hybrids	Hybrids were different from parents	78	Yu et al. (2012)

A method of transformation and regeneration was patented which involves the cocultivation of cotton explants (hypocotyls from 8-day-old seedlings)with *Agrobacterium* for 2 days on callus-initiation media (Murashige and Skoog salts, glucose 30 g L^{-1}, myo-inositol 100 mg L^{-1}, nicotinic acid 1 mg L^{-1}, pyridoxine-HCL 1 mg L^{-1}, thiamine HCL 10 mg L^{-1}, magnesium chloride 1.87 g L^{-1}, potassium nitrate 1.90 g L^{-1} and gelrite 4 g L^{-1}) without plant growth regulators. The transformed tissues are screened over the 12.5–50 mg L^{-1} kanamycin and 150 mg L^{-1} cefotaxime. Surviving cells are then continuously cultured on embryo genic calli inducing media and finally germinated over the shoot inducing media (Strickland 1998). The cell suspension culture or embryo axis is also bombarded with high

density particles coated with plasmid using a biolistic gun (Finer and McMullen 1990). The recombinant plasmid contains a hygromycin resistant gene which is used to screen the transgenic cells over culture media (Finer and McMullen 1990).

In planta methods were devised to avoid complicated regeneration protocols (Kalbande and Patil 2016). The in planta pollen tube pathway is also a popular method of transformation. The principle of this method is to use the pollen tube formed by the pollen to insert the gene into the embryo sac post pollination/fertilization. The steps are like pollinating the flower, cutting the stylar tissue (10–12 h post pollination) and injecting the vector solution carrying the gene of interest. The desired genes are directly inserted in pollen or injected into the developing embryo via pollen tube pathways. The in planta method of transformation in seedlings includes a vertical cut in 4-day-old seedlings at the junction of cotyledonary leaves to expose the apical meristem (Kalbande and Patil 2016). The exposed apical meristem is then treated with *Agrobacterium* transformed with the gene of interest. A transformation efficiency of 6.89% was obtained in cv. LRK-516. The highest efficiency of transformation was obtained through *Agrobacterium*-mediated transformation as the gene of interest was inserted in the cells of L3 layers which produce germline tissues. Biolistic transformation produces chimeric tissue due to insertion in non-targeted layers.

Some 58 transgenic cottons carrying insect and herbicide resistance with a maximum of three genes (two insect resistance+ one herbicide resistance) along with marker genes, which have been released for general cultivation in various parts of the world. Transgenic cotton adaptation is increasing at a rate of about 5% year^{-1} containing either of *cry* genes or herbicide-tolerant genes (Anderson and Rajasekaran 2016). Transgenic cotton is the second major commercial success, after soybean, of *Cry* genes (*Cry1Ac, Cry2Ab2, Cry2Ae, Cry1Ab, Cry1A*) along with herbicide resistant genes (*BAR,EPSPS, BXN, DMO*). Two transgenic cvs. NuCOTN^{33} and NuCOTN^{35} with the trademark Bollgard™ were released for general cultivation in 1996 through a joint venture between the Monsanto and Delta & Pine companies (Traxler et al. 2001). These cultivars were also subsequently released in Argentina, China, Australia, South Africa and Mexico. Later, several new companies introduced the Bt cotton (transgenic cotton transformed by various *Cry* genes which encode crystal protein δ-endotoxin to kill lepidoptera class insects) cultivars and local varieties in cotton growing countries were incorporated with various *Cry* genes through *Agrobacterium*-mediated transformation (Strickland 1998) or biolistic genetic transformation (Finer and McMullen 1990) or backcrossed resulting in the wide spread of Bt cotton cultivars containing *Cry* genes encoding toxin which provides protection against the bollworm complex (Wu et al. 2008).

In comparison to the non-Bt cotton, Bt cotton occupies 85% of the total of cotton cultivation area in the USA, 90% in India and Pakistan and 65% China (Anderson and Rajasekaran 2016). Bt cotton has had great success in India which nearly doubled the cotton production due to reduction of yield losses and protection against boll worm. In Pakistan, farmers are still awaiting the benefits of the Bt cotton due to several factors including weak expression of *Cry* genes in local genotypes and their poor adaptation due to adverse climatic conditions, cotton leaf curl virus infestation

and sucking pests. Bt technology has reduced the pesticide spray by 40%, reduced yield losses by 20% and farmer profitability was increased by 70%. In future, multiple Bt or Bt-like genes will be staked together along with herbicide resistant genes to increase the efficacy, durability against the boll worm complex and to reduce the yield losses due to weeds by various multinational or local companies in countries like the USA, China, India and Pakistan (Naranjo 2010). The staking of RNAi genes in Bt cottonwas used to interfere with the metabolism of juvenile hormone acid methyl transferease in *Helicoverpa armigera*. The staking of genes increased the efficacy of the Bt cotton against the Bt resistant insects (Ni et al. 2017). *Cry9C* gene and *Cry 2A* or *Cry 1Ac* were pyramided (staked) in Bt cotton to increase the efficacy against the two lepidopetran insects i.e. *Spodoptera litura* and *Heliothis armigera* (Li et al. 2014).

Gene pyramiding strategy (incorporation of diversified sources of resistance in a single genotype to reduce yield losses) has been adapted to kill insects due to host ability to produce various types of toxins which may delay the buildup of pest resistance (Brévault et al. 2013). Initial selection exposure over *Cry1Ac* increased the survival of *Helicoverpa*over two-toxin cotton (Brévault et al. 2013). Some other events of transgenes are under trial or in developmental process with fascinating results (Table 2.5). However, biotechnological products are put under a high watch list and a heavy load of formalities which slows down the research from laboratories to the commercialization success.

2.9 Improvement of Cotton Against Abiotic Stresses

Cotton yield is threatened by various abiotic and biotic stresses on lint yield. Heat and drought stress are the major threats for future cotton production due to rapid increase in the CO_2 which may increase the day and night temperatures by 1–5 °C (Singh et al. 2007). Higher temperatures reduce the boll and flower retention of the plant and cause abscission of 40% of the bolls (Singh et al. 2007). Moreover, heat stress also has a repressing effect over boll size, number of seeds per boll, and oil and fiber quality traits (Pettigrew 2008). High temperature also ameliorate the evapotranspiration losses, which increases the water requirements of the crop.

Breeders have made significant efforts to develop heat or drought tolerant breeding material (Khan et al. 2008; Ullah et al. 2008; Ur Rahman et al. 2004). The efforts of plant breeders are generally aimed at improving seed cotton yield under a targeted environment. However, seed cotton yield per se as a selection criteria is complicated due to dependence over wide range of yield components in non-stress conditions and is also dependent on the plant resistance under stress conditions. For instance, yield under non-stress condition is the product of higher boll number, size of boll and number of fruiting points, while under high temperature, yield is the product of gametophytic fertility, canopy architecture (foliage position, hairiness), delayed leaf senescence, photosynthesis efficiency, lower respiration rate and harvest index (Jha et al. 2014; Kakani et al. 2005). A second approach is to screen the

elite and wild germplasm and target the physiological or morphological traits which may be introgressed within advanced breeding lines. QTL mapping of the traits related to abiotic stress could further help to speed up the introgression and to decrease the linkage drags in the elite breeding material. Various transgenes have been identified which may further help to enhance the cotton crop tolerance to various abiotic stresses (Table 2.6). These transgenesare not negatively associated with yield, thus transformation dos not induce any yield drags.

2.10 Improvement of Cotton Against Biotic Stresses

Cotton plant biotic stress resistance is conventionally dependent on several morphological traits such as frego bract, nectriless, gossypol glands, red canopy color, leaf trichome, glabrous leaf, okra leaf shape and small leaf area. These morphological traits are linked tothe insect defense umbrella. However, their utilization in practical plant breeding to create insect resistant cotton is limited due to their effect on plant morphology and yield. Plant biotechnology has been used to introduce transgenes against various abiotic factors. Bt transgenic cotton containing *Cry*genes having resistance to bollworms has gained popularity and replaced the conventional cotton cultivars in many parts of the world. Research is in progress to introduce transgenic cotton for disease and sucking pest resistances. Hen egg white lysozyme, GAFP4, *FreB* gene (VDAG-06616) has been characterized as resistant to *Verticilium* wilt upon introgression in cotton (Table 2.5). The genes *Galanthus nivalis agglutinin* (GNA) and *Amaranthus caudatus agglutinin* (ACA) were found resistant against aphid infestations (Yang et al. 2017).

2.11 New Emerging Technologies

Genome editing techniques such as CRISPR/Cas (clustered regularly interspaced short palindromic repeats: associated protein) is one of the emerging technologies to knock down undesirable genes at a specific site. This technique is used to edit the genome through nuclease guided by the RNA to target a specific site in the genome provided that target site has a known sequence. CRISPR were found to be present in the bacteria which was used to inactivate viral invasions (Aqeel and Raza 2017). The CRISPR sequences are activated after the invasion by a virus utilizing its associated protein (Cas9). As a result of activation of CRISPR, it deactivates the viral genome and keeps a small part of the viral genome in its own genome as a spacer sequence (Aqeel and Raza 2017). The guider RNA and spacer sequence in the edited CRISPR/Cas9 system is expected to be widely utilized in the modification of the cotton genome. However, the CRISPR/Cas9 genome modification technique requires a protospacer adjacent motif. Cas9 proteins induce double-stranded breaks at the target site which can be NHEJ (non-homologous end-joining) or HDR

(homologous directed repair) by causing indels (insertion and deletions) in the genome. This technique has been attempted to induce resistance against cotton leaf curl virus and *Verticilium* wilt in cotton as a replacement for RNAi technology (Iqbal et al. 2016). Optimization of the CRISPR/Cas9 technique in cotton is in process (Long et al. 2018). High CRISPR/Cas activity was observed in targeted genomic sites such as*GhMYB25*-likeA and *GhMYB25*-likeD. The targeted sites showed 50% editing through sgRNA of the transgenic allotetraploid cotton plants (Li et al. 2017a, b). A mutation efficiency of 47.6–81.8% in two genes i.e. *Cloroplastos alterados 1 (GhCLA1)* and *vacuolar H^+-pyrophosphatase (GhVP)* was induced through two guide RNAs (Chen et al. 2017). The promoter GHU6 was successfully cloned and provided 6–7 times more expression for sgRNA than the AtU6-29 promoter (Long et al. 2018). Multi-site genome editing was done through two sgRNA in a single vector which targeted two genes *Discosoma red fluorescent protein2*(*DsRed2*) and *GhCLA1* in cotton. CRISPR/Cas9 successfully targeted both loci and a transformation efficiency of 66.7–100% was observed (Wang et al. 2018). The albino expression of endogenous gene *GhCLA1* was observed in 75% of the transgenic plants. It is difficult to target both loci in genome A and D controlling single traits due to the polyploidal nature of cultivated cotton (Janga et al. 2017). The application of CRISP/Cas9 is severely handicapped due to the absence of efficient genetic transformation (Long et al. 2018). The application of CRISPR/Cas9 in cotton genome editing is detailed in a separate chapter in this book.

Genome-wide association (GWAS) mapping is another emerging alternative method for detecting QTL and dissection of quantitative traits in cotton, such as plant canopy architecture (Su et al. 2018), agronomic traits i.e. yield and its components (Gapare et al. 2017; Huang et al. 2017), fiber quality traits (Gapare et al. 2017) and diseases (Li et al. 2017a, b). GWAS has several advantages over the biparental linkage mapping such as high density mapping covering the whole genome, robust, time efficient, cost effective and there was no need to create mapping populations for QTL mapping (Huang et al. 2017).

2.12 Conclusions and Prospects

Cotton is an important world fiber and oilseed crop. It belongs to the genus *Gossypium* which has four cultivated species, two diploid and two tetraploid species. *Gossypium hirsutum* is a widely cultivated species for the spinnable medium length fiber in the textile industry and occupies 90% of the world cotton primarily located in the Americas, Asia and Africa. Traditional plant breeding (based on basic principles and selection methods i.e. pedigree, bulk and recurrent selection for cotton) led to a substantial increase in fiber yield and quality. However, cotton species especially, *G. hirsutum* and *G. barbadense*, are affected by insects and diseases which increase production cost. Therefore, Bollgard cotton was introduced to reduce yield losses due to boll worm complex infestation. Introduction of Bt cotton has provided a novel method for insect resistance in cotton and reduced yield losses

from lepidopteron insects. On the other hand, viral diseases continue to challenge sustainable cotton production in various parts of the world and introgression of resistance against diseases has proved difficult through traditional plant breeding due to rapid emergence of new pathogens. It was hoped that antisense and RNAi technology will provide solutions to combat cotton disease; however, they have failed to develop disease-resistant cotton. CRISPR/Cas is a new emerging technology that may be applied to correct or modify the genome region associated with susceptibility to indeterminate growth habit, boll losses, diseases and insects. Continuous emergence of new viral strains could continue the battle between pathogen and breeder. Climate changes due to environmental pollution and the accumulation of greenhouse gases may threaten key cotton production regions such as the Indo-Pak subcontinent. It has been observed that heat, salinity, mineral deficiencies and drought stress pose serious threats to cotton production. Breeding strategies such as incorporation of resistance genes for the development of climate resilient cotton crops are important to combat future climate changes.

Appendices

Appendix I: Research Institutes Relevant to Cotton Breeding and Biotechnology

Institution	Specialization and research activities	Contact information and website
Central cotton research institute, Multan, Pakistan	Varietal development, cotton yield management	http://www.ccri.gov.pk/
Cotton research Institute, Nanjing University China	Cotton genomics and genetics	http://mascotton.njau.edu.cn/
Central Institute for Cotton Research, India	Cotton varietal development, Integrated Pest Management	http://www.cicr.org.in/
Institute of Cotton Research, China	Genetic breeding, germplasm resources, farming cultivation, plant protection, molecular biology	http://www.caas.cn/en
Cotton Research Institute, Zimbabwe	Cotton varietal development, Integrated Pest Management	https://www.gfar.net/organizations/cotton-research-institute-1
Australian Cotton Research Institute, Narrabari, USA	Biopesticide, Insecticide Resistance, Cotton Nutrition and Irrigation, Cotton Pathology	https://www.dpi.nsw.gov.au/about-us/research-development/centres/narrabri
ACSA International Cotton Institute, USA	Basic education on all aspect of cotton	https://bf.memphis.edu/cotton/index.php/main/instructions
Cotton Research Institute, Egypt	Cotton breeding research, production and technology transfer	http://www.arc.sci.eg/InstsLabs/Default.aspx?OrgID=2

(continued)

Institution	Specialization and research activities	Contact information and website
International Cotton Advisory Committee, USA	Cotton research and development policies formation	Icac.org
Cirad Agriculture Research Institute, France	Cotton germplasm, data bases	https://www.cirad.fr/en
Uzbekt Research Institute, Uzbekistan	Cotton germplasm resources	https://en.yellowpages.uz/company/uzbek-scientific-research-institute-of-cotton
Nazili Cotton Research Institute, Turkey	Cotton research and development	administrator@nazilli.tagem.gov.tr

Appendix II: Cotton Genetic Resources

Cultivar	Important traits	Cultivation location
NexGen 5711 B3XF	Bollgard3 XtendFlex Cotton Technology, Smooth leaf, bacterial blight tolerance, and fiber	AMERICOT, USA
ST 5517GLTP	Three-gene Bt technology of TwinLink Plus, bacterial blight resistance, and good storm tolerance	Stoneville, USA
FM 1953GLTP	An early/medium maturity glyphosate tolerance LibertyLink TwinLink Plus variety, bacterial blight resistance, bollworm resistance and fall armyworm	Bayer, USA
PHY 300W3FE	Early maturing, moderate water stress resistant, superior fiber quality	Phytogen, USA
IUB2013, FH142, MNH886	High yield potential, Cry1A genes, heat resistance, increased boll retention under heat stress	South Punjab, Pakistan
Sicot 71 4B3F	High yield, fiber quality, wide regional adaptability, three transgenic trait Monsanto's Bollgard II and RRFlex transgenic traits, providing both pest resistance and herbicide tolerance	CSIRO, Australia
GIZA86	High yield extra-long cotton having longest and thinnest fiber	Egypt
ICS105	High yield and adaptable varieties	Maharashtra, India
FM 1944GLB2	Broadly adapted to all cotton-growing region, Liberty® and glyphosate herbicide tolerant, Lepidopteran resistant	USA
Gloria	High yield potential	Turkey

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Chapter 3
CRISPR/Cas9: A New Genome Editing Tool to Accelerate Cotton (*Gossypium* spp.) Breeding

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Abstract Cotton has a tremendous economic value worldwide due to its high-quality fiber, edible oil and protein contents. However, the intensifying scenario of human population expansion and global environmental changes demand a proportionate increase in cotton production. In the past, several successful attempts have been made by introgression of many quality- and yield-related traits into elite cotton cultivars through conventional breeding. However, those measures are time consuming due to the reliance on introgression of naturally-existing genetic variation through extensive backcrossing. Nonetheless, plant breeding can be accelerated through modern genome editing (GE) tools. Various GE techniques including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced palindromic repeats and CRISPR-associated proteins systems (CRISPR/Cas)-based approaches have been successfully employed for various crop plants. Among them, CRISPR/Cas-based approaches hold great GE potential due to their simplicity, competency and versatility. In cotton, this system can regulate the gene expression associated with quality traits, to circumscribe phytopathogens and/or to stack molecular traits at a desired locus. In gene stacking through site-specific endonucleases, the desired genes can be introduced in close proximity to a specific locus in the cotton genome with a low risk of segregation. However, such executions are tedious to achieve through classical breeding techniques. Moreover, through the CRISPR/Cas-based approaches, transgene-free cotton plants can easily be produced by selfing or backcrossing to meet the current

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genetically modified organisms (GMO) guidelines. In this chapter, we address the potential application of CRISPR/Cas-based approaches in available whole cotton genomes to sustain cotton productivity, and achieve genetic improvement, pathogen resistance and agronomic traits. Future prospects of GE applications in cotton breeding are also addressed.

Keywords Cotton · CRISPR/Cas · Genome editing · Plant breeding · Targeted mutations

3.1 Introduction

Cotton (*Gossypium hirsutum* L.) is a major fiber crop and the sixth largest source of vegetable oil in the world. The genus *Gossypium* encompasses more than 52 species, but only 4 have been domesticated over a period of 1–2 million years of polyploidization (Wendel 1989). The widely cultivated New World (NW) allotetraploid (2n = 52), *G. hirsutum* and *G. barbadense*, consist of a pair of A and D sub-genomes; whereas, the Old World (OW) *G. arboreum* and *G. herbaceum* have diploid (2n = 26) A or D genomes (Chen et al. 2015). The primary source of more than 90% of fiber output is the most widely cultivated *G. hirsutum*.

The post-genomic era in agriculture faces a major challenge in the improvement of crop cultivars for higher productivity and protection against insects, pests and diseases. A continuous depletion of plant genetic resources coupled with global environmental calamities favors various biotic and abiotic stresses related to crop plants (Arzani and Ashraf 2016). The genetic manipulations of native genes to develop resistance in crop plants requires an efficient tool. Reverse genetics has been widely used to elucidate the functions and regulation of various genes and the metabolic pathways they control in crop plants (Canas and Beltran 2018). However, these approaches have limited application in plant breeding due to various shortcomings including instability and imperfect silencing, which have produced blurry interpretation of the phenotype. Unavailability of resistant germplasm against major biotic and abiotic stresses is the main reason for low productivity in different parts of the world. In this scenario, conventional plant breeding has been sharing its role to control insect, pests and diseases to sustain food security. However, the use of modern biotechnological tools has been integrated in different breeding programs to produce better crop varieties enabling plant breeders to cross unrelated species to introduce foreign genes into crop plants (Zhang 2015).

Genetic variation is an indispensable part of resistance breeding to enhance host plant resistance against various stresses. Random mutations with various physical and chemical mutagens and irradiations have long been employed to create genetic variation in crop plants (Laskar and Khan 2017). However, such mutations are random and generate a huge background mutation load on crop plants under study. In the modern era, different genome editing (GE) techniques are in use to create genetic variation in crop plants; however, clustered regular interspaced palindromic repeats and CRISPR-associated proteins system (CRISPR/Cas) is a versatile, robust

and more suitable technique than its other counterparts. Various CRISPR-based GE systems are highly promising in crop genetic improvement because of their precision, specificity, multiplexing ability, minimized off-target activity, robustness and simplicity (Ding et al. 2016).

CRISPR is an RNA-based adaptive immune system of archaea and bacteria, which confront the invaded DNA phages and viruses. CRISPR/Cas system comprised of CRISPR-associated Cas genes, which encode RNA-based single-guide RNA (sgRNA) endonucleases, palindromic repeat sequences and non-coding short variable protospacers RNAs. The protospacers are created by inserting the short variable part of the invading nucleic acids that help the invaded cells in component recognition and to chop the foreign nucleic acids. Such CRISPR-based defense approaches have three fundamental steps: adaptation, expression and interference (Fig. 3.1). In adaptation, the spacer sequences are acquired and are chronologically arranged within the CRISPR-array. The expression phase involves the expression of *Cas* genes and precursor CRISPR-RNA to generate mature crRNA. The integration of crRNA and Cas proteins results in the cleavage of the foreign nucleic acids during the interference phase. The protospacer adjacent motif (PAM) is another important component of the CRISPR-system; PAM is the sequence which is recognized by the Cas protein to perform its functions and it is located either upstream or downstream of each protospacer sequence. It is not actually a part of the CRISPR locus, but represents a conserved sequence of the invading nucleic acids (Demirci et al. 2018). Depending upon their origin, various Cas orthologues recognize different sequences and vary for their requirement of PAM sequences. The Cas protein is guided to digest the target nucleic acid and results in a double-stranded break (DSB) ~3 bp upstream to the PAM sequence. This DSB is repaired by either non-homologous end joining (NHEJ) or homologous recombination (HR).

3.2 Different Versions of CRISPR/Cas Systems and Their Applications

Ease of use and versatility of the CRISPR system has made it a rapidly- expanding programmable GE tool. The earlier, original and simple form of site-specific GE tool is CRISPR/Cas9 system. The Cas9 orthologue of CRISPR/Cas-system was obtained from *Staphylococcus aureus* (SaCas9) (Steinert et al. 2015). The CRISPR/Cas9-based system has been extensively used in plants to improve qualitative and quantitative traits and to confer resistance against various phytopathogens (Li et al. 2016; Nekrasov et al. 2017; Steinert et al. 2015). In order to exploit the unique binding ability of Cas9 to any complementary sequence, Cas9 was engineered to abolish its endonuclease activity and resulted into catalytically inactive protein, called dead Cas9 or *dCas9*. This *dCas9* was successfully implicated to regulate the genes. Later, a transcriptional activator domain VP64 was fused to *dCas9* and this system was applied to promote transcriptional activation in plants (Perez-Pinera et al. 2013).

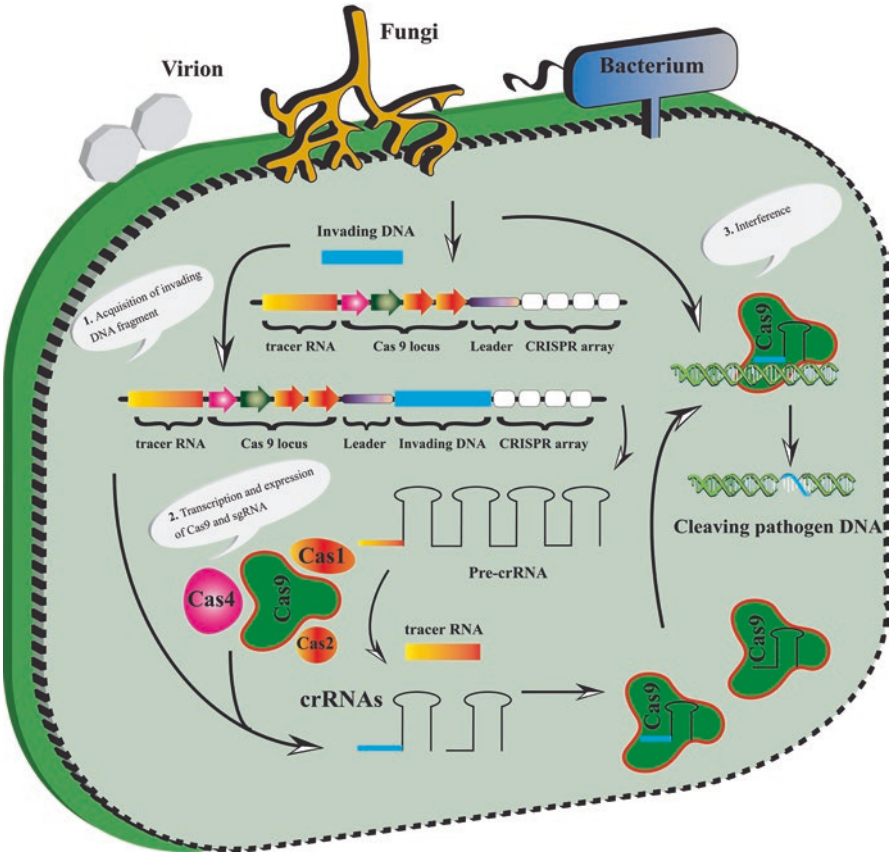

Fig. 3.1 A generalized model for inducing CRISPR/Cas-based resistance in cotton. Invading nucleic acid is identified, recruited and cleaved by Cas protein. The whole model is based on three fundamental steps of acquisition, expression and interference. During the first step, the CRISPR-locus integrates and duplicates the invaded DNA, which triggers the expression of pre-CRISPR RNA machinery in the invaded cell during the expression step. The third and final step involves cleavage of the invaded nucleic acid in coordination with the CRISPR RNA machinery

Recently, a second CRISPR-based system, CRISPR/Cas12a (also referred as CRISPR/Cpf1), was introduced. This system belongs to the class II CRISPR systems, which differs from CRISPR/Cas9. In contrast to Cas9, Cpf1 requires a small crRNA to mediate its activity; it recognizes T-rich PAM and induces staggered double-standard breaks (DSBs) distal from the PAM (Lei et al. 2017). The successful application of Cas12a-mediated mutagenesis in rice and tobacco induced biallelic heritable mutation, proving its potential for mutagenesis in plants (Xu et al. 2017a, b). In rice, by using two different Cas12a versions i.e. FnCas12a and LbCas12a, targeted genomic insertions (via HR) were achieved with higher mutation rates as compared to SpCas9-based experiments (Begemann et al. 2017). Cas12a is an excellent choice for gene regulation as it can be fused with a variety of

activators (such as VP16, p65, and Rta) to achieve excellent activation. Thus, CRISPR/Cpf1 system can provide an extremely important tool for cotton GE to induce alterations in the genome via HR.

Recently, a useful addition of another Cas version, Cas13 (previously known as C2C2 and C2C6), have been made into the CRISPR family. Cas13 is a class II effector protein that exclusively targets RNA molecules by its two higher eukaryotes and prokaryotes nucleotide-binding (HEPN) domains (Abudayyeh et al. 2017). An important feature of this system is promiscuous RNA cleavage upon activation. Furthermore, similar to Cas12a, Cas13 proteins have the autonomous ability to process pre-crRNA without a tracrRNA. Such abilities of Cas13 have made it a best choice to target a wide range of RNA manipulations. Two of the most active orthologues are Cas13a from *Leptotrichia wadei* and Cas13b from *Prevotella* spp. (Abudayyeh et al. 2017; Cox et al. 2017). The superiority of RNA knockdown has been established in terms of specificity as compared to RNA interference (RNAi) technique. In rice plant protoplasts, more than 50% RNA knockdown at only 48 h after transformation has been achieved (Abudayyeh et al. 2017). Importantly, Cas13 has the matchless ability to target specific RNA splicing isoforms at the post-transcriptional level, while it indifferently affects all isoforms at the transcriptional level. This ability of Cas13 makes it an excellent choice to target RNA genome of various pathogens without affecting wild-type transcripts in crop plants (Mahas et al. 2018).

3.3 Functional Genomics and Limitations in Cotton Breeding

Up to now, a number of tools and resources have been applied to investigate large-scale functional genomic studies in cotton. During the past couple of decades, efforts to develop transgenic cotton cultivars have progressed impressively, to confer genetic resistance to insect, pests and various diseases (Yu et al. 2016). However, advancements to improve the overall physiology, plant architecture, early maturity, fiber traits and yield parameters are progressing at a slow pace. The D-genome of diploid cotton species *G. raimondii* was completely sequenced in 2012 (Wang et al. 2012), followed by 1694 Mb A-genome of *G. arboreum* (Li et al. 2014). Soon after the sequencing of A and D-progenitor species, the complete genomes of allotetraploid *G. hirsutum* and Sea Island cotton species (*G. barbadense*) were also sequenced (Li et al. 2015). After advancements in NGS technologies, different reference draft genomes are available for diploid and tetraploid *Gossypium* species. However, different erroneous interpretations have been observed in those genome assemblies regarding chromosomal lengths and the gene annotations (Ashraf et al. 2018). Such errors in genome assemblies need to be dealt using bioinformatics, transcriptomics and epigenomics tools to precisely address mutations, gene annotations and differential protein expression.

After whole-genome cotton sequencing, the major challenge could be the functional annotations and empirical investigations of the genome biology. Unlike other crop and model plants, a few genetic linkage maps are available for cotton (Li et al. 2016). Such genetic maps may provide a base for genetic marker development, gene mapping and effective characterization of different genes. During the last decade, marker-assisted selection has helped to identify ~2000 quantitative trait loci (QTLs) in the interspecific populations of *G. hirsutum* and *G. barbadense* (Said et al. 2015). However, those QTLs represented large genomic regions, and possibly, may miss several informative genes during functional genomic analysis based upon the QTL-studies. It is thus a prerequisite for cotton functional genomics to have a fine map of the crucial genomic regions along the cotton genome with an effectively large number of markers. In some recent studies, fine mapping of some crucial genes have been reported for the glandular gene (Cheng et al. 2016), leaf architecture (Andres et al. 2014) and some fiber-quality related traits (Xu et al. 2017a, b). The presence of copy-number variations (CNVs) in a plant genome may alter the genetic structure, gene dosage, gene regulations and ultimately may affect important traits. Fang et al. (2017) reported 989 CNV-affected genes in cotton, associated with the cell wall, plant morphology and translational regulation. Whole-transcriptome profiling is another important tool, which can be helpful for functional genomics in cotton. The transcriptome profiling for leaf senescence, fiber development and biotic and abiotic stresses (Zhang et al. 2016a, b) have been also studied. However, the comparative analysis and RNA-seq tools could not provide complete insight into the gene functions. Thus, functional genomic studies on a larger scale can efficiently assign gene functions through empirical corroborations.

Epigenetic modifications such as DNA-methylation may affect gene expression in plants and have a role in morphological diversity. In cotton, DNA-methylation has been reported to be affected by seasonal variations and fiber development (Osabe et al. 2014). Song et al. (2017) reported ~500 genes, which were epigenetically modified between wild and cultivated cotton cultivars.

The development of omics data in cotton functional genomics necessitates the development of a comprehensive database so that all genomic information may readily be available to cotton breeders. Many such databases have been developed (Table 3.1) and among them CottonGen offers a curated platform, which is easily accessible and has a user-friendly interface. Likewise, CottonFGD is another quick and readily available database to access whole genome sequences, functional annotations and transcriptomic data of most of the whole genomes of cotton. The ccNET database has been designed for coexpression networks and to display ~1155 *G. arboreum* and ~1884 *G. hirsutum* functional modules, respectively.

Innovations in omics platforms and the availability of diploid and tetraploid cotton whole genome sequences have significantly facilitated efforts to find candidate genes for many commercially-valuable traits. Nevertheless, understanding of the molecular basis of important traits (e.g. lint and fiber-quality traits) is very limited due to the complex nature of polyploidy (Chen et al. 2017). One major feature of polyploidy is an enormous variation in gene expression and functionality. For example, genome duplication in polyploid cotton may also duplicate the genes. Thus,

Table 3.1 Major databases useful for functional genomic studies in cotton (*Gossypium* spp.)

Database	Web address	Specialty
CottonGen	https://www.cottongen.org	Genomic, genetic and breeding resources
CottonFGD	https://cottonfgd.org	Cotton functional genomics
CGRD	http://cgrd.hzau.edu.cn/index.php	Cotton genome resource
CottonDB	http://www.cottondb.org	Cotton genome
CGP	http://cgp.genomics.org.cn/page/species/	Cotton genome project
GraP	http://structuralbiology.cau.edu.cn/GraP/	*G. raimondii* functional genomics platform
CEGC	http://cottonevolution.info/	Comparative evolutionary genomics
JGI	http://jgi.doe.gov	Join genome institute
ccNET	http://structuralbiology.cau.edu.cn/gossypium/	Coexpression networks with function modules

either gene copies may be retained for a balance or one copy may be lost or silenced to create new functions (Wendel 2015). In allopolyploid cotton species, some of the homologous gene pairs (duplicated genes) are unequally expressed, possibly due to asymmetrical evolution (Zhang et al. 2015). These homologous genes may be expressed variably across species, tissues or even cells and represent a basic feature of allopolyploidy. Therefore, the genomic and transcriptomic information, together with modern biotechnological tools such as genome editing, can reshape future breeding programs to produce superior cotton cultivars.

Crop performance always depends on genotype, the environmental growing conditions and the interaction between genotype and environment. Genotypic expression of a cultivar in various environmental conditions is a main challenge faced by plant breeders as stability of the genotypic potential can be affected under diverse environments. The narrow genetic base of upland cotton germplasm being used in breeding programs is the main challenge to developing new cotton cultivars (Zhang et al. 2015).

Cotton stability and adaptability have been studied and diverse levels of variations in different morphological and yield related traits have been observed. In this regard, AMMI analysis (additive main effects and multiplicative interactions) is useful in supporting breeding program decisions such as specific adaptations to target (tolerance to disease, heat, drought and cold) and selection of environments or test-site locations (Maleia et al. 2017; Riaz et al. 2013). Heritability and the genetic potential of different cultivars for different morpho-yield traits are earnestly needed for the selection of parental breeding lines. Yield is a highly complex character and is directly influenced by different morphological and yield-contributing traits (Khan et al. 2009). Therefore, a thorough knowledge of the nature and genetic potential of different genotypes, inheritance pattern of different traits and the extent of relationship and correlation of yield with various agronomic characters is crucial for breeders to tackle the breeding challenges and to increase yield successfully (Thiyagu et al. 2010; Tonk et al. 2018)

3.4 Difference Between Genome Editing and Classical Breeding of Cotton

Conventional cotton breeding involves combining desired traits from elite cotton or wild types of *Gossypium* (Zhang 2015). There are certain drawbacks in using the conventional approach in cotton breeding such as the intraspecific crossing barrier between *G. barbadense* and *G. hirsutum*. Although these species have no barrier in terms of reproduction within the parental lines, and a hybrid F1 generation can be propagated, such a cross encounters hybrid breakdown in the F2 and subsequent generations (Zhang 2015).

Inadequate achievement in pioneering desired genes from diploid species to *Gossypium hirsutum* is because of obscurity in the F1 hybrid production, chromosome number resurgence due to recurring backcrossing and chromosome doubling. Various breeding approaches coupled with the classical genetics tools could help cotton breeders to improve cotton fiber, seed quality and enhance yield. In addition, coupling of these technologies can lead to early development of tolerant or resistant cultivars and better adaptability of cotton crops to local environments. CRISPR/Cas GE technology could be a promising tool in addressing these issues of classical breeding, which are still unresolved or need improvement to expedite breeding programs. There are many candidate genes, which need investigation such as genes involved in biosynthesis of cotton gossypol, development of cotton fibers with respect to its negative regulator and resistance against *Verticillium* wilt. CRISPR/Cas technology holds promise in investigating these candidate genes and can advance research based on functional genomics, and ameliorate the prospective for the molecular breeding of cotton (Chen et al. 2017; Li et al. 2017a, b), which conventional breeding approaches may not achieve in a short time span.

However, the CRISPR/Cas system has been successful in achieving genetically-edited mutants for gene function study in cotton as well as in allotetraploids, with high specificity and high efficiency, both in terms of protoplast level and in the generation of stable cotton transgenics (Chen et al. 2017).

Compared to conventional mutation breeding, CRISPR/Cas technology can create specific mutations at the desired loci and some number of off-targets loci can be envisaged. Additionally, in contrast to the conventional mutation breeding program, CRISPR/Cas technology has little unintended load of mutation in the gene-edited crops. With the help of the CRISPR/Cas method, just as a plant-breeding approach, backcrossing is always possible in the case of an off-target mutation. In conventional breeding as well as mutation breeding, nevertheless, unknown and unintended mutations arise similarly and can be eliminated with several crosses in successive cycles. Homology recombination facilitated CRISPR/Cas can lead to the introduction of traits for cotton improvement. The technology carries an advantage over the use of transgene insertion methods for the development of transgenic products. Thus, CRISPR/Cas is a promising plant-breeding tool with huge potential that can result in precision cotton breeding by working with native traits available within the cotton crop.

3.5 Layout Plan of CRISPR/Cas-Based Genome Editing

The application of CRISPR/Cas-based GE has already been applied in a few studies to genetically engineer the cotton genome (Table 3.2). However, successful execution of the CRISPR/Cas system in cotton GE necessarily requires the generalized mechanism discussed in the preceding section (Fig. 3.2).

3.5.1 Data Mining and Single Guide RNA (sgRNA) Designing for Target Sequence

For successful execution of cotton GE, selection of the target region of the genome is of primary importance. While selecting the target region in the cotton genome, major challenges could possibly be off-targets, genome polymorphism, polyploidy, transposons, single nucleotide polymorphisms (SNPs) and introns (Iqbal et al. 2016; Klein et al. 2018; Zhang et al. 2018). Although a very few studies are available to gauge off-targets in plants, these problems can be overcome in three steps by using different computer-based programs: (i) target genome site selection for the design of sgRNA, (ii) predicting off-target activities of the designed sgRNA and (iii) evaluation of on- and off-target cleavage rates (Chen et al. 2017; Lei et al. 2014). Several web-based tools and bioinformatics programs are now available to ascertain the cotton GE (e.g. ATUM, Alt-RTM CRISPR/Cas9 System, Cas-OFFinder, CCTop, CHOPCHOP, CRISPRpred, CRISPR-P, CROP-IT, GT-Scan, MIT CRISPR design and sgRNA Designer).

Table 3.2 List of genome editing (GE) studies and their potential targets in cotton (*Gossypium hirsutum*)

Target genes	Description of genes	sgRNA promotor	Binary vector	Reference
GhCLA1	*Cloroplastos alterados 1* gene for chloroplast development	U6	pRGEB32	Wang et al. (2018a, b)
GhMYB25-like A, GhMYB25-like D	Transcription factors for fiber and trichome development	AtU6-26	pCBC through gateway cloning	Li et al. (2017a, b)
GhCLA1, GhVP	GhCLA1 for chloroplast development and vacuolar H + -pyrophosphatase (GhVP)	AtU6	pP1C.4	Chen et al. (2017)
GhPDS, GhCLA1, and GhEF1	Phytoene desaturase (PDS), GhCLA1 commence chloroplast development, elongation factor-1 protein catalyze the binding of aminoacyl-tRNA	AtU6-29 and/or AtU3b	pYLCRIPSR/Cas9	Gao et al. (2017)
GhARG	Arginase gene	NtU6	pBIGFP/Cas9	Wang et al. (2017a, b)

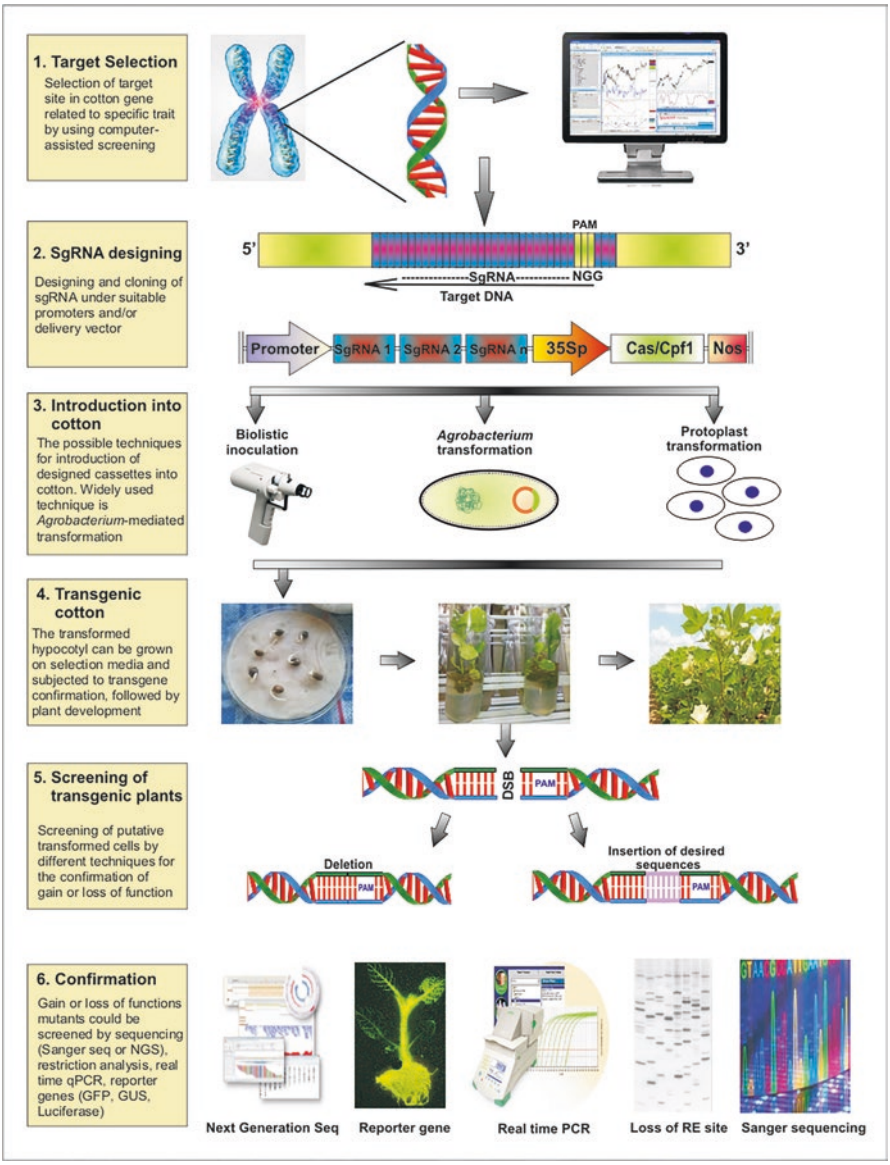

Fig. 3.2 Schematic execution of CRISPR/Cas genome editing system in cotton. Online web-based tools are used to select and design ~20 nucleotides (nt) sgRNAs complementary to the targeted region in the cotton genome. The specific sgRNAs and Cas protein are expressed either from a single binary vector as one cassette or expressed separately from different binary plasmids. The assembled cassettes are then transfected into the plant genome through a suitable transfection technique such as *Agrobacterium*-based inoculations, particle bombardment using gene guns and/or protoplast transformation. After the successful transfection, the mutants are evaluated for the targeted mutations and are analyzed further for the presence of any off-target activity through the expression of reporter genes, nuclease enzymes, gel electrophoresis and/or next generation sequencing approaches. The successfully screened mutants are then employed directly for downstream applications

3.5.2 Choice of CRISPR/Cas System

Selection of an appropriate CRISPR/Cas system, sgRNA promoter, choice of binary vector for sgRNA delivery and mode of transformation are some of the prerequisites to successfully engineer the cotton genome. Expression of bacterial Cas into cotton requires the codon optimized Cas and a suitable plant promoter. Different constitutive and/or inducible promoters such as CaMV 35 s, CMV, EF1A, LTR and UBO have been successfully utilized to drive Cas protein in plants. Beside these, many plant RNAIII-based promoters, such as U3p and U6p, have also been used successfully (Belhaj et al. 2013).

Selection of an efficient Cas protein is another important requirement. Based on the experiment need, a codon optimized Cas protein has efficient GE ability with improved efficacy. Besides GE, gene silencing [CRISPR interference (CRISPRi)] and gene activation [CRISPR activation (CRISPRa)] has also been achieved by using the codon optimized dCas9 version lacking nuclease ability (Qi et al. 2013). Recently, a new endonuclease Cpf1 has been employed to cleave the target DNA via a staggered DNA double-stranded break, producing cohesive ends with 4–5 overhangs of nucleotides with much higher efficiency and specificity, as compared to its predecessor Cas9. Availability of different CRISPR-based GE tools will broaden the applications of cotton genome engineering.

3.5.3 Delivering CRISPR/Cas Cassettes into Cotton Genome

After evaluating the designed sgRNAs and the selection of a CRISPR system, the next step is to assemble the whole cassette (including sgRNA, CRISPR, Cas/Cpf1) into a suitable plant-based binary vector. Delivery of the resultant recombinant vector into the cotton genome is a very crucial step as the success of entire system is dependent on the choice of a successful transformation system. Several techniques are being used to execute stable plant transformation such as polyethylene glycol (PEG)-mediated transformation, protoplast transformation, biolistic inoculations and transit peptides; besides these techniques, successful transformation can also be achieved by using various plant virus-based vectors (Butler et al. 2016). Nonetheless, the most tested way of stable genetic transformation in cotton is *Agrobacterium*-mediated transformation, and this method has been opted successfully in CRISPR-based cotton genome engineering (Chen et al. 2017; Li et al. 2017a, b; Wang et al. 2017a, b). In *Agrobacterium*-mediated stable transformation, cotton seeds are surface sterilized followed by germination on wet filter papers at 28 °C in darkness. After a couple of days, when the length of the seed root reaches 1–2 cm, the root apex is injured with a sterile syringe and are suspended in *Agrobacterium* culture. Afterward, the infected seedlings are cultivated for 48 h in darkness and 48 h in light and then transferred to the greenhouse. Transgenic plants are selected by growing them on suitable antibiotics.

3.5.4 Manipulation of Targeted Mutagenesis Through In Vitro Regeneration and Screening of Transgenic Plants

Stable transformation of cotton can be achieved by agro-inoculating the hypocotyl or cotyledonary petiole. After successful transfection, the inoculated material can be grown on the selection media and resulting transgenic plantlets subjected to confirmation either by Southern blotting or by sequencing the PCR amplicons (Fig. 3.2). However, the phenotypic and genotypic screening requires detection and confirmation of the targeted mutations, which may include, but are not limited to, resistance to insects, pests and diseases, tolerance to abiotic stresses, and improvement of agronomic features such as yield and quality traits (Hua et al. 2017).

3.5.5 Detection and Confirmation of Successful Genome Modification

The verification of a mutation induced by the CRISPR system and efficiency of GE is a crucial step for downstream processing of the transformants. An easy way to detect the CRISPR/Cas-induced putative mutation in the target plants is to amplify the target gene by using specific primers. The achieved PCR amplicons then can be purified and cloned into a vector. To map all the possible mutations, more than ten clonal amplicons of each transgenic plants should be sequenced by the Sanger method. (Chen et al. 2017; Li et al. 2017a, b). Sanger sequencing, developed by Fredrick Sanger et al. (1977), is based on the selective incorporation of dideoxynucleotides (ddNTPS) by DNA polymerase during in vitro DNA replication. This is quite simple, inexpensive and a convenient approach to determine simple or complicated chimeric mutations. Another approach to verify the GE event is the use of a reporter gene, like GUS, fluorescent proteins (GFP, YFP, RFP) and luciferase, where a reporter gene bearing a mutation could be corrected by the CRISPR/Cas system and vice versa (Feng et al. 2014). Use of T7 Endonuclease I is another promising way to evaluate and calculate the GE efficiency. In this method, PCR amplicons of the target region are annealed and digested with T7 Endonuclease I, only non-perfectly mismatched DNA fragments are digested, which are quantified through gel electrophoresis (Wang et al. 2017a, b). Alternatively, at the target loci, a restriction endonuclease site can be targeted (either corrected or introduced) during Cas9/sgRNA cleavage (Xie and Yang 2013). Polyacrylamide gel electrophoresis (PAGE) can also be used to confirm the GE events by single-stranded conformation polymorphism. In this technique, the single-stranded DNA (of the target gene) with mutation will show a different migration rate in the gel because of different DNA conformations (Zhang et al. 2016a, b). High throughput or next-generation sequencing of the whole genome is highly sensitive, robust and an efficacious method to detect the mutation in the target region and to evaluate any off-targets during a GE event (Feng et al. 2014).

3.6 Potential Applications of CRISPR/Cas in the Post-Genomic Era of Cotton Breeding

The availability of whole genomes of diploid and tetraploid cotton species has enabled scientists to assign gene functions to a few genes by comparative analysis with the known genes (Liu et al. 2018). These comparative analyses cannot provide an exact insight into the specific roles of the genes. Their precise roles can only be elucidated empirically and by reverse or forward genetics tools, which is the most difficult task in cotton breeding. To tackle this problem, GE can be a good solution to elucidate the functions of the genes, especially those which are involved in insect, pest and disease resistance, abiotic stresses, agronomic features, yield and quality traits. Among potential application of GE, the targeted interference (CRISPRi) or activation (CRISPRa) of a specific gene in upland cotton can provide precise role of the gene.

Cotton fibers are seed trichomes, which are made from long single integument cells and represent a good model to study plant growth. Although cotton fibers are biologically the most important trait in cotton development, understanding the cellular mechanisms regarding fiber initiation and elongation are still scanty. Genetic evidence has revealed that among 28 members of HD-ZIP IV subfamily the transcription factor GhHOX3 in the GL2 type domain is crucial for fiber elongation (Shan et al. 2014). According to Deng et al. (2016), phytoseterols and a balanced ratio between campestosterol:sistosterol plays a pivotal role in cotton fiber development. Several other studies also identify fiber-related genes such as *E6*, *GhExp1*, *GhSusA1*, *PIP2s*, and *GA20ox* (Bai et al. 2014). Moreover, many transcription factors in MYB, C2H2, bHLH, WRKY and HD-ZIP gene families are also differentially expressed during cotton fiber development.

The role of transposable elements (TEs) in cotton fiber development cannot be ruled out, apart from their role to diverge the genome sizes in cotton species. Cotton fiber development is thus a complex program and is a trait of primary importance. At this stage, GE can contribute to elucidate the mechanisms of fiber development in cotton. The gene knocking capability of CRISPR-based approaches can be utilized for the identification and functional characterization of certain genes in cotton. It has been successfully employed to knockdown *OsERF922* gene against rice blast and TMS5 to study temperature sensitivity in rice (Wang et al. 2016a, b).

A robust CRISPR/Cas9 system has been developed for site-specific mutagenesis in *G. hirsutum* by generating multiplexed sgRNAs with 66–100% GE efficiency (Wang et al. 2018a, b). Li et al. (2017a, b) also successfully targeted two identical genomic regions *GhMYB25-like A* and *GhMYB25-like D* in upland cotton with >98% mutation efficiency and no off-target activity. Similarly, Chen et al. (2017) obtained 47.6–81% transformation efficiency by adopting GE in upland cotton.

Gossypol biosynthesis pathways enable cotton plants to resist against insect and pests. The GaWRKY1 transcription factor has recently been shown to be involved in the gossypol biosynthesis pathway (Tian et al. 2016). Moreover, Wu et al. (2017) identified at least nine genes directly involved in gossypol biosynthesis. The

complete regulatory network for gossypol biosynthesis necessitates the CRISPR/Cas9 system to unravel the molecular basis of the whole pathway and to produce glandless cotton cultivars.

Cotton leaf curl disease (CLCuD) is a devastating threat caused by CLCuD-associated begomoviruses (genus *Begomovirus*, family *Geminiviridae*) to global cotton production (Sattar et al. 2013, 2017). CLCuD has been reported to be associated with at least five different begomovirus species and various DNA-satellites in cotton. Numerous conventional and nonconventional strategies have been utilized to circumvent this disease complex; however, none could achieve successful control. In this situation, a comprehensive CRISPR/Cas-based resistance strategy can be devised to control this important cotton disease (Iqbal et al. 2016).

3.7 Multiplexed Gene Stacking Using CRISPR/Cas9

Simultaneous modification of multiple genes and controlling a particular trait can be a useful method in functional genomics study and resistance against various pathogens. CRISPR system is a powerful tool, which can target member of multiple gene families in one go. Several successful examples are known where multiplex GE has been employed in different plant species such as rice, maize, *Arabidopsis*, tomato, tobacco and wheat (Li et al. 2017a, b; Wang et al. 2017a, b). The majority of these studies followed the polycistronic tRNA-sgRNA (PTG)-based approach (Ma et al. 2015; Xie et al. 2015). In this approach, sgRNAs scaffold constructs are arrayed with a specific spacer, which can be separated by conserved tRNA for multiplexing (Fig. 3.3). Varying efficiencies of GE have been obtained through the PTG-based system; PTG showed a 3–31 fold higher GE efficiency with 15–19% higher mutation as compared to other CRISPR/Cas9-based multiplexing approaches (Xie et al. 2015). However, a new multiplexing method referred as simplified single transcriptional unit (SSTU) CRISPR system has been developed (Wang et al. 2018a, b). In the SSTU-based method, different endonucleases such as FnCpf1, LbCpf1 orCas9 and their sgRNA array were coexpressed in rice from a single Pol II promoter, without any additional processing machinery. A higher magnitude of GE efficiency was achieved by this simple and efficient method. The SSTU-based multiplex CRISPR system can be an option for cotton GE as it is advantageous because of the simplified construction of the cassettes and higher efficiency compared to other multiplexing techniques. By opting for the SSTU-based approach, successful manipulation of different cotton genomic loci can be engineered with improved efficiency against different abiotic and biotic stresses (Fig. 3.3).

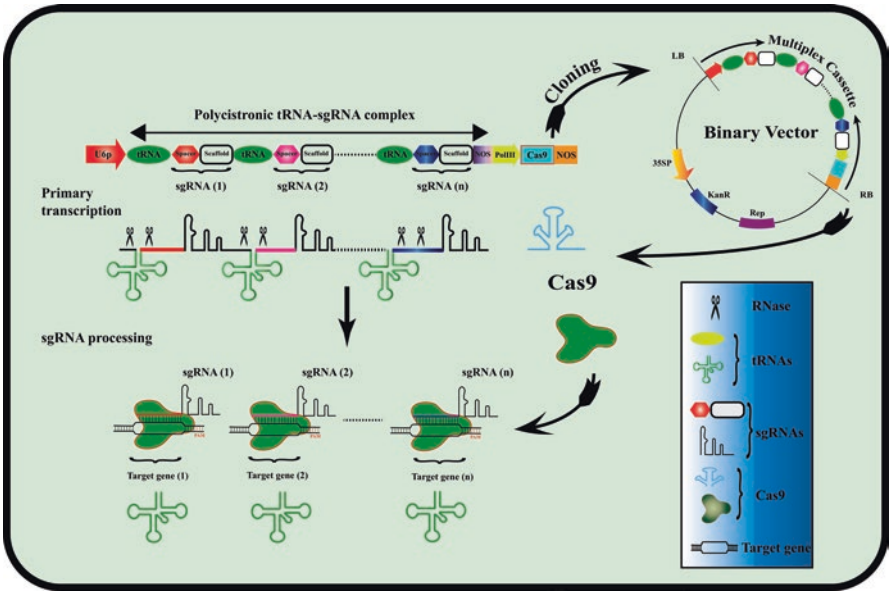

Fig. 3.3 A schematic representation of multiplex CRISPR/Cas-based genome editing in cotton. The multiplex cassette can be assembled for multiple sgRNAs following the tRNA, with spacer sequences insertions followed by the NOS terminator at the end. Finally, the Cas protein can be expressed through a separate Pol-III promotor and NOS terminator sequences sharing the same cassette in the binary vector. These multi-components will be transcribed and expressed independently from each other in the cotton genome to commence GE

3.8 Countering Off-targets During Genome Editing

Off-target activity is a major concern during GE events, which causes chopping of non-targeted sequences in the plant genome leading to the disruption of normal functioning of non-target genes. Many web-based tools (such as CROP-IT, CCTop and/or CRISPOR) are available now to predict and evaluate the off-target activity of the designed sgRNAs. Genome-wide off-target mutations have been implicated to the *seed sequence* in the single-guided RNA, which is specific for the intended target DNA, due to the observation that the first eight nucleotides at the distal end to PAM motif of *seed sequence* are relatively tolerant to the target site compared to the 12 nucleotides at the proximal end to PAM motif. Off-target incidence in plants has been rare according to the data available in the literature; however, it has been suggested that elimination of off-target mutation can be achieved with the use of backcrossing. Whole genome analysis of sgRNA can be performed in order to minimize the chance of off-target mutation and to achieve highly specific GE (Tang et al. 2018).

In cotton, whole genome analysis of sgRNA during its design has been done to avoid potential off-targets. Potential off-targets of various sgRNAs with few base pairs of mismatches in the cotton genome in GE transgenic cotton plants have been

checked using online analysis, such as CRISPR-P website with the database containing the *G. raimondii* genome and the *G. hirsutum* genome database website; this was validated using the restriction enzyme digestion-suppressed PCR (RE-PCR) assay and sequence analysis. The verified mutations in the plant genes were only used for the analysis of off-target mutations. No off-target mutation was observed for these sgRNAs using CRISPR/Cas9 technology in cotton (Chen et al. 2017; Wang et al. 2018a, b). Li et al. (2017a, b) also reported that there was no off-target mutation after sequencing the putative off-target sites for the sgRNA1 and sgRNA2 of GhMYB25-like gene with 3 and 1 mismatched nucleotides, respectively.

Other GE strategies have been developed to offset off-target repercussions, such as a D10A mutation in the RuvC nuclease domain of Cas9, which can only create a single strand break using two gRNAs, simultaneously. FokI nucleases can be fused to catalytically inactive Cas9 protein to form dimers in order to cleave the DNA. These strategies will effectively address the common and widespread practical issues to adopt CRISPR/Cas systems in crop plants (Bortesi and Fischer 2015).

In rice, it has been shown that the Cas9-paired nickases effectively suppress off-target mutations, as two guide RNAs are used simultaneously. However, it has been reported that decreased frequency of on-target mutation has occurred (Mikami et al. 2016). The use of ribonucleoproteins (RNPs) has been known to mitigate the effect of off-target repercussions during GE application. In plants, it has been shown that the use of SpCas9 in amalgamation with a single sgRNA has been shown to lessen the effect of off-target repercussions. The RNP strategy has also been shown to eliminate the cytotoxicity effect, which is often associated when performing the transfection of DNA. In addition, RNPs also eliminate the possibility of small DNA fragments arising from the plasmids to be integrated (Kim et al. 2017).

3.9 Production of Transgene-Free Cotton

Unlike genetically modified (GM) crops, the genetic makeup of GE crops is not disturbed by a native genetic change in the form of deletion or insertion and thus resembles the naturally-mutated crop plant (Nekrasov et al. 2017). However, the GE crops will have to satisfactorily overcome many regulations to be labelled as non-GMO. In general, the complete CRISPR/Cas-based system is expressed in the plant cell through intermediate DNA machinery, including T-DNA of the vector, Cas9 protein and adjoining components, which act as transgenes. The social acceptance of GE crops demands the production of stable lines of heritable CRISPR-mutated genes free of the expression cassettes. Genetic segregation is used during T_0 to T_2 generations for stable inheritance of the mutants of interest, free of transgenes. Yang et al. (2017) reported that the T_1 *Brassica napus* GE plants were free of T-DNA (76.2%) and *Cas9* (11.3%), respectively. The more advanced approach is the use of DNA-free GE using RNP complex delivery system (Ricroch et al. 2017) to get transgene-free plants in a single generation. The RNP approach is equally efficient as plasmid-based expression cassettes to knockout or edit a gene in those plants

where protoplast transfection is successful, such as *Arabidopsis*, lettuce, petunia, rice, tobacco and wheat (Zhang et al. 2017). Alternatively, fluorescent proteins can be utilized as marker genes to assist in the selection of transgene-free crop plants in which protoplast transfection is difficult.

GE crops can be obtained by either selfing or backcrossing to the original parental line where the segregation of gametes takes place and homozygous GE mutants with desired alteration in the intended loci of gene can be obtained without any RNA-guided GE nuclease transgene construct. The GE0 plants can be selected for the selfing process wherein the GE1 generation, the transgenes become segregated during the segregation of gametes. RE-PCR genotyping as well as the DNA sequencing method can be used to select the GE plants, whereas the transgene-free plants can be obtained by negative selection with intended modification only in the first generation. The conventional molecular approaches are known for the elimination of transgenes with the use of Cre/loxP piggyback transposon systems and FLP/FRT systems (Khatodia et al. 2016; Zaidi et al. 2018).

Argonaute 18 (*ZmAgo18a* and *ZmAgo18b*) gene has been studied in maize using CRISPR/Cas9 technology via the selfing method to eliminate the *Cas9* transgene. To remove the *Cas9*/gRNA trasngene, selfing in the T0 population was conducted. The segregation of *Cas9* transgene is expected to occur at 3:1 (*Cas9* positive: *Cas9* negative) ratio. This ratio was veracious in Ago18a #2, Ago18b #19, Ago18b #20, but not in Ago18a #15 where all 20 seedlings from Ago 18a#15 were found to contain *Cas9*/gRNA transgene (Char et al. 2017). The selfing method was conducted by Tang et al. (2018) in rice to eliminate the *Cas9* transgene where they elucidated the role of *OsNramp5* mutation on accretion of cadmium and other related metals.

Moreover, He et al. (2018) used a set of suicide transgenes, which eliminates all the pollen as well as the embryos produced by T0 plants that contain the CRISPR/Cas9 transgene. The authors point out that this offsets the time required in the labor-intensive selection of GE crops free of transgenes through selfing and backcrossing. The GE crops and the GMOs have been compared over time and many studies concluded that the GE crops must be declared as non-GMOs. In many other countries, the fate of GE crops regarding GMO regulation is still under review (Davison and Ammann 2017). The highest court of the European Union (EU) has recently adjudicated stringent regulations for GE crops, as has been practiced for GMOs, such as routine regulations, health assessment, impact on environment and proper labeling. Responding to this decision, many scientists presumed plummeted funding on GE crop research. This may unfortunately provoke similar regulations in other countries however, US officials are not prepared to impose such a regulation for GE crops so far (Callaway 2018; Stokstad 2018).

The production of transgene-free cotton is particularly important to convince the public about it being a non-GMO product and to obtain cotton crops using the CRISPR/Cas9 system to commercialize the GE crop without encountering any GMO laws in the future. The use of backcrossing, selfing, as well depending upon breeders' choice, and other methods mentioned above could be crucial in obtaining GE cotton plants without the presence of the Cas9 T-DNA cassette transgene.

3.10 Genome Editing Bottlenecks in Polyploid Cotton

In contrast to rice and *Arabidopsis*, cotton functional genomics is intricate due to its allopolyploid genetic structure. Additionally, cotton's relatively inefficient genetic transformation compared to other crops, and low number of mutants, position cotton as a challenging crop in generating stable cotton transgenics and mutants.

Due to polyploidy, homologous sequences and the presence of larger repeat sequences in the allotetraploid cotton genome, many economically-significant traits governing fiber quality, resistance to biotic stresses, tolerance to abiotic stresses and/or yield related traits are regulated by multiple genes or alleles. The polyploidization of A and D-diploid genomes generate highly repetitive DNA contents in the tetraploid *Gossypium hirsutum* genome (Li et al. 2015). Therefore, it is a prerequisite to select multiple homoeoalleles for site-directed mutagenesis with the CRISPR/Cas9-based system. It is a critical step to choose the best candidate sgRNAs for targeted mutagenesis in cotton. Moreover, the experimental validation of the sgRNAs is also another crucial step towards the generation of a GE-cotton crop. Gao et al. (2017) has recently introduced a reliable transient transformation assay to validate sgRNAs in cotton within a stipulated short time period.

Most genes involved in various pathways have not been assigned any proven functions and their functional annotation has largely been inferred by comparison with known genes from other plants. Only a few studies are available in which most of the functional genomics relied upon RNA-interference. Somehow, such studies were limited due to gene redundancy and the presence of highly homologous genes. The effective application of the CRISPR/Cas9 system necessitates stable targeted mutation to accomplish the generation of stable homozygous mutants. The selection of an appropriate sgRNA is a key factor, which may affect the mutagenic efficacy of CRISPR/Cas9 because use of different sgRNAs targeting a single gene may produce variable results (Ma et al. 2016). One of the major bottlenecks in cotton transformation is the generation of stably-inherited cotton mutants, which itself is a laborious and lengthy process. Moreover, stable transformation is particularly needed for practical application of the CRISPR/Cas9-based system to study important agronomical traits. The genetically-stable execution of GE events can only be validated by producing successive generations of transformed cotton plants.

3.11 Conclusions and Prospects

Since the advent of the first CRISPR/Cas-based GE system, many new versions of CRISPR/Cas have been developed, which have revolutionized biotechnology. Contemporary CRISPR-based technologies have opened new horizons in disease management, insect resistance and functional genomics, especially in assigning gene(s) functions through a gain- and/or loss-of function approach. Although conventional breeding has developed many new elite, disease-resisting and high-yielding

cotton varieties, the pace of these approaches is insufficient to meet the present-day requirements. Henceforth, the post-genomic era challenged scientists to develop efficient tools for quick, high-yielding and abiotic/biotic-stress resistant crops. Given the whole-genome sequence of *G. hirsutum*, much information is now available to engineer the cotton genome, study gene functions and improve different traits pertinent to biotic and abiotic stresses. The majority of the genes have multiple copies, homologous sequences and a large proportion of repeats. Therefore, cotton genome engineering poses a tedious task, which can produce undesirable results due to high similarity and gene redundancy. It demands careful and precise investigations to engineer the cotton genome. However, the availability of cotton genome information will promote cotton genome engineering and functional genomic research. CRISPR/Cas-based GE approaches can help in promoting yield, lint amelioration, mitigating major biotic (such as fungal, bacterial and viral diseases) and abiotic stresses (Fig. 3.4). Additionally, CRISPR/Cas-based systems can be explored to activate resistance genes, and to curtail susceptible genes involved in fungal, bacterial and viral diseases of cotton. Additional applications may include the regulation of secondary metabolites to downregulate the gossypol production and for the hyperproduction of genes involved in lint amelioration. Likewise, other characters such as upregulation of antioxidants, fiber length, pests and disease resistance can be promoted through GE in cotton. Thus, the CRISPR/Cas-based GE approaches hold sub-

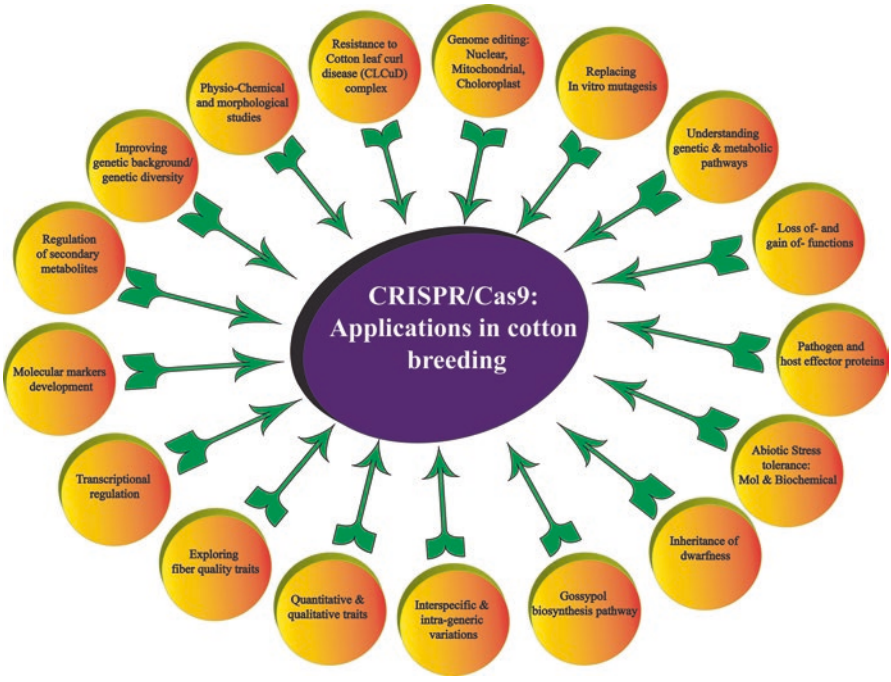

Fig. 3.4 The potential application of current and future CRISPR/Cas based genome editing in cotton

stantial potential to accelerate the genetic improvements in cotton. Together with conventional breeding, it will be a valuable addition in the cotton breeder's toolbox.

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Appendix I: Some Research Institutes Relevant to Cotton Molecular Breeding

Institution	Specialization and research activities	Contact information and website
Australian Cotton Research Institute	Cotton pathology and Biotechnology, Insecticide resistance management, Integrated pest management	Locked Bag 1000Narrabri NSW 2390 Narrabri, NSW, Australia Email: narrabri.office@dpi.nsw.gov.au https://www.dpi.nsw.gov.au/about-us/research-development/centres/narrabri
The Institute of Cotton Research (ICR), CAAS	Cotton molecular biology, genetic and breeding, cotton pathology	Chinese Academy of Agricultural Sciences, No.12 Zhongguancun South St. Haidian District Beijing, China Email: diccaas@caas.cn http://www.caas.cn/en
Cotton Research Institute	Cotton breeding and Genetics; biotechnology, cytogenetics	Nanjing Agricultural University NO.1 Weigang, Nanjing 210,095, China Email: moelab@njau.edu.cn http://mascotton.njau.edu.cn/Contact_us.htm
Central Institute for Cotton Research (CICR)	Cotton breeding and genetics, pathology, molecular breeding	PB.No.2, Shankarnagar PO, Nagpur 440,010, India. Email:cicrnagpur@gmail.com http://www.cicr.org.in/index.html
Central Cotton Research Institute (CCRI)	Cotton breeding and genetics, cytogenetics, pathology, molecular breeding	Central Cotton Research Institute (CCRI) Old Shuja Abad Road, Multan 60500, Pakistan. Email: dtmalik@gmail.com http://www.ccrim.org.pk/
Cotton Research Institute (CRI)	Cotton breeding, molecular genetics	9 El-Gamaa St., Giza, Cairo, Egypt. Email: naserradwan1936@yahoo.com http://www.arc.sci.eg/
Nazilli Cotton Research Institute, Ministry of Agriculture	Cotton breeding and Genetics and molecular breeding	Directorate, Bozdoğan Yolu Üzeri 5. Km Nazilli, Aydin, Turkey Email: pamuk@tarim.gov.tr https://arastirma.tarim.gov.tr/pamuk
USDA-ARS, Southern Regional Research Center	Cotton breeding and molecular genetics	New Orleans, LA 70179, USA Email: jpjordan@nola.srrc.usda.gov http://www.msa.ars.usda.gov/

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Chapter 4
Jute (*Corchorus* spp.) Breeding

Liwu Zhang, Aminu Kurawa Ibrahim, Sylvain Niyitanga, Liemei Zhang, and Jianmin Qi

Abstract Jute (*Corchorus* spp.) has more than 100 species, but only white jute (*C. capsularis*) and dark jute (*C. olitorius*) are commercially cultivated diploid (2n = 2x = 14) crops traditionally used to make coarse cloth, burlap, rope and paper. Jute is one of the world's important sources of natural fiber, but a distant second to cotton. This chapter focuses on the achievements in jute breeding, which include germplasm biodiversity and utilization, breeding methods, genetics of important agronomic traits, genomics and cultivation. Besides conventional breeding methods, the biotechnologies of tissue culture, transgenic technology and molecular marker-assisted selection have made great contributions to jute breeding. The whole genome sequencing for *C. olitorius* (~361 Mb) and *C. capsularis* (~336 Mb) has advanced jute improvement into the genomic era to become part of genomics-based crop research. The development of the germplasm variome, breeding genomics and cultivation genomics will lead to revolutions in jute improvement. However, jute cultivation has declined significantly with the development and use of synthetic fibers over the past few decades. Future prospects and priorities in jute breeding, such as fiber quality, genetic improvement and mechanization of production, are proposed. Emphases were given to the combination of biotechnology, genomics, germplasm innovation and mechanization of production.

Keywords Bast fiber · Biotechnology · *Corchorus* · Cultivation · Genetics · Genomics · Germplasm

4.1 Introduction

Jute (*Corchorus* spp.) is an annual fibrous crop of the *Corchorus* genus in the Malvaceae family, earlier classified in the Tiliaceae family or Spermanniaceae family (Heywood et al. 2007). More than 100 species of *Corchorus* exist and are distrib-

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uted in the tropics and subtropics, as well as warmer regions in the world. Among these, only two species *C. olitolius* (dark jute, tossa jute or jute mallow) and *C. capsularis* (white jute), are commonly used as important sources of natural bast fiber, which is obtained from the phloem or outer bark (Xiong 2008). The diploid number in the two cultivated jute species is 2n = 14. Far from only generating natural fiber, *C. olitolius* or jute mallow is used as vegetable crop (Benor et al. 2010). According to Xiong (2008), jute cultivation began 5000 years ago. At present, it is mainly grown in Asia, Africa, Eastern Europe and the Americas. The two cultivated species (*C. olitolius* and *C. capsularis*) vary with respect to morphology (seed color, silique shape and length, etc.) and physiology (reproductive habit, etc.) properties (Zhang et al. 2015a, b, c, d). *Corchorus olitolius* is an often cross-pollinating species, while *C. capsularis* is self-pollinating. Additionally, they differ in respect to genome size; *C. olitorius* has a genome size of ~361 Mb while *C. capsularis* has ~336 Mb (our group's data).

Jute has both economic and environmental significance. Various component parts of jutes serve as raw materials in bioindustries to make biodegradable and biocomposite materials that are not harmful to the environment. The leaves of dark jute are important source of nutrients such as proteins, vitamins, fibers, carbohydrates, essential amino acids and mineral nutrients (Nyadanu and Lowor 2015). Consumption of jute leaves provides valuable antioxidants required for good health and the leaves are also used as a demulcent and purgative (Khan et al. 2006). Seeds, roots and leaves of jute are herbal medicines. Local peoples in different parts of the world use jute mallow as a remedy for diseases, tumors, gonorrhea, etc. (Zeghichi et al. 2003). The stem bark or lingo cellulosic bast fibers, withdrawn from the stem of jute plant, is fully biodegradable and is used for a diversity significant economic products such as rope, twine and cordage for tying, knotting and binding, especially of agricultural commodities. Furthermore, the fiber is woven to make fabrics such as hessian, gunny bags and canvas. Its fiber is a substitute for nonbiodegradable fibers with a large range of commercial applications (Benor et al. 2012; Islam 2013).

The jute plant requires hot and humid climates. The two cultivated species are widely grown under relative humidity of 60–90% and temperatures of 24–37 °C (Ghosh and Dutta 1980). It performs well in environments with well-distributed annual rainfall of 2500 mm, temperature of 15–34 °C and a mean relative humidity of 65%. Areas with rainfall less than 1000 mm, soil waterlogging, and incessant rainfall, temperatures below 15 °C and above 34 °C are detrimental to jute. Dark jute cannot tolerate waterlogging while white jute can tolerate it, but prolonged water stagnation impairs its bast fiber quality. A temperature below 10 °C restricts seed germination in both cultivated jute species. However, white jute can tolerate temperature above 32 °C at germination, while dark jute is adversely affected at that temperature.

Commercially, white and dark jute species are significant sources of natural fibers. Jute provides about 80% of total bast fiber production in the world (http://www.fao.org/statistics/). Cultivated jute varieties give an average yield of 2.2 mt/ha and have a growth cycle of 120–150 days. Almost all global jute fiber growth and production is concentrated in Southeast Asian countries, dominated by India,

Bangladesh and China; it is cultivated on a smaller scale in Thailand, Myanmar, Pakistan, Nepal and Bhutan.

Despite the significant contributions of jute to the environment, economy, medicine and nutrition being known, jute is still neglected by the national agricultural institutes of many countries (Nyadanu and Lowor 2015). Jute cultivation has declined significantly with the development and use of synthetic fibers over recent decades. In addition, there are limited genetic resources to improve agronomic traits such as high yield, good quality bast fiber as well as biotic and abiotic stresses. Therefore, it is imperative to promote consumption and improve genetic resources through breeding approaches in jute.

This chapter focus on: (a) germplasm biodiversity and utilization; (b) genetics of important agronomic traits and genomics; (c) breeding objectives and main methods and (d) major cultivars in the world and cultivation.

4.2 Germplasm Conservation, Biodiversity and Utilization

4.2.1 Germplasm Conservation

A balanced application of conservation methods is needed in both ex situ and in situ conservation; furthermore, balanced methods are needed ex situ for seed, pollen, field gene bank, in vitro DNA and gene storage which rely on the biological influences, availability of accessions as well as level of infrastructure development. For jute, conservation seed storage is a safe and suitable method that enables short- and medium-term conservation of genetic diversity. If properly monitored, this can be a suitable form of dynamic conservation of seed storage, renewed every 5–10 years.

In recent decades, jute and related fiber crop germplasm has been collected and conserved in different germplasm repositories or gene banks in most of the jute fiber producing countries, such as Bangladesh, India and China (Appendix I). In China, the Laboratory of Bast Fiber Crop, Fujian Agriculture and Forestry University (FAFU) (Fuzhou, China) and the Institute of Bast Fiber Crop of the Chinese Academy of Agricultural Sciences (Changsha, China) reported that the germplasm accessions of bast or phloem fiber crops increased to 10,970, among which 2804 accessions were identified for some agronomic traits (Xiong 2008). Based on the statistics as of December 31, 2010, the total number of jute germplasm accessions, consisting of 11 species, was 1946, out of which 641 accessions were introduced from overseas. In India, the Indian Council of Agriculture Research (New Delhi, India) has been conserving up to 1450 accessions of dark jute and 830 white jute accessions in the International Jute Organization (IJO), since 1993 (Palit et al. 1996), while about 655 accessions consisting of both landraces and wild relatives were gathered from distinct agro-ecological regions between 1999 and 2004. In Bangladesh, 313 wild species, 647 accessions of *Corchorus capsularis* and 939 accessions of *C. olitorius* (Mahapatra and Saha 2008) are maintained; in addition, they maintain 5936 accessions consisting of 22

species of *Hibiscus*, 15 of allied genera, as well as 15 species of *Corchorus* yet to be characterized (Haque et al. 2007).

4.2.2 Taxonomy and Germplasm Classification

Jute belongs to the genus *Corchorus* and the family Tiliaceae, according to Benor et al. (2012); later, based on molecular study of the chloroplast genome and phylogenetic analysis done on the combination of 13 single copy gene families of 15 sequenced plant genomes, it was integrated into the Malvaceae family. The genus *Corchorus* has more than 100 species, of which 50–60 are the most valuable (Mahapatra and Saha 2008) (Table 4.1). The genus contains annual and short-lived perennials (Benor et al. 2010) that are concentrated in tropical and subtropical, as well as warmer regions in the world. At present, jute (*Corchorus*) is represented by two commercial species (*C. olitorius* and *C. capsularis*) consisting of diploids (2n = 2x = 14) (Fig. 4.1).

To classify jute germplasm, a primary collection of 54 typical varieties, dark (20 accessions) and white (34 accessions) jute respectively, were sampled from 11 countries and regions. Diversity analysis was investigated on the basis of 46 morphological traits at the experimental farm of Fujian Agriculture and Forestry University (FAFU) in 2016 and 2017. Data analysis of these traits in 2 years revealed that 23 quality or pseudo-quality traits basically were unchanged. These 23 traits could be taken as monotonic investigating characters for each tested variety. Among the 23 traits, silique shape, axillary bud, location of flower and fruit, color of stem and color of petiole are grouping traits.

Statistical analysis of these 54 different varieties showed that the coefficient of variation (CV) of 18 agronomic traits varied from 11.89% to 38.50% (Table 4.2). Among them, the CV of stem diameter, days to seeds mature, plant height, fresh bark thickness and days to technical mature, were less than 20%, indicating that the traits were less affected by environment. The CV of days to flowering, nodes of main stem, number of branches, days to buds, branching height, dry bark weight per plant and fresh stem weight per plant were between 20% and 30%, signifying that these traits were moderately affected by environment. However, the CV of fresh bark weight per plant and bark rate were more than 30%, indicating their vulnerability to environmental influence.

4.2.3 Origin, Dispersal, and Evolution

The continent of Africa, Indo-Burma countries and southern China are the homeland of *Corchorus*, or jute. Kundu (1956) suggested that the origin of *Corchorus* accessions were based on morphological data and relationships. Later, different

Table 4.1 Jute (*Corchorus* spp.) taxonomy where all species have x = 7 chromosomes

Cultivated *Corchorus* species	Wild species	Chromosomes number in 2 n	Geographical distribution
C. olitorius	*C. aestuans*	14	Africa, China and India
C. capsularis	*C. africanus*	–	Tanzania
	C. angolensis	–	Angola, Namibia
	C. asplenifalais	14	Southeastern Africa
	C. axillaris	–	China
	C. baldaccii	–	Eastern Africa
	C. brevicarnutus	–	Eastern Africa
	C. capsularis	14	Asia, Tanzania, South America, Europe
	C. cavalerici	–	Southern China
	C. cinerascens	–	Eastern Africa
	C. confusus	–	Southeastern Africa
	C. depressus	–	Northeastern Africa, India
	C. elachocarpus	14	–
	C. erinoceus	–	South Africa, Somalia
	C. erodiades	–	Socotra
	C. fascicularis	14	Africa, India
	C. gilettii	–	Kenya
	C. hitus	28	–
	C. junodis	–	South Africa, Mozambique
	C. kirkis	14	Southern Africa
	C. longgipedunculatis	–	Southern Africa
	C. merxmuelleri	–	Namibia
	C. olitorius	14	Asia, Africa, South America, Europe
	C. onotheroides	–	Southern China
	C. pascuorum	28	Australia
	C. pinnatipartitus	–	South Africa, Botta Weiner
	C. polygonatum	–	South of China
	C. psammophilus	–	–
	C. pseudo-capsularis	–	Africa
	C. pseudo-oblitorius	–	Eastern Africa
	C. saxatilis	–	Zaire, Zambia
	C. schimperi	–	Southeastern Africa
	C. sidoides	14	–
	C. siliguosus	28	–
	C. stenophyllus	–	Somalia
	C. tridens	14	Africa, India
	C. trilocularis	14	Africa, India
	C. uriticifolius	–	Southeastern Africa, India

(continued)

Table 4.1 (continued)

Cultivated *Corchorus* species	Wild species	Chromosomes number in 2 n	Geographical distribution
	C. velutmais	14	Southeastern Africa
	C. walcolta	14	–

Note: Information unknown

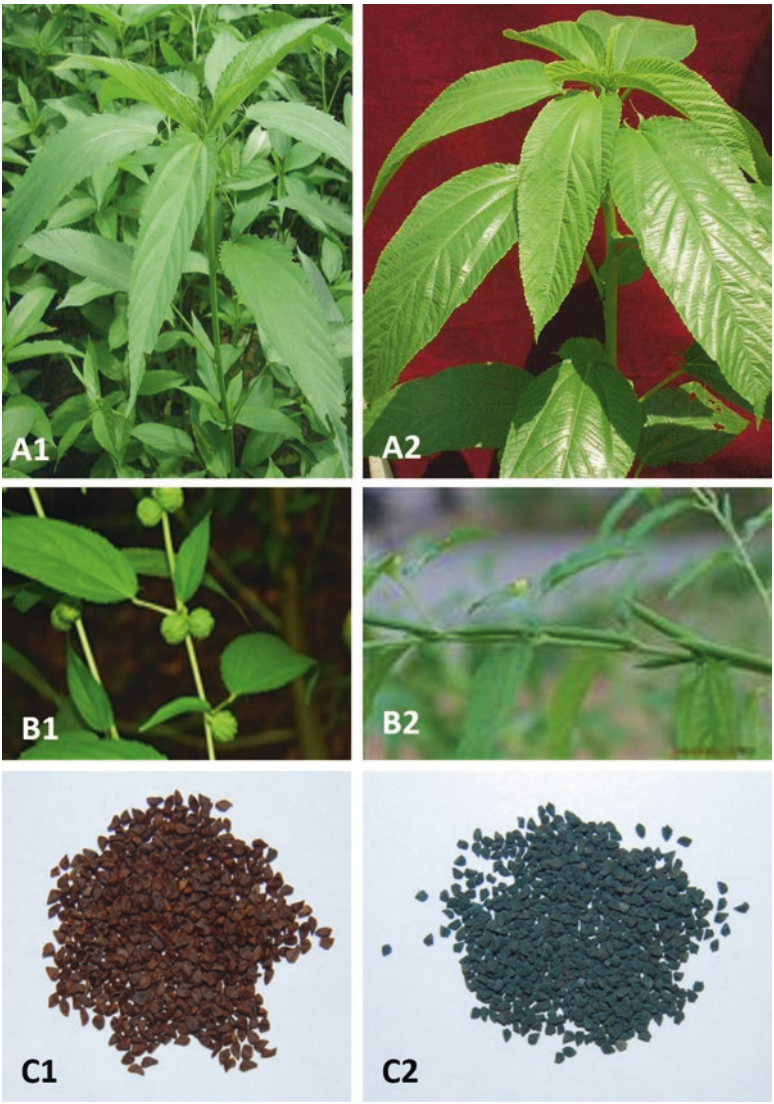

Fig. 4.1 Typical morphological traits of two cultivated species, white and dark jute. **(a1, a2)** Leaves, (**b1, b2**) Leaves, siliques, (**c1, c2**) Seeds, white and dark jute, respectively

Table 4.2 Statistical analysis of main quantitative traits in jute

Quantitative traits	Jute germplasm resource population		
	Mean ± SD	Range	CV (%)
Days to buds (d)	60.47 ± 14.86	28–86	24.57
Days to flowering (d)	69.38 ± 14.83	36–94	21.38
Days to technical mature (d)	90.12 ± 14.95	55–113	16.59
Days to seeds mature (d)	138.65 ± 16.48	100–169	11.89
Dry bark weight per plant (g)	34.64 ± 10.28	10.0–58.0	29.68
Plant height (cm)	343.16 ± 41.64	236.0–425.0	12.13
Branching height (cm)	250.57 ± 66.57	59.0–362.5	26.57
Stem diameter (cm)	1.70 ± 0.20	1.10–2.24	11.71
Fresh bark thickness (mm)	1.01 ± 0.15	0.65–1.40	14.85
Fresh stem weight per plant (g)	386.02 ± 114.72	139.0–830.0	29.72
Fresh bark weight per plant (g)	152.54 ± 49.88	39.0–340.0	32.70
Nodes of main stem (No.)	50.28 ± 11.10	12.0–79.0	22.08
Number of branches (No.)	3.15 ± 0.70	2.2–5.0	22.22
Bark rate (%)	8.39 ± 3.23	2.7–23.0	38.50
Cellulose content of fiber (%)	53.29 ± 5.85	44.25–66.64	10.98
Lignin content of fiber (%)	12.21 ± 2.22	9.37–17.80	18.22
Fiber strength (N/g)	341.67 ± 57.53	239.3–472.7	16.84
Fiber fineness (m/g)	388.76 ± 95.92	256.2–584.0	24.67

SD Standard deviation, *CV* Coefficient of variation

geographical origins of *Corchorus* species have also been supported with molecular data of RAPD and AFLP (Basu et al. 2004). As suggested by (Kundu 1956), *C. capsularis* originated in the Indo-Burma region including southern China; however, no archeological evidence is available, other than historically it was known to be utilized in China and India. *Corchorus olitorius* is known to be of African origin (Roy et al. 2006); this is supported by microsatellite diversity analyses information of two cultivated jute species (white and dark species) and archaebotanical results (Kundu 1956). Molecular phylogenetic relationship analyses of different member species of jute with specific regions of chloroplast DNA (cpDNA) found both species to share similarity through maternal inheritance.

Thus far, no substantial efforts have been made to unravel the evolution of all members of the *Corchorus* genus. Most studies have relied on the available biological resources of commercially-cultivated species, which includes: *C. olitorius* and *C. capsularis*. Phylogenetic analyses were conducted using an internal transcribed spacer and concluded that *C. olitorius* and *C. capsularis* have the same ancestor; the same conclusions have also been made from the analysis of chloroplast DNA (Kundu 1956). Moreover, morphological analysis (Maity and Datta 2008) has asserted a close relationship between dark and white jute, and between *C. aestuans* and *C. urticifolius* that further showed that the former is an ancestor to dark jute and was likely introduced through natural crossing with *C. urticifolius*. Mir et al. (2008a) demonstrated natural crossing between the ancestral species of *C. olitorius* and

C. aestuansis, most of them cultivated and growing over a diverse range and introduced into different continents of the world (Benor et al. 2010). Therefore, the African tropics are believed as the place of origin for *Corchorus*. Later it dispersed to India through Egyptian commerce routes and other routes through Somalia and via southwest monsoon trade routes. From there, it was distributed to other countries.

4.2.4 Genetic Diversity and Molecular Analysis

Genetic diversity describes the set of genetic traits in the genetic constitution of a species; it is the key to developing new crop germplasm and breeding. Usually, genetic diversity analysis in crops requires both phenotypic and DNA-marker approaches. Phenotypic markers contribute significantly to the studies of crop evolution, germplasm assessment and detecting the distinction between genotypes. Palit et al. (1996) evaluated 235 accessions of both cultivated species (2016 from white jute and 192 from tossa jute) based on morpho-physiological traits. However, the evaluation of genetic diversity with the morphological traits approach may not give accurate results because field evaluation takes a long time and it is usually subject to environmental influence. These challenges of genetic variability analyses with morphological approaches should be alleviated by the use of unlimited molecular markers.

Currently, molecular markers that have been developed for jute include: inter-simple sequence repeat (ISSR) (Roy et al. 2006), random amplified polymorphic DNA (RAPD), sequence-tagged microsatellite site (STMS) (Roy et al. 2006), simple sequence repeats (SSR) and INDELs (Zhang et al. 2017). Out of these markers, microsatellites (SSRs) or INDELs, were affirmed as desirable markers for genetic variability studies due to their advantages of low cost, codominant Mendelian inheritance, high polymorphism, being rapid and convenient for detection (Mir et al. 2009). Therefore, microsatellites have been employed by different researchers to analyze genetic variability in *Corchorus*. Other researchers have described genetic diversity in *Corchorus* species using available molecular markers. Benor et al. (2012) evaluated 292 accessions, from indigenous and exotic landraces that are independently from white and dark jute, for genetic variability studies using 172 SSRs. It was concluded that most of the 292 accessions could be characterized into different groups as an indication of the existence of variability among the accessions.

Studies of genetic diversity in jute have been conducted using SSRs for DNA fingerprinting. Zhang et al. (2015a, b, c, d) developed DNA fingerprinting, with 58 jute varieties using SSRs and found that 58 varieties were clustered into 2 groups which indicated the presence of high genetic diversity between the white and dark varieties (Fig. 4.2).

Molecular studies of jute involving genetic diversity analysis, using different molecular markers (Haseena et al. 2008) and construction of genomic and cDNA

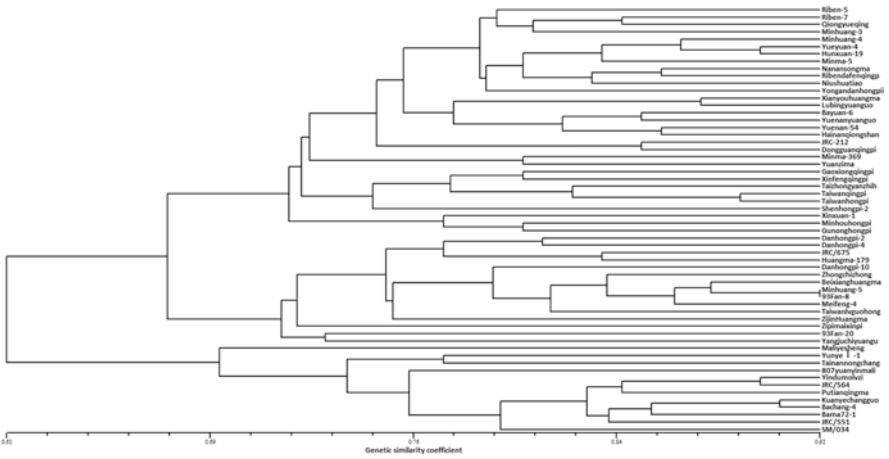

Fig. 4.2 Genetic diversity of different jute accessions based on molecular markers. Dendrogram constructed from cluster analysis by unweighted pair group of arithmetic means (UPGMA). (Source: Zhang et al. 2015a, b, c, d)

libraries followed by subsequent sequencing of randomly selected clones (Wazni et al. 2007) have been utilized and documented. Roy et al. (2006) used ISSRs, STMS and DNA (RAPD) markers to study genetic diversity analysis of 20 exotic germplasm lines and 20 commercial varieties of the two cultivated species (*Corchorus olitorius* and *C. capsularis*) and two wild relatives of jute (*C. aestuans* and *C. trilocularis*). Furthermore, Hossain et al. (2002) investigated the *C. olitorius* (12 accessions) and *C. capsularis* (9 different varieties) by DNA fingerprinting using RAPD and generated species-specific RAPD markers which relate molecular-marking data with current genetic classification.

The genetic diversity of 49 genotypes of *Corchorus olitorius* and *C. capsularis* were previously evaluated via amplified fragment length polymorphism (AFLP) as well as SSR analyses where a significant degree of variation between them was reported (Basu et al. 2004). It was documented that RAPD and ISSR markers were efficient in revealing intra- and interspecific genetic variations. Mir et al. (2008b) used SSR markers from *C. olitorius* to analyze the genetic diversity among 81 genotypes of *C. capsularis* and *C. olitorius* for important traits including fiber yield. Previously, morpho-physiological traits such as protein profile, stomata size, plant height and harvest index, were the only traits that were considered in *Corchorus* for genetic diversity analysis. However, these traits are not only subject to environmental influence but are also limited in number, thereby making them unfit for effective assessment of genetic diversity. The use of molecular markers can overcome this constraint since they are independent of the environment and unlimited in number. Furthermore, molecular markers serve as the most reliable and advanced parameter for evolutionary prediction, phylogenetic analysis as well as taxonomic characterization of all living organisms, including jute.

4.3 Genetic Studies

4.3.1 Inheritance of Quality and Pseudo-quality Traits

The genetic control of quality or pseudo-quality traits was studied in the crosses of 36 recombinations, involving 17 parents (Lu et al. 1980) (Table 4.3). The joint segregation analysis revealed that axillary bud, color of petiole, and stipule shape were controlled by one dominant gene. Frequency distributions of axillary bud and color of petiole as well as stipule shape of the F_2 generation fit showed a 3:1 ratio, which could be inferred that the presence of the axillary bud was a complete dominant over the absence of axillary bud; red petiole was dominant over white petiole; and normal stipule was dominant over irregular stipule. The inheritance of anthocyanin pigmentation in the stem was investigated via 12 crosses from 6 Chinese landraces. The segregating ratio in F_2 populations varies with the cross and may be 3:1, 9:3:4, 9:7 or 13:3.

4.3.2 Genetic Studies of Main Quantitative Traits

The inheritance of main quantitative traits of seven crosses was also observed and studied by (Qi et al. 1991). The broad heritability varied from 41.6% to 97.1%, while the narrow heritability varied from 29.4% to 94.3% (Table 4.4). Bast fiber yield had significant positive correlations with these quantitative traits, among which the relative high correlation coefficients between fiber yield and fresh bark weight per plant, plant height and days to flowering were 0.814, 0.760, and 0.648, respectively. The regression equation between bast fiber yield and fresh bark weight per plant, plant height, fresh bark thickness, bark rate were significant, with standard regression values of 0.443, 0.437, 0.291 and 0.113, respectively. Path coefficient analysis indicated that fresh bark weight per plant and plant height played a leading role in the determination of bast fiber yield. Also, the correlation coefficient (0.253) and standard regression coefficient (0.291) of bark rate were very close, indicating that bark rate directly affects bast fiber yield, with a significant positive

Table 4.3 Genetic analysis of quality traits in the F_2 populations in jute

Traits	Dominant	Recessive	F_2 progeny Theoretical ratio
Axillary bud	Presence of axillary bud	Absence of axillary bud	3:1
Stipule shape	Normal stipule	Irregular stipule	3:1
Color of petiole	Red	White	3:1
Location of flower and fruit	Node	Middle of node	3:1

Table 4.4 Heritability of main quantitative traits in jute

Quantitative traits	Broad heritability	Narrow heritability
Days to flowering (d)	73.9	58.7
Dry bark weight per plant (g)	88.3	79.0
Plant height (cm)	73.9	58.7
Branching height (cm)	97.1	94.3
Stem diameter (cm)	74.4	59.7
Fresh bark thickness (cm)	41.6	26.3
Fresh stem weight per plant (g)	85.9	75.3
Fresh bark weight per plant (g)	58.4	41.3
Bark rate (%)	45.4	29.4
Fiber strength	55.3	38.2
Fiber fineness	73.9	58.7

correlation between bast fiber yields and bark rate. Therefore, in breeding jute varieties with high bast fiber yield, it is imperative to take days to flowering, fresh bark weight per plant, plant height, bark rate and fresh bark thickness as main selection criteria for effective improvement of comprehensive traits. These findings will facilitate the design of breeding strategies for main quantitative traits improvement in jute.

4.3.3 Cytogenetics

Cytogenetics is the science concerned with the structure, number, function, and movement of chromosomes and the numerous variations of these properties as they relate to the transmission, recombination and expression of the genes. Olawuyi et al. (2014) studied chromosomes in jutes and found that 2n = 14 as the diploid number. However, many of the cells revealed chromosome clumping which were more prevalent at the metaphase stage. There were also similarities among the chromosomes in terms of morphology in the two cultivated species. The similarity in chromosome morphology suggests that they have a common ancestry.

Further cytogenetic analysis of karyotype and chromosome numbers of *Corchorus* were computed from some species and wild relatives of *Corchorus* using the root tip squash method Chen et al. (2011) (Fig. 4.3). The number of chromosomes in these materials was 2n = 14 and the karyotype formulas were as follows: Kuanyechangguo, a cultivar of *C. olitorius*, 2n = 2x = 14 = 14 m (4SAT); Nanyangchangguo, wild species of *C. olitorius*, 2n = 2x = 14 = 14 m (2SAT); Tanzaniachangguo, wild species of *C. olitorius*, 2n = 2x =14 = 2 M + 12 m; Minma No. 5, a cultivar of *C. capsularis*, 2n = 2x = 14 = 12 m + 2sm; Aidianyehuangma, wild species of *C. capsularis*, 2n = 2x = 14 = 14 m; Lianjiangyehuangma, wild species of *C. capsularis*, 2n = 2x = 14 = 4 M + 10 m; *C. aestuans*, 2n = 2x = 14 = 2 M + 12 m; *C. pseudo-olitorius*, 2n = 2x = 14 = 2 M + 12 m; Tianma,

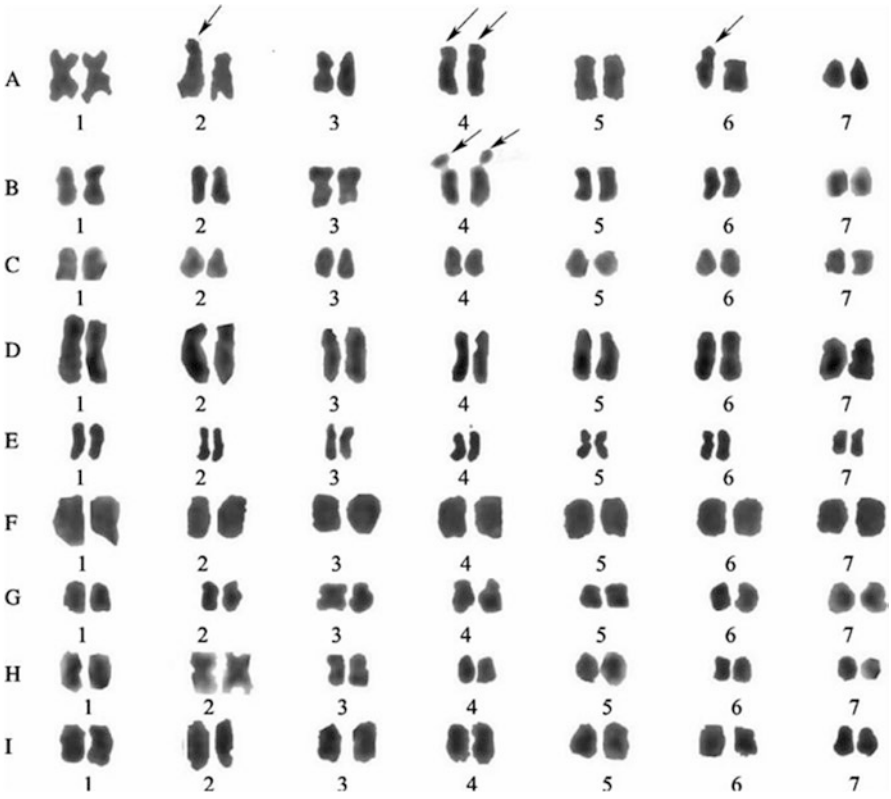

Fig. 4.3 Chromosomes karyotypes of (**a**) Kuanyechangguo, *Corchorus olitorius*, (**b**) Nanyangyeshengchanguo, *C. olitorius*), (**c**) Tansangniyayeshengchangguo, *C. olitorius*, (**d**) Minma 5, *C. capsularis*), (**e**) Aidianyeshenghuangm, *C. capsularis*), (**f**) Lianjiangyeshengyuangguo, *C. capsularis*), (**g**) Jiahhuangm, *C. aestuans*), (**h**) Jiachangguozhong, *C. pseudo-olitorius*), (**i**) Tianma, *C. olitorius* (arrows indicating satellite chromosome). (Source: Chen et al. 2011)

wild relatives of *C. olitorius*, 2n = 2x = 14 = 14 m. All of these karyotypes belonged to the 1A type except for Kuanyechangguo which were 1B type. However, chromosome clumping was observed in virtually all the cells at the metaphase stage with morphological similarities among the chromosomes.

4.4 Breeding Methods and Technologies

General breeding objectives of jute include: high bast fiber yield, good quality, multi-resistance, suitable growth period and mechanized production. Considering these breeding objectives, jute researchers have bred many cultivars in the world in recent decades (Xiong 2008). In India, the major jute cultivars are, JRO-632, JRO-878, JRO-7835, JRO-524, JRO-66, JRO-3690, JRO-8432, TJ- 40, KOM- 62

for *Corchorus olitorius*; and JRC-321, JRC- 212, JRC-698, JRC-7447, JRC-4444, UPC-94, Kc-1, KTC-1 for *C. capsularis*. In Bangladesh, the major jute cultivars are, O-4, O-9897, OM-1, O-72 or *C. olitorius*, D154–2, CVL-1, CVE-3, CC-45, BJC-7370, BJC-83 for *C. capsularis* (Xiong 2008).

4.4.1 Conventional Breeding

Introduction, selection and sexual hybridization (intra- and interspecific) has often been the popular choice of breeders (Xiong 2008) for developing improved jute varieties that have good agronomic traits such as resistant to both biotic (pests and diseases) and abiotic (drought, floods, heat etc.) stresses, and high quality yield. Since 1949, the major jute cultivars are: Guangfengchangguo, Kuanyechangguo, Bachang 4, Maliyeshengchangguo for *Corchorus olitorius* (Table 4.5); and D154, Lubinyuanguo, Yueyuan 1, JRC-212 and C46 for *C. capsularis* (Table 4.6). Jute genetic resources in China are presented in (Appendix II).

From the information of the major jute (*Corchorus capsularis*) cultivars obtained in China since 1949, it is found that intraspecific hybridization was the main method for breeding new cultivars. For example, cv. Huangma 179 of *C. capsularis* was obtained from the cross of Meifeng No. 2 and Minma No. 5. According to the practice of jute breeding in previous decades, it can be concluded that there are several principles of parental selection for the method of intraspecific hybridization in jute, i.e. prepotency, complementary strategies of targeted traits, wide adaptability, abundant genetic differences and good combining ability.

4.4.2 Interspecific Hybridization

As part of intraspecific hybridization, some scientists have reported on hybridization between the two cultivated fiber jute species: white and dark jute. Patel and Datta (1960) performed hybridization between a wide range of cultivated and wild types which resulted in a few nonviable seeds, a reciprocal grafting of the parental species and the use of x-ray irradiated pollen for hybridization was also conducted, but the resulting fruit did not reach maturity. Islam and Rashid (1960) performed hybridization by smearing the pedicel of the flower with indole acetic acid (IAA) and found that the F_2 and F_3 generations revealed female parent traits. A normal crossing between two cultivated species was also carried out by Srinath and Kundu (1952) which resulted in premature embryo growth. Interestingly, interspecific hybridization using a wide range of wild species and cultivars which produced a hybrid (F_1) maternal and no segregation in F_2 was observed (Arangzeb and Khatun 1980). Islam (1964) has also performed interspecific hybridization between the *Corchorus capsularis* and *C. olitorius* using growth hormones and embryo culture. The resulted F_1 and F_2 generations indicated maternal inheritance only. Many

Table 4.5 The information of major dark jute (*Corchorus olitorius*) cultivars in China

Cultivar name	Pedigree	Bred (introduced) units	Bred (introduced) year
Cuilv	Introduced from India	India	1919
Bama 72–1	Introduced from Pakistan	Pakistan	1972
Bama 72–2	Introduced from Pakistan	Pakistan	1972
Bama 72–3	Introduced from Pakistan	Pakistan	1972
Bachang 4 (0–4)	Introduced from Pakistan	Pakistan	1966
Maliyeshengchangguo	Introduced from Mali	Mali	–
Zhema 1	Selected from Cuilv	ZJAAS	1955
Changguo 751	Mutant from Guangfengchangguo	ZJAAS	1972
Heganhuangma	Selected from local variety	IBFC	–
Yuanjiang 101	Selected from Cuilv	IBFC	1963
Guangfengchangguo	Selected from local variety	IBFC	1965
Changguo 134	Selected from Yuanjiang 101	IBFC	1970
Tupihuang	Selected from F1 (Guangfengchangguo × Jiegan No. 1) treated with Co^{60}	IBFC	1972
Guangbaai	Guangfengchangguo × Bama 72–2	IBFC	1973
Xianghuang 2 (Changguo 277)	Selected from Guangfengchangguo	IBFC	1975
Kuanyechangguo (070–36)	Guangfengchangguo × Bachang 4	IBFC	1980
Xianghuangma 1 (075–22)	(Maliyeshengchangguo × Guangbaai) × Maliyeshengchangguo	IBFC	1986
Xianghuangma 2 (078–13)	Bama 72–2 × Kuanyechangguo	IBFC	1990
Xianghuangma 3 (089–1)	Kuanyechangguo treated with Co^{60}	IBFC	1997
Y007–10	Xianghuangma 1 × Bachang 4	IBFC	2007
Funong 1	Taizi 4 treated with Co^{60}	FAFU	2009
Funong 4	Cuilv × Bama 72–3	FAFU	2010

Source: Zhang et al. (2015a, b, c, d)
FAFU Fujian Agriculture and Forestry University, *IBFC* Institute of Bast Fiber Crops, Chinese Academy of Agricultural Sciences, *ZJAAS* Zhejiang Academy of Agricultural Sciences

attempts using conventional sexual hybridization techniques between the commercially cultivated species (white and dark) jute have been done for improvement. However, hybrids could not be attained because of the absence of intermediate features in the F_1 hybrid. Whereas there is a possibility that the two species can be sexually crossed, F_1 and F_2 plants showed only maternal traits.

Interspecific hybridizations were targeted with the aim to integrate fine fiber traits from the donor (wild species) to cultivated members of jute between *Corchorus aestuans* and *C. capsularis*, *C. trilocularis* and *C. capsularis* (Arangzeb and Khatun 1980) and (*C. trilocularis* and *C. capsularis* or *C. olitorius*). It was recorded that fine fiber trait was donated from *C. trilocularis* to *C. capsularis* in Tri Cap, a native

Table 4.6 Information of major white jute (*Corchorus capsularis*) cultivars in China

Cultivar name	Pedigree	Bred (introduced) units	Bred (introduced) year
D154	Introduced from India	INDIA	1915
JRC-212	Selected from D154	INDIA	–
Hongtiegu (Tieguma)	Selected from local variety	–	1958
Hepingzhuhaoma	Selected from local variety	–	–
Hainanqiongshan	Selected from local variety	–	–
Lubinyuanguo	Selected from local variety	–	–
Xinfeng	Selected from local variety Xinfengqingpi	ZJAAS	1950
Yuanguo 564	Selected from Meifeng 4	ZJAAS	1973
Huangma 971	715 × Meifeng 4	GDAAS	–
Xinyuan 1	Selected from D154	GDAAS	1952
Xinyuan 2	Selected from JRC-212	GDAAS	1957
Yueyuan 1 (Xinxuan 1)	Selected from Taiwan local variety	GDAAS	1955
Yueyuan 2 (Xinxuan 2)	Selected from Xinxuan 1	GDAAS	1964
Yueyuan 3	Selected from Xinxuan 1	GDAAS	1961
Yueyuan 4	Yueyuan 1 × Xinxuan 1	GDAAS	1963
Yueyuan 5	Yueyuan 1 × Yueyuan 2	GDAAS	1963
Yueyuan 6	Yueyuan 2 × 57–1289	GDAAS	1968
681	Yueyuan 5 × 63–161,111	GDAAS	1972
713	57–172,321 × Yueyuan 5	GDAAS	1973
715	57–172,921 × Yueyuan 5	GDAAS	1973
716	Yueyuan 5 × Yueyuan 6	GDAAS	1975
Xuan 46 (C46)	Selected from local variety Liuanhuangma	FAFU	1962
Kuaizaohong	Selected from a mass population	FAFU	1962
Meifeng 1	C46 × 1	FAFU	1969
Meifeng 2	Yueyuan 1 × C46	FAFU	1969
Meifeng 4	Xinxuan 1 × Lubinyuanguo	FAFU	1969
Minma 5 (Minge 5)	Yueyuan 1 × Lubinyuanguo	FAFU	1970
Fuma 1	(Yueyuan 1 × Xinxuan 1)F_1 × Xinxuan 1	FAFU	1973
Huangma 179 (77-17P)	Meifeng 2 × Minma 5	FAFU	1979
912	Selection from Huangma 179 treated with gamma ray	FAFU	1991
C2005–43	Selection from Zhonghuangma 1 treated with gamma ray	IBFC	2007
Minma 91	Selected from local variety Pinghezhuhaoma	ISSFJ	1972
Minma 396	Selected from Yueyuan 5	ISSFJ	1972

(continued)

Table 4.6 (continued)

Cultivar name	Pedigree	Bred (introduced) units	Bred (introduced) year
Minma 273	Yueyuan 5 × Yueyuan 4	ISSFJ	1972
Minma 407	Selected from Yueyuan 5	ISSFJ	1973
Minma 603	Yueyuan 5 × Hongtiegu	ISSFJ	1973
Qiongyueqing	Qiongshan × Yueyuan 5	IBFC	1974
Huangma 71–10	Yueyuan 5 × Hainanqiongshan	IBFC	1981
Zhonghuangma 1 (C90–2)	[(71–8 × 79–51) × 79–51] × 79–51	IBFC	1990
Fuhuangma 3	Meifeng 2× Minma 5	FAFU	2011

Source: Zhang et al. (2015a, b, c, d)
FAFU Fujian Agriculture and Forestry University, *IBFC* Institute of Bast Fiber Crops, Chinese Academy of Agricultural Sciences, *GDAAS* Guangdong Academy of Agricultural Sciences, *ZJAAS* Zhejiang Academy of Agricultural Sciences, *ISSFJ* Institute of Sugarcane Sciences, Fujian Academy of Agricultural Sciences

of Bangladesh. Other perspective crossings were made as *C. sidoides* (female parent) × *C. siliquosus* (male parent) where 19.05% pod setting was obtained compared to 8.7% in the reciprocal cross but the F_1 pod seeds were nonviable, indicating that the species were phylogenetically unrelated and getting viable hybrids rather impossible. A spontaneous amphidiploid in the F_3 progeny of a cross between *C. olitorius* × *C. depressus* was also isolated (Islam et al. 1973). (Islam et al. 1981) was unsuccessful in developing plantlets while attempting to induce polyploidy using anther culture of spontaneous amphidiploid *C. olitorius* × *C. depressus* hybrid. However, viable F_1 were generated from the cross of C. *capsularis* × *C. trilocularis* and were further assessed cytomorphologically (Maity and Datta 2008). Despite poor fiber-yielding nature of *Corchorus* wild species, their germplasm is reported to be a genetic resource for disease resistance (Palve et al. 2004). Exploitation and conservation by interspecific hybridization between wild and cultivated species is an efficient strategy for creating genetic diversity in jute.

Mosaic disease resistant strains have been generated from *Corchorus trilocularis* (wild species) and *C. capsularis*. However, this approach has certain limitations when introducing desired genes for specific purposes. Conversely, *C. capsularis* and *C. olitorius* need to be hybridized even though they possess these traits in their own right. A number of studies reported unsuccessful hybridization between *C. capsularis* and *C. olitorius* (Datta et al. 1960). In another perspective, Mia and Shaikh (1967) claimed to have succeeded in generating such hybrids. However, all the female parents in the F_1 and F_2 hybrids were dominant. Another independent study claimed to have generated up to F_3 hybrid progenies but no further reports on the exploitation of those hybrids, probably because the entire population had complete resemblance of the female parent (Raut and Naik 1983). Generating the entire hybrids was possible only when *C. olitorius* was used as a pistil late parent. Hybrids could not be obtained using *C. capsularis* as a female parent, although one successful hybridization is reported (Bhaduri and Bairagi 1968). Further investigations,

however, are required to reveal whether the *C. capsularis* and *C. olitorius* cytoplasmic factors could inhibit fruit set when *C. capsularis* serves as the female parent.

4.4.3 Mutation Breeding

Research on the effects of X-rays on organisms received great attention following Muller's X-ray experiments on genetic mutation of *Drosophila* in the 1920s. The first commercial mutant was produced in tobacco in 1934. Mutation breeding in jute has been going on for a long time and many mutants have been produced affecting different parts of the plant such as the stem, leaf, floral organs, pods and fiber character (Ghosh and Sen 1971). Different morphological changes in the leaves and stems and variation in the flowering time were obtained after irradiating dry and moist seeds of *C. capsularis* and *C. olitorius* with X-rays (Patel and Datta 1960). Sengupta and Palit (2004) used jute (*C. capsularis*) mutant lines (CMU) generated by X-ray treatment with the accession CMU 013, having the most undulated phenotype, to compare with the normal parent (JRC212) for growth analysis, which aimed to identify a phloem fiber mutant with reduced lignin. They observed that the normal and mutant plants showed similar leaf photosynthetic rates but also noted that the mutants were slower in growth with shorter internodes and possessing less fiber. Also, cellulose content from the mutants was more than that in the JRC212, but their fibers contained 50% less lignin than the normal type. Again, there was no obvious differentiation of primary and secondary vascular tissue between the CMU 013 stem and that of the JRC212, but unlike the JRC212, the mutant lacks secondary phloem fiber bundles. Instead, the phloem wedges of the middle stem of the mutant were less lignified and few thin-walled fiber cells with uni- or bi-seriated radial rows. They concluded that the mutant was deficient in lignified phloem fiber (*dlpf*), and this could be used to engineer jute strains with low lignin fiber and may also be a good model to study the mechanism of secondary wall thickening in fiber cells.

Generally, due to the lack of genetic variability, systematic breeding programs in jute have been inadequate (since the early 1940s). However, there was an extensive application of physical mutagens such as X-ray and gamma-ray to obtain genetic variations within a short period of time and this led to documentation of both basic and applied mutagenesis in jute over time (Thakare et al. 1973).

4.4.4 Tissue Culture

Many attempts have been made to improve jute crop through traditional breeding methods including both intra- and interspecific hybridization among the two primary fiber jute species (dark and white jute). However, these approaches experienced limitations: introducing specific genes for specific purposes, failure to produce viable offspring due to existence of narrow genetic diversity (Benor et al. 2012), and

strong cross-incompatibility between the two cultivated species of jute (Swaminathan and Iyer 1961) also limit the improvement of jute using conventional breeding (Mir et al. 2008b). For this reason, biotechnological approaches are needed to develop varieties by the insertion of specific genes besides the traditional approaches.

Some developments have been achieved in jute due to the application of biotechnology, including somatic hybridization via protoplast fusion, *Agrobacterium*-mediated transformation and plant regeneration.

4.4.5 Application of Molecular Markers in Breeding

For successful plant improvement, breeders must have a clear understanding of the nature of genetic variations among the plant species in question. The use of molecular markers is therefore necessary to achieve a number of important steps in plant breeding (Acquaah 2012), which include: gaining a better understanding of breeding materials and the breeding system, rapid introgression of simply inherited traits, early generation testing, unconventional problem solving and plant cultivar identification.

Molecular markers in jute, such as RAPDs, chloroplast-SSRs, gSSRs, ISSRs and AFLPs have been utilized to assess genetic diversity in both cultivated and wild species (Mir et al. 2008b). Sarkar et al. (2016) observed a reduction in the genome sizes of *Corchorus* which led to the validation and development of expressed sequence tag-derived simple sequence repeat (EST-SSR) markers. An association mapping panel has also been developed for *C. olitorius*, and a number of RAD-SNP (restriction-site-associated DNA single nucleotide polymorphism) markers are being used for genome-wide association mapping of complex bast fiber quality traits. Again, 4509 SSR loci have been identified from more than 34,000 unigene sequences of *C. capsularis* in order to develop a set of flanking primer pairs, where trinucleotide repeats had the highest frequency (60%) (Saha et al. 2017). The authors also established a mean PIC value of 0.34 (though few markers indicated PIC values greater than 0.5), suggesting that these markers can be efficient in measuring genetic diversity as well as mapping of quantitative trait loci (QTLs) in jute.

4.4.6 Genetic Transformation

A sexual incompatibility barrier was observed between the two species of jute that has hindered the development of an improved variety possessing beneficial traits of both species by conventional breeding (Patel and Datta 1960); therefore, improvement of this crop may be achieved through the application of genetic transformation. The genetic transformation of a number of crops largely involves direct delivery of foreign DNA to the explants, with subsequent regeneration of the plant from its callus. However, this same procedure cannot be employed in the case of

Corchorus, because many of its cultivars resist in vitro regeneration, thereby making its genetic manipulation almost impossible. However, some reports established a genotype-dependent response to in vitro regeneration for *C. capsularis* (Naher et al. 2003) and *C. olitorius* (Khatun et al. 2003). Hitherto only a few reports of *Agrobacterium tumefaciens*-mediated transformation and subsequent in vitro regeneration were available for the aforesaid species and all of them are inconclusive regarding transient gene expression, and also there exists insufficient evidence for transgene integration and genetic inheritance. Until now, shoot tips of many plant species, such as *Glycine max* (Liu et al. 2004), *Vitis vinifera* and *Poa pratensis* have been successfully used and employed.

A direct transgenic plant regeneration protocol was developed from *Agrobacterium tumifaciens* infected explants (Khatun 1993). In another approach, Shafrin et al. (2015) conducted research with the aim of reducing the amount of lignin present in jute by down-regulating the genes encoding coumarate 3-hydroxylase (C3H) and ferulate 5-hydroxylase (F5H) via amiRNA gene silencing. The content for acid insoluble lignin was reduced by about 25% while fiber lignin was reduced by 12–15%, compared to the normal plants.

A mean transformation efficiency of 4.09% was achieved in an *Agrobacterium tumefaciens*-mediated transformation of *Corchorus capsular* where stable expression of the intron harboring GUS transgene was achieved (Tables 4.7 and 4.8) (Saha et al. 2014).

Another protocol for plant regeneration from jute was developed from explant cotyledonary nodes (Fig. 4.4). Induction of transgenic roots was also achieved by Zhang et al. (2015a, b, c, d).

4.5 Genes and Genomics Available for Breeding

Due to the fact that jute is a minor crop, the number of genes identified so far are very limited (Samira et al. 2010). Islam et al. (2005) documented sequences of 15 jute genomic and cDNA clones which are significantly homologous to that of *Arabidopsis* genes. Sixteen expressed sequence tags (ESTs) were observed by Wazni et al. (2007) indicating significant similarity to *Arabidopsis*. However, it was observed that, ESTs from *Corchorus capsularis* were RNA polymerase C1 (*rpoC1*) gene and putative phosphate transport ATP binding protein, and that from *C. olitorius* was chloroplast 18S ribosomal RNA gene, respectively. The three gene products caffeoyl-CoA-O-methyltransferase, cinnamyl alcohol dehydrogenase and 4-coumaryl-CoA ligase are enzymes involved in lignin synthesis. A leucine rich repeat receptor-like protein kinase (LRR-RLK) gene from *C. olitorius* has been identified and sequenced and was analyzed as being involved in a number of stress response-related pathways in the species (Basu et al. 2004). With the development of next generation sequencing, RNA-seq of *C. olitorius* and *C. capsularis* has advanced gene mining and annotation.

Table 4.7 The various growth media and their compositions for genetic transformation of the shoot tips of *Corchorus capsularis* cv. JRC 321 explants

Purpose of use	Medium code	Content
Basal medium	BM	MS salts + vitamins +0.1% (w/v) myo-inositol +3% (w/v) sucrose +0.8% (w/v) agar, pH 5.8
Seed germination	GM	1/2 MS salts and vitamins +0.05% (w/v) myo-inositol +1.5% (w/v) sucrose +0.8% (w/v) agar, pH 5.8
Explant preculture	PCM	MS salts and vitamins +0.1% (w/v) myo-inositol +2% (w/v) sucrose +2.22 μM BAP + 5.71 lM IAA + 0.58 lMGA3 + 50 μM acetosyringone, pH 5.6
Cultivation with *Agrobacterium*	CCM	MS salts and vitamins +0.1% (w/v) myo-inositol +2% (w/v) sucrose +2% (w/v) glucose +2.22 μM BAP + 5.71 μM IAA + 0.58 μMGA3 + 100 μM acetosyringone, pH 5.4
Shoot induction	SM	MS salts and vitamins +0.1% (w/v) myo-inositol +3% (w/v) sucrose +1 lM BAP + 5.71 μM IAA + 0.7% (w/v) agar, pH 5.8
Shoot elongation	SEM	MS salts and vitamins +0.1% (w/v) myo-inositol +3% (w/v) sucrose +1 μM BAP + 5.71 μM IAA + 1.44 μMGA3 + 0.8% (w/v) agar, pH 5.8
Shoot growth	SGM	MS salts and vitamins +0.1% (w/v) myo-inositol +3% (w/v) sucrose +4.44 μM BAP + 2.85 μM IAA + 0.8% (w/v) agar, pH 5.8
Rooting of shoots	RM	1/2 MS salts and vitamins +0.05% (w/v) myo-inositol +1.5% (w/v) sucrose +1.476 μM IBA + 0.65% (w/v) agar, pH 5.8
Agrobacterium culture	ACM	MS salts and vitamins +0.1% (w/v) myo-inositol +2% (w/v) glucose +100 μM acetosyringone, pH 5.4

Source: Saha et al. (2014)

The draft genomes of the two cultivated jute species differ with respect to genome sizes. *Corchorus olitorius* has a draft genome size of ~448 Mb while *C. capsularis* has ~404 Mb (Islam et al. 2017). However, our group sequenced *C. capsularis* var. Huangma 179 and *C. olitorius* var. Kuanyechangguo genome by integrating whole-genome shotgun reads, Pacbio sequences and Hi-C as well as high-density genetic maps. We assembled 361 Mb for *C. olitorius* and 336 Mb for *C. capsularis*, and annotated 28,479 *C. olitorius* genes and 25,870 *C. capsularis* genes (Fig. 4.5). The different genome size of the two cultivated jute species suggests independent evolution. These chromosome-scale genome sequences provide a resource for engineering superior jute lines.

4.6 Production and Cultivation

Jute is one of the more important world natural fibers, but a distant second to cotton with respect of cultivation and consumption. (http://www.fao.org/statistics/). Jute cultivation depends on season, climate and soil. It is mainly concentrated in a fertile geographical region (Ganges Delta) shared with Bangladesh and India. In addition,

Table 4.8 A generalized scheme of stable genetic transformation in jute

Steps	Content	Time
1	Explant and Preculture	1 day
	Shoot tips (2–3 mm) from 5-day-old seedlings cultured on PCM medium	
2	Explant injury	Immediate transfer
	Shoot tips longitudinally sliced and punctured at 1 or 2 points in the apex	
3	Inoculation and co-cultivation	3 days
	Explants in *Agrobacterium* suspension for 30 min, vacuum infiltrate for 10 min	
	Coculture at CCM medium under continuous dark condition at 26–27 °C	
4	Recovery	5–7 days
	Culture on SM medium (with timentin)	
5	1st Selection	10 days
	Culture on SM medium(with 8 mg/L hygromycin, timentin)	
6	2nd and 3rd selection	2 passages, 14 days each
	Culture on SM medium(with 12 mg/L hygromycin, timentin)	
7	Regeneration	10 days
	Regeneration shoots to SEM medium for 20 days	
	Regeneration on BM medium (with timentin)	
8	Rooting	
	Regeneration bearing 2–3 leaves to RM medium (with timentin)	
	Plantlets at Yoshida solution for 30 days, transfer to soil	

Source: Saha et al. (2014)

China also has a modest area of jute cultivation. About 85% of the global jute production is from India, Bangladesh and China (Fig. 4.6 and 4.7). The remaining 15% is shared with all the remaining jute-producing countries in the world, in Africa, Eastern Europe, the USA and South America.

4.7 Conclusions and Prospects

Jute, like other world crops, is faced with agricultural, environmental, social and economic problems. Besides the development and use of synthetic fibers, the challenges related to jute production include: lack of mechanized production, limited land for crop production, lack of planting materials, lack of adequate knowledge of improved cultivation technology, inadequate product grading knowledge by farmers and lack of high-yielding varieties at sowing time (Hossain et al. 2002). Economic constraints of jute cultivation are lack of cash resources, low market price and low demand of jute and others (Hossain et al. 2002). Also, jute faces environmental challenges, such as biotic stresses (insect pests and diseases), abiotic stresses

Fig. 4.4 Regeneration of jute plant (*Corchorus capsularis* cv. Huangma 179) from cotyledonary node as an explant. (**a**) Callus induced from cotyledonary nodes, (**b**) A shoot differentiated from the callus; (**c**) Clearly differentiated root system developed on an MS medium, (**d**) Plants adjusting to soil condition in greenhouse-acclimatized potted plants. (Source: Zhang et al. 2015a, b, c, d)

including drought, salinity, heat, flooding, cold, depleting water resources (this affects the retting process), unpredictable weather conditions and others. Regarding social constraints, jute is neglected by researchers and national agricultural policies development of many countries in the world, and for this reason is classified under neglected crops. Its leaves are underutilization considering the crop's significance (Nyadanu and Lowor 2015). Most urbanized people and the youth tend to relate their consumption with poverty and the past.

Keeping in view the abovementioned challenges, new improved jute cultivars that possess desired traits should include: improved quality, high yield, resistance to both abiotic (drought, flood, heat, cold etc.) and biotic (insects and pests) stresses; all these are required to meet the challenges. Hence, breeding approaches must be the core of this demand. Furthermore, development of new cropping system which efficiently use scarce resources and promotion of jute consumption are also needed.

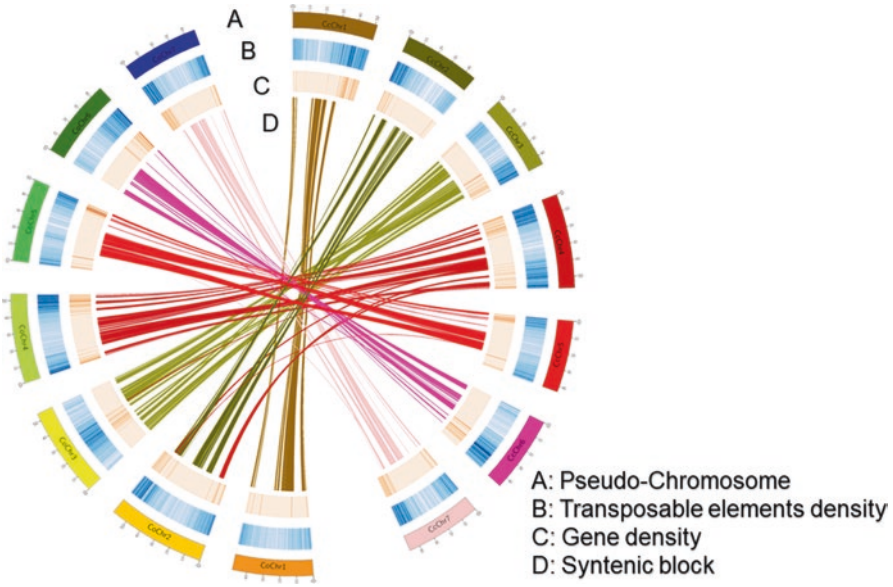

Fig. 4.5 Basic genome information, including pseudo-chromosomes, transposable elements density, gene density and syntenic block, in *Corchorus capsularis* var. Huangma 179 and *C. olitorius* var. Kuanyechangguo, respectively

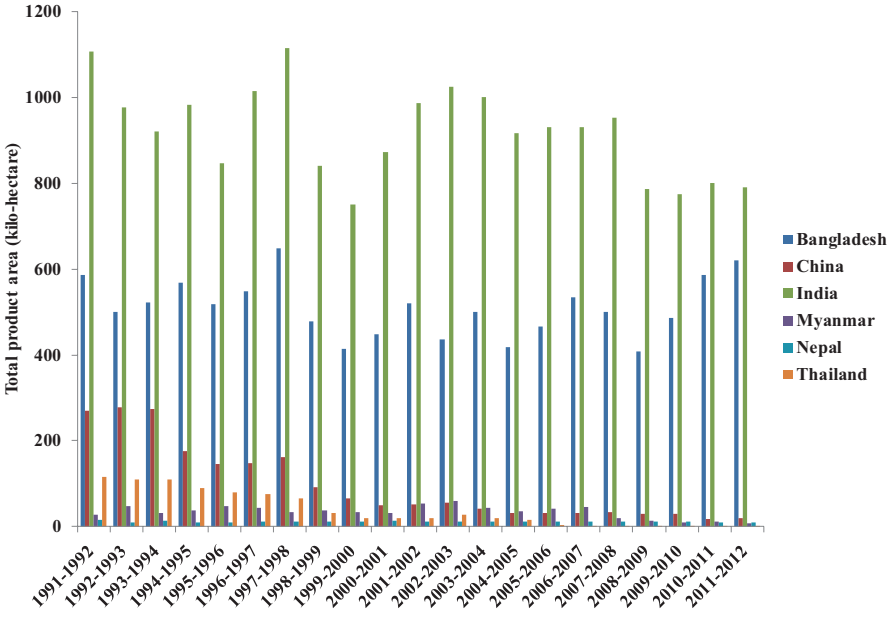

Fig. 4.6 Major jute fiber producing countries by area, 1991–2012 (unit: kilo hectare). (Source: http://www.fao.org/statistics/)

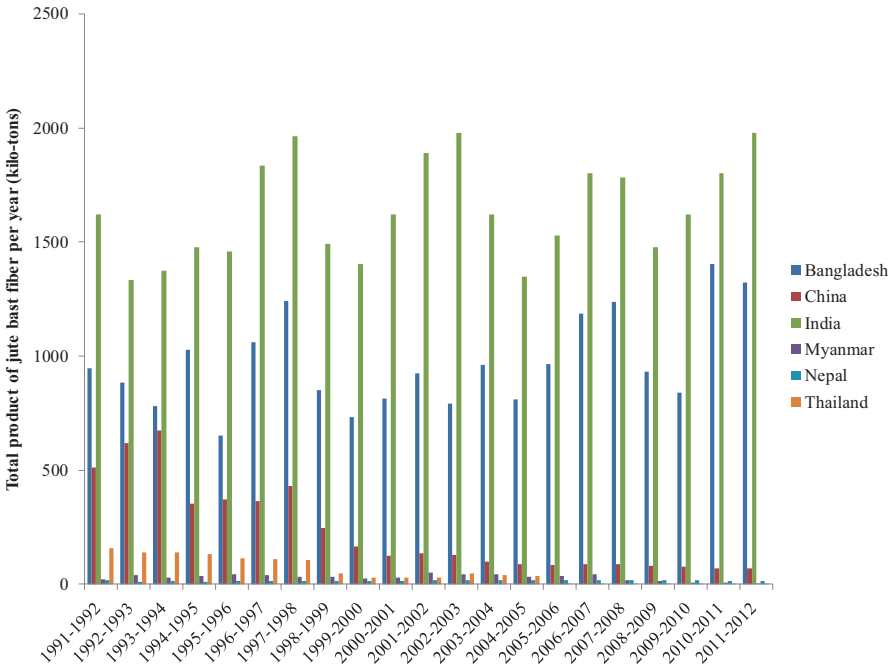

Fig. 4.7 Major jute fiber producing countries by volume, 1991–2012 (unit: kilotons). (Source: http://www.fao.org/statistics/)

Less attention is devoted to the improvement of fiber crops in comparison to cereals and vegetables. For better exploration of the genetic diversity in jute, molecular analysis of *Corchorus* species is very important and this will provide improved precision when more information is available. Collection and utilization of germplasm from jute have significantly increased productivity and this should be further assessed to improve desirable traits and fulfill market demands; hence, there is serious need for introducing trait-specific germplasm. In addition, fiber qualities are increasingly important due to their global impact on textile manufacture, processing and end-product value. Improvements of fiber quality and uniformity are likely to involve changes in fiber initiation, low lignin contain, elongation, diameter, maturation, strength and plant architecture. These changes will be made with knowledge gained from construction of core collections, genes conferring agronomic important traits, and transcriptome expression during the critical periods for fiber development. Remarkably, the complete genome sequencing for *C. olitorius* and *C. capsularis* has advanced jute improvement into the genomic era. The development of germplasm variome, breeding genomics and cultivation genomics will lead to a revolution in jute improvement.

Appendices

Appendix I: Research Institutes Interested in Jute

Institutes	Area of specialization and research activities	Website
College of Crop Science, Fujian Agriculture and Forestry University, Fuzhou 350002, China	Jute and allied fiber crop improvement and multiple utilization	http://www.fafu.edu.cn; http://bastfiber.fafu.edu.cn
	Release of important varieties, such as Fuhuangma 360, Funong 560, Huangma 179, Meifeng No. 4, Funong No. 5. Whole genome sequence of jute; Whole genome sequence of kenaf; jute and kenaf improvement and multiple utilization	
Institute of Bast Fiber Crops, Chinese Academy of Agricultural Science, Changsha 410,006, China	Bast fiber crops improvement and multiple utilization. Bast fiber crops improvement and multiple utilization	http://www.chinaibfc.com/
Basic and Applied Research on Jute Project, Bangladesh Jute Research Institute, Dhaka 1207, Bangladesh.	Jute and allied fibers	www.jutegenome.org
	Jute and allied fibers improvement and multiple utilization	
Central Research Institute for Jute and Allied Fibres (CRIJAF), Barrackpore, Kolkata 700,120, West Bengal, India	Jute and allied fibers	www.crijaf.org.in
	Jute and allied fibers improvement and multiple utilization	

Appendix II: Jute Genetic Resources in Main Countries

Cultivars	Important traits	Cultivation location
Yueyuan No.5	Stems, petioles, buds, and capsules are all green with axillary buds, lodging resistance, and late flowering stage	Guangdong Province, China
Yueyuan No.4	Stem green, petiole and calyx are red, axillary buds, short and less branches, late-maturing stage	Guangdong Province, China
MeiFeng No.4	Stem green (light red at seedling stage), petiole, calyx, and capsule are light red. Without axillary buds, the stems are clearly spirally curved	Fujian Province, China

(continued)

Cultivars	Important traits	Cultivation location
Huangma 179	Stem green (light red at seedling stage), petiole, calyx are red; axillary buds	Fujian Province, China
Minma No.5	Stem green (light red at seedling stage), petiole and calyx are red, axillary buds, resistant to anthracnose, medium-maturing	Fujian Province, China
Minma No.407	The plants are tall, the skin is thick, late-maturing stage	Fujian Province, China
Zhonghuangma No.1	Stem, stipules, and petiole are green, dark green leaves, few branches stage	Hunan Province, China
Zhejiang Jiatouma	Stems, petioles, calyx, and capsule are green, with axillary buds, medium-maturing stage	Zhejiang Province, China
Guangfengchangguo	Stems, petioles, calyx, and capsule are green, with axillary buds, medium-maturing, drought resistance.	Zhejiang Province, China
Kuanyechangguo	Stems, petioles and capsules are green, with axillary buds, oval leaves	Hunan Province, China
Xianghuangma No.1	Stem and petiole are green, mid-late maturing stage	Hunan Province, China
Xianghuangma No.2	Stems and leaves are green, with axillary buds, stable yield	Hunan Province, China
Xianghuangma No.3	Stems and leaves are green, with axillary buds, few branches	Hunan Province, China
BJRI Tossa-4O-72	High yielding. Can be sown early. Proper sowing time: 15 March–30 April. Maximum yield 4.96 mt/ha and average yield at farmers' field 2.90 mt/ha	Bangladesh
BJRI Tossa-2O-9897	High yielding. Can be sown early. Proper sowing time: 30 March–30 April. Maximum yield 4.61 mt/ha and average yield at farmers' field 2.73 mt/ha	Bangladesh
BJRI Tossa-3OM-1	High yielding. Can be sown early. Less photo sensitivity. Proper sowing time: 20 March–30 April. Maximum yield 4.62 mt/ha and average yield at farmers' field 2.49 mt/ha	Bangladesh
BJRI Tossa-1O-41967	High yielding. Late maturing type. Can be sown in highland. Proper sowing time: 15 April–15 May. Maximum yield 4.51 mt/ha and average yield at farmers' field 2.32 mt/ha	India, *C. olitorius*
BJRI deshi-2CVL-11977	High yielding variety. Very popular. Sowing time: 30 March–15 April. Maximum production 5.16 mt/ha and production at farmer's field is 2.46 mt/ha	India, *C. olitorius*
BJRI deshi-3CVE-31977	Early maturing type. Quick growing. Suitable for 3 crop rotation. Sowing time: 30 March–15 April. Maximum yield 4.51 mt/ha and average yield at farmers' field is 1.97 mt/ha	India, *C. olitorius*

(continued)

Cultivars	Important traits	Cultivation location
JRC 7447 (Shyamali)	Pods are non-shattering type, capable of utilizing higher dose of N2 fertilizer, suitable for mid-March to mid-April sowing	India, *C. capsularis*
JRC 4444 (Baldev)	Pods are non-shattering type, optimum sowing time early-March to mid-April	India, *C. capsularis*
UPC94(Reshma)	Pods are non-shattering type, suitable for late February to late March sowing	India, *C. capsularis*

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Chapter 5
Ramie (*Boehmeria nivea* L. Gaud) Genetic Improvement

Pratik Satya, Sabyasachi Mitra, and Deb Prasad Ray

Abstract Ramie (*Boehmeria nivea* L. Gaud) is a wonderful fiber-producing plant species, but it has not received adequate care and attention from researchers. Ramie fiber is considered the longest, strongest and most durable of all known plant fibers. The fiber is composed of pure cellulose being resistant to microbial attacks. Ramie leaves are highly nutritious and can be used food as well as animal feed. In addition, different parts of the plant are used in traditional medicine in many Asian countries. Despite its high value as fiber, food, feed or medicine, the cultivation of ramie has been declining over the past 50 years. Ramie cultivation has principally been limited to China under traditional farming system. The productivity and profitability of ramie farming systems are declining gradually, although industrial demand remains high. Under this backdrop, we discuss the status of genetic improvement and cultivar development in ramie that helped to sustain production despite a decrease in area under cultivation. Also, recent progress on genetic and genomic resources have been reviewed, including genome sequencing, transcriptome characterization, diversity analysis, genetic map construction and transgenic cultivar development, which provide new opportunities to improve the genetic make-up of the cultivars for better productivity, higher resistance to biotic and abiotic stresses and improvements in fiber quality. Integration of conventional and molecular breeding methodologies are also emphasized for development of new, end-use specific cultivars.

Keywords Biotechnology · Cultivar · Fiber · Genetic improvement · Genomics · Molecular breeding · Productivity

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5.1 Introduction

Ramie (*Boehmeria nivea* L. Gaud), a premium fiber-producing crop species, is a special gift of nature having several unique properties. Ramie fiber has great merit for special applications in the textile sector. It is the strongest, most oriented and most durable of all known natural fibers. In addition, it transmits heat, absorbs moisture, and becomes stronger when wet. Ramie fiber is pure cellulose (96%), exhibiting high luster, resilience and resistance to bacterial degradation. The fiber is formed in the phloem (bast) region of the stem and is known to be the longest plant cell that can reach up to a length of 60 cm. Use of ramie fabrics has long been part of Asian cultural heritage, such as translucent traditional mosi fabric of South Korea, designated as an UNESCO heritage (UNESCO 2011) or traditional clothes of common people in medieval China (Benn 2002). Ramie fabric is also considered as an integral component of heritage fashion of Asia.

Ramie is no less interesting as a plant than as a fiber. It is a perennial species that can live more than 100 years, multiply by both clonal and sexual propagation, can be harvested 3–5 times annually and has a unique mechanism of pollen dispersal. The plant produces high biomass, which is being used as an excellent animal feed, support material for growing plants, production of biofuel, mulching, substrate for cultivation of mushrooms and as input in many other novel applications including prevention of erosion in river banks or highlands. Although the crop uptakes a high amount of nutrients from the soil, a good fraction is returned back in the form of shaded leaves that help to increase soil organic carbon. Apart from the major economic uses, ramie biomass fixes substantial amounts of carbon dioxide from the air; thus, its role in abating environmental pollution cannot be ignored. More surprisingly, ramie leaves are also used as human food and beverages; a number of recipes are available for cooking rice-ramie cake and making ramie tea.

Despite being a commercially important crop, ramie cultivation for the past 50 years has declined considerably. Shifting of ramie farmers to other crops or other occupations may be one of the major reasons for this decline. Moreover, profitability issues of this crop have squeezed the scope of promotion and uses due to several other factors: complexity in fiber separation and processing, slow pace of research and development due to limited resource allocation and inadequate industrial investment. However, a renewed interest for bringing back natural fiber holds good promise for increase in utilization of this amazing gift of nature, which may boost the production of ramie in the coming days.

5.1.1 Importance of Ramie

Ramie is principally grown as a fiber crop worldwide. It is cultivated on about 500,000 ha; over 95% of this area is present in China. Apart from China, the crop is grown in Brazil, Laos, Japan, South Korea, India and the Philippines. Since 2000,

the area under ramie has decreased considerably, from 966,000 to 521,000 ha in 2016 (Fig. 5.1). Consequently, production of fiber has decreased from 160,000 to 110,000 mt during this period. The productivity of the crop has remained stationary for a long period with an average of about 20 q/ha.

From ancient times, ramie fiber has been used in making clothes, upholstery, canvas, fishing nets, sewing thread and marine packages. Ramie spinning is also mentioned in NungShu, a book on agriculture written in 1333 by Wang Chen (Zhen), a pioneer agronomist official of Yuan Dynasty (1271–1368 AD) of China. According to Korean literature dated 1479, the people of Suri and Noha wore white ramie clothes (Hendrickx 2007). The short fibers are used in making cigarette papers and currency notes. In the textile industry, particularly in Japan and China, ramie is used as blend with cotton for making various high-value fabrics. Ramie fabric has several advantages, including high moisture absorbance that provides comfort in wearing clothes, particularly during the hot summer, resistance to bacteria, fungus, moth and other insects, resistance to strong alkali and mild acid and increased strength under wet conditions. The fabric has high luster and reflectance and is amenable to dye, bleach and hot water treatments.

The problems of using ramie as a fabric are low elasticity and brittleness, although several finishing techniques are available to improve the quality. Because of high demand, ramie fiber fetches a good market price. Presently it is priced at about 800 USD/mt (FAOSTAT 2018), although like other natural fibers, the price fluctuates considerably. Since the textile and apparel export is expected to grow at a compound annual growth rate of 6.3% with an export projection of 1600 billion

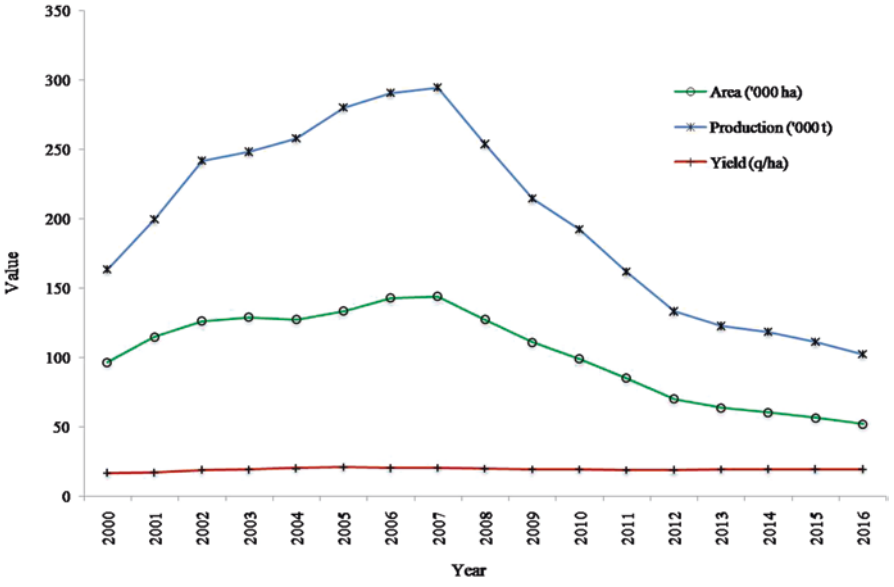

Fig. 5.1 Worldwide estimate of area, production and yield of ramie 2000–2016. (Source: FAOSTAT 2018))

USD by 2025 (UN Comtrade 2018), demand for ramie and ramie-cotton blended fabrics are expected to increase in the near future. Ramie is also blended with other natural fibers like silk to produce high-quality fabrics. In addition to its traditional use the fiber can also be used for production of fiber composites.

For sustaining the profitability of the ramie cultivation system, alternate use of ramie fiber and the whole plant have been explored at various levels. Only 15–18% of the ramie biomass is harvested for fiber extraction; about 60% of this is further lost during decortication (fiber strand separation) and chemical processing. At the end, only 4–5% of the total biomass is captured in the fiber. The residue left after decortication can be utilized for preparation of animal feed, mulch material, bedding material for mushroom cultivation or input for bioethanol production. Ramie stalks and leaves are highly nutritious animal feed, suitable for ruminants, pigs and poultry. The leaves contain high (17.0–25.6%) protein along with iron, manganese, calcium and vitamins and are excellent source of phenolics and flavonoids. Digestibility of ramie leaf was found superior to pigeon pea hay (Ferreira et al. 1997) (Table 5.1). Further improvements in nutrition can be obtained by silage making and pellet preparation. Studies showed that ramie feed increases milk production in goats by 15% (Dos Santos et al. 1995). Ramie is also consumed by Koreanswith rice-cake, muffin, cookies, or as a beverage (Lee et al. 2010). Ramie tea, made from concentrated dried ramie leaves is marketed in Korea, where it is claimed to have high calcium content and help the digestive system. It has been also observed that dried powder of ramie leaves added to wheat bread increases antioxidant activity and shelf-life (Lee and Joo 2012).

Ramie is also used as traditional medicine for hepatoprotective, antifungal, antipyretic, antivenom and diuretic purposes in China (Huang et al. 2006; Lin et al. 1998; Xu et al. 2011a, b). It has been observed that 10–100 mg/L root extract of ramie has anti-hepatitis B virus activity (Huang et al. 2006). Ethanol extract of ramie leaves also have anti-diabetic effect. In addition, ramie efficiently removes heavy metals, particularly cadmium from soil. Cadmium is a serious pollutant, particularly in the industrial belts causing itai-itai disease leading to bone softening and kidney failure. In Japan, this disease is considered one of four most important pollution-related health hazards. Ramie has a high potential for bioremediation in areas polluted with heavy metals, such as electroplating and battery manufacturing industries.

Table 5.1 Digestibility coefficients of ramie hay analyzed from feeding experiments on rabbits

Parameter	Ramie hay (%)	Pigeon pea hay (%)
Dry matter	49.80	24.00
Crude protein	59.49	25.64
Crude fiber	22.78	27.41
Ether extract	36.14	32.26
Nitrogen free extract	63.04	20.98
Gross energy	47.93	24.62

Source: Ferreira et al. (1997)

5.1.2 Taxonomy, Distribution and Natural Habitat

The genus *Boehmeria* belongs to tribe Boehmerieae, family Urticaceae. The Dutch botanist, Rumphius first discovered the plant on Banoa Island of the Malay Peninsula and named it *Ramium magus* (Carter 1910); it was commonly known by its Malay name *rami.* It was described by Linnaeus in 1737 under genus *Urtica.* The genus was designated as *Boehmeria* by Thunberg in 1794, to honor the German botanist and physician George Rudolph Böhmer (Wilmot-Dear and Friis 2013). The species name, *nivea* is Latin for *snow*, describing the whitish appearance of the underside of the leaf. The plant was also discovered by Hamilton from Bengal and Assam of India in 1807. Some of the early botanists described the Indian species as *B. tenacissima* or *rhea*, while the Chinese and Malay Peninsula type was designated as *B. nivea* or *China-grass*. However, as per the present-day classification, these two types are considered to be variants of species *B. nivea* (Wilmot-Dear and Friis 2013).

Boehmeria is a major genus of the family Urticaceae. According to *The Plant List*, a comprehensive working list of all plant species (http://www.theplantlist.org/), there are 387 known species entries under the genus *Boehmeria*, of which 96 species have been accepted, while another 164 were found to be synonymous to the accepted species. However, 126 species recorded still need to be assessed, suggesting a possible increase in the number of species under *Boehmeria*. On the basis of morphological characters, Wang (1995) identified five sections of *Boehmeria*. Further karyotypic and DNA sequence studies confirmed the basic chromosome number (n = 14) and revealed two distinct evolutionary paths in this section (Liao et al. 2009). On the other hand, Wilmot-Dear and Friis (2013) classified only 47 species under genus *Boehmeria*, with 33 indigenous species in the Old World and 14 indigenous species in the New World. Of these species, only one, *Boehmeria nivea* (L.) Gaudich, or white ramie, is cultivated as a fiber crop. Various authors have described this species as *B. tenacissima*, *Urtica. nivea*, *B. nipononivea*, *B. nivea* var. *reticulata*, *B. nivea* var. *concolor* and *B. nivea* var. *viridula*, which are synonymous to *B. nivea*. The species *B. tenacissima* or *B. nivea* var. *tenacissima* is also known as green ramie, which is an ecotype of ramie and is cultivated sporadically. The major wild relatives of ramie are *B. virgate* (*B. virgata* ssp. *macrophylla* (syn. *B. macrophylla*) and *B. virgata* ssp. *virgata*), *B. japonica* (syn. *B. spicata*) (Japanese false nettle), *B. cylindrica* (small spike false nettle), *B. grandis, B. clidemioides and B. siamensis.* Of these, *B. virgata* is the most widespread species of *Boehmeria*, thus a considerable variation is observed within this species. The species is observed in almost every continent of the Old World, from Africa to Australia. Only a few wild *Boehmeria* species are used by native people, primarily for making ropes, nets and cloths. Leaves of a few species are used as animal feed. However, a number of species have been identified to have antibacterial and anti-cancer properties. Preliminary investigations suggest that many wild *Boehmeria* species have high potential for medicinal use. For example, an alkaloid boehmeriasin A has been isolated from *B. siamensis* that targets topoisomerases to stop DNA synthesis in proliferating cells, which may have use in cancer treatment (Christodoulou et al. 2015).

Fig. 5.2 Global distribution of *Boehmeria.* Source: Distribution data was retrieved from GBIF (Rivas Pava et al. 2017), base map was downloaded from Google Earth and distribution map was created in RStudio (Version 1.0136)

Based on records of *Boehmeria* available at the Global Biodiversity Information Facility (GBIF), we generated an occurrence distribution map of *Boehmeria*, (Fig. 5.2), which shows that the genus is distributed in both the Old World and New World. In the Old World, The distribution is concentrated in Indo-Burma, China and Southeast Asia, Japan, parts of Central Africa and Australia. In the New World, *Boehmeria* is distributed primarily in the Caribbean islands, South America and Central America. Several species including *B. macrophylla*, *B. platyphylla*, *B. rugulosa* and *B. sidaefolia* have been reported in India, particularly in the Northeast region, upper Himalaya and South India.

The species *Boehmeria nivea* is distributed in forest margins, roadsides, moist places around streams and hills in China (Jiangxi, Gansu, Hubei, Hunan, Henan, Shaanxi and Sichuan provinces), Japan, India, almost all the Southeast Asian countries, Central Africa and Madagascar. Ramie is known as *chou-ma*, *chu-ma* or *dz*' in China; as *riha, kunkhura* or *puya* in India; *karamushi*, *karea* or *tsjo* in Japan; *ra-mi* in Malaysia and as *China-grass* in Europe and the USA. However, ramie was introduced in Europe only in the eighteenth century (Chen et al. 2003) and was introduced in the USA in about 1855 (Maiti et al. 2010).

5.1.3 Botanical Description

Ramie is a tall perennial shrub or sub-shrub, usually reaching a height of 1–4 m (Fig. 5.3a, b). Several stems are formed from the underground rhizomatous root. These stems are called canes, where fiber is formed in the phloem (bast) region. The canes are covered with dense, soft hairs. The diameter of the stem varies from 0.5–2 cm based on growth conditions and plant age. The leaves are stipulate, alternate, broadly ovate to elliptical and vary extensively in size with a length of 5–25 cm

Fig. 5.3 Different parts of a ramie plant. (**a**) Ramie plant, (**b**) General view of a crop, (**c**) Leaves of white and green ramie, abaxial surface, (**d**) Male and female inflorescence, (**e**) Inflorescence bearing seeds, (**f**) Seed, (**g**) Female flower with receptive white stigma, (**h**) Fruit

and breadth of 4–20 cm. The leaves are green, with indentations at the margins, and narrow abruptly at apex, containing a long petiole of 3–11 cm. The most marked morphological character of ramie is the presence of white tomentum of tangled hairs on the abaxial surface that give a feathery feel and in most cases, a white shining appearance of the lower leaf (Fig. 5.3c). The adaxial surface is also covered with shorter, dense hairs that feel more rough when touched.

The morphology of the reproductive organs of ramie is quite interesting and has a profound effect on its breeding behavior. The species is cross-pollinated, as it bears unisexual flowers borne on distinct inflorescences (Fig. 5.3d). Surprisingly,

the female inflorescence is borne on the top of the cane, while male inflorescence is present on the lower side.The male inflorescence is a proximal raceme, arising from the cane progressively from bottom to the top. In a study with over 50 germplasm accessions we have observed that the male inflorescence can spread through 70–150 cm of the cane. The male flowers are borne on the branched axes as clusters of 6–15 flowers. The female inflorescence is a terminal raceme, appearing later than the male inflorescence, has a length of 8–40 cm. We observed that the number of nodes in the male inflorescence was almost double that of female inflorescence, while average number of flowers per flower cluster was four to five times more in the female inflorescence compared to that of the male inflorescence. A few axes bear both male and female flower clusters. The male flowers are pubescent, each with a four-lobed perianth and short pedicel. Four stamens are present in the male flower bearing numerous pollen grains on each anther. Average anther filament length is about 1.8 mm, while average diameter of the anther is 0.7 mm. Each female flower contains a single ovary with long pointed style covered with soft hairs at one side (Fig. 5.3g). A fully mature style measures up to 0.5 mm. The length of the ovary varies from 0.3 mm to 0.75 mm, while the average diameter was 0.27 mm. Stigma is persistent during seed development. Each fruit is single seeded (Fig. 5.3h). Seeds are microscopic; thousands of seeds are produced from a single cane (Fig. 5.3e, f).

5.1.4 Cytology

Due to their small size, chromosome identification and karyotype determination is difficult. The basic chromosome number of the genus *Boehmeria*is n = x = 14 (Acharya et al. 2003; Liao et al. 2009; Pierozzi and Benatti 1998). Most of the species are diploid, but polyploidy exists in some species under section *Duertia*. The chromosome number of ramie was first determined by Chatterjee and Bhattacharya in 1957 as 2n = 28. They estimated the size of the mitotic metaphase chromosome to be 2.0–2.5 μm in length. Recently, Pierozzi and Baroni (2014) reported that the size of the chromosomes in the range of 1.0–1.7 μm. Karyotype analysis revealed that all the chromosomes except chromosome 4 are submetacentric (karyotype formula-1 m + 12sm + 1sm^{s}). Cytological observations in some varieties recorded a prevalence of subtelocentric chromosomes. It has been suggested that the present diploid ramie might originate from an ancestral species with 2n = 14 through whole genome duplication (Pierozzi and Baroni 2014).However, the genome sequence data did not reveal any sign of whole genome duplication.

5.1.5 Fiber Structure and Properties

Ramie fiber develops in the primary phloem as single fiber strands; each strand is oval or elliptical in cross-section (Fig. 5.4a) (Maiti 1979; Maiti et al. 2010). Ramie fiber content (fiber weight/harvested biomass) is about 3.5–5%. The fiber strands

Fig. 5.4 Ramie fiber: structure and properties. (**a**) Cross-sectional view of ramie fiber cells in phloem, (**b**) Decortication of ramie fiber, (**c**) Single fiber strand (ultimate fiber), magnification 100x, (**d**) Decorticated fiber, (**e**) Degummed fiber, (**f**) Ramie yarn

are connected to each other by pectin and hemicellulose polymers, along with wax and lipids. These polymeric substances are collectively termed gum. Mechanical decortication of the bark is performed to separate the fiber strands (Fig. 5.4b). Gum content of the decorticated fiber bundles varies from 20 to 35% of the total fiber weight (Fig. 5.4d). The process of gum removal from the fiber is known as degumming. Degumming is commercially done by hot alkali treatment, although microbial degumming methods have also been developed, which reduces the extent of chemical degumming. The presence of gummy material makes the fiber brittle,

Table 5.2 Chemical composition of raw and degummed ramie

Component	Decorticated (raw) ramie (%)	Degummed ramie (%)
Cellulose	73–75	90–96
Hemicellulose	12–14	2–3.5
Pectin	8–10	0.5–1.0
Lignin	1–1.5	–
Wax	0.2–0.6	–
Ash	3.0–3.5	0.5
Water soluble compounds	6–7	–

Source: Pandey (2007a), Sarkar et al. (2010)

susceptible to microbial degradation and natural shine and color of the fiber is lost. Several studies have been undertaken to develop safer degumming techniques to reduce the environmental hazards of alkali treatment, but this method is still predominant for separation of gum from fiber at a commercial scale. Decorticated ramie fiber has 75% cellulose, while the degummed fiber (Fig. 5.4e) is made of pure (>90%) cellulose (Table 5.2).

The unicellular fiber strands (Fig. 5.4c) are 20–120 mm long, although fiber length of up to 600 mm is reported. The breadth of the fiber is 10–80 µm. The length/breadth (L/B) ratio of ramie fiber is the highest among plant fiber cells, being in the range of 2000–3600 (Sarkar et al. 2010). The birefringence value of the fiber is also the highest among all the natural fibers. Ramie fiber can be spun into fine yearn, which can be used for both textile and non-textile purposes (Fig. 5.4f). The physical and chemical properties of ramie fiber has been reviewed in detail by Pandey (2007a, b). Ultra-structural studies showed that ramie fiber cells have a single primary cell wall and multiple layers (4–6) of secondary cell walls. Three different types of cellulose microfibril layers, sexine, middle and internal layers were identified. The orientation of cellulose microfibrils is transverse, while in the middle layer it is axial (Yan 2000). The major physical properties of ramie fiber are listed in Table 5.3.

The tenacity of ramie fiber is the highest among natural fibers. The fiber shows a tenacity of 40–65 g/tex, with a gravimetric fineness of 0.4–0.8 (Pandey and Krishnan 1990). Ramie cellulose is highly ordered, oriented and has little void space. However, the fiber is comparatively rigid and has lower elasticity than other vegetable fibers, which is an undesirable character for yarn making. However, the elasticity can be improved by physical and chemical treatments.

5.1.6 Domestication, Selection and Early Improvements

Historically, ramie is one of the oldest examples of human-plant association. Its use has been recorded since the Bronze Age. China has a long heritage of cultivation and diversified use of ramie. The remains of ramie fabric of the Bronze Age have

Table 5.3 Physical properties of ramie fiber

Property	Value	Comparative status
Ultimate fiber length	20–120 mm	Longest plant cell
Ultimate fiber diameter	15–80 μm	Thickest fiber cell
Length/breadth ratio	2400–3600	Highest among fiber cells
Circularity of fiber	0.78	Similar to other fibers
Cell wall thickness to fiber cell width ratio	0.6	Highest among fiber cells
Birefringence	0.07	Highest among plant fibers
Moisture regain (at 65% RH)	10.1%	Lower than jute, sunnhemp and flax
True density (g/cc)	1.56	Highest among plant fibers
Water accessible pore volume (ml/g)	0.09	Much lower than cotton
Fiber strength /tenacity (g/tex)	40–65	Strongest of all plant fibers
Gravimetric fiber fineness (tex)	0.4–0.8	Finest of all plant fibers
Energy to break (J/g)	5.94	Almost three times more than flax
Breaking elongation[a] (%)	2.8%	Much lower than cotton

[a]Measure of elasticity
Source: Pandey (2007b)

been recovered from Liangzhu in eastern China (Kuhn 1988). Ramie fibers have also been identified from the earthen plasters of Taoist temples of the Wudang Mountains, built during 1412–1417, suggesting that in addition to textile purpose, the fiber was also used as a composite for construction (Ma et al. 2017). However, a matting shroud woven from ramie fibers of the Bronze Age was unearthed from the Dongson site of Dong Xa in northern Vietnam (Bellwood et al. 2007), suggesting ramie was also known as a fiber plant in ancient Viet Nam. Ramie fiber was used as a wrapping material for mummification and was considered as a symbol of wealth and richness in Egyptian civilization (Kozlowasky et al. 2005; Maiti et al. 2010). Ramie has been also mentioned in ancient epic Indian literature such as the *Ramayana* and *Sakuntala* (Anonymous 1989), which suggest that the fiber was also used in Ancient India. Furthermore, the old name of Guwahati, the capital of Assam, is Rheabari, which literally means the homeland of ramie (Sarma 2008). This clearly signifies the influence of this crop in the culture of northeastern India. Although ramie was not known in Europe and the Americas until recently, wild relatives of this species have been used by Native Americans for making bows and arrows (Kozlowaski et al. 2005). Moreover, clothes made from nettle fiber, a relative of ramie, was recovered from a 2800 year old Bronze Age cremated body placed in an urn in Denmark (Bergfjord et al. 2012), suggesting that fiber plants like ramie and nettle were known to humankind since the dawn of civilization.

Based on genome sequence data, the mulberry tree (*Morus notabilis*) was found to be a close relative of *B. nivea*. The two species probably have diverged about 51.4 million years ago (Liu et al. 2017). Taxonomic and DNA evidence suggests that the cultivated white ramie, *B. nivea* originated from green ramie, *B. tenacissima* (Jiang and Jie 2005).Comparative transcriptome analysis revealed that 10,745 unigenes of these two subtypes shared over 96% identity (Liu et al. 2014). Of these, 1002 genes

had a positive selection pressure, indicating they have been favored during domestication of ramie. Combined with archeological evidence of the presence of ramie in Liangzhu in China during the period 3300–2200 BC (Kuhn 1988), high genetic diversity of ramie in China, particularly in the populations of the Yangtze River, Liao et al. (2014) suggested that the ramie was domesticated in and around the middle and lower regions of the Yangtze Valley about 5000 years ago. However, recovery of ramie fabric in Viet Nam during Bronze Age suggests multiple independent domestication events. Current molecular evidences suggest that selection during domestication favored high fiber content and traits associated with wider adaptability in cultivated ramie. Functional annotation of genes revealed that protein kinases related to abiotic stresses received positive selection pressure during domestication (Liu et al. 2014). Another interesting revelation was that *WAT1 (WALLS ARE THIN 1)*, a phloem specific gene associated with secondary fiber cell development was under positive selection pressure during domestication, which may be a driving force for an increase in quantity of cellulosic fiber in cultivated types.

5.2 Cultivation and Traditional Breeding

5.2.1 Current Cultivation Trends and Practices

Ramie is a subtropical crop, although it can be grown well in the tropical regions. Cultivation practices vary widely depending on the climate and soil conditions. In China, ramie is harvested three times in a year. The first crop season is the longest, starting from early March to early June, with a duration of 85–90 days. The second crop season starts in mid-June and the crop is harvested in early August after 50–60 days. The third season continues from mid-August to early or mid-October, depending on the onset of winter. In regions of low temperature, the third crop season is further shortened to 50–60 days. Production of the first cut is much higher than the second and third cut. On average 1000–1200 kg raw fiber/ha can be obtained from the first cut, while the second and the third cut give raw fiber output of 500–600 k/ha and 400–500 kg/ha, respectively, producing an average fiber yield of 2000–2300 kg/ha/year. The agronomic requirements of the ramie crop for optimum yield are as follows:

(a) Climate – Warm, humid, rainfall around 1500–2500 mm, temperature 23–32 °C, relative humidity 80%, free from frost.
(b) Soil – Well-drained, deep, fertile loam or sandy loam, rich in organic matter, pH 6–7.
(c) Propagating material – By seed, rhizome, stem cutting, waste stock or young shoot. Both seedling derived and young shoot derived seedlings are planted first in the nursery and then transplanted in the main field after 3–5 months. Rhizomes can be directly planted in the field. Rhizomes from 1 ha area can be used to

plant 20 ha of land. To prevent termite attack, the rhizomes and cuttings should be treated with insecticides.

(d) Planting method – Ridge and furrow method is preferred over flatbed sowing. The planting density varies according to cultivation practices. A row-to-row distance of 60 cm and a plant-to-plant distance of 30 cm can give good yield. Ridges should be 1 m apart to facilitate mechanical interculture operations. Chinese farmers maintain a plant population of 25,000–45,000/ha for good yield depending on soil and climatic conditions, the optimum being 30,000 plants/ha.

(e) Nutrition management – In China, three cuttings are taken per year. Fertilizer requirement depends on the cutting season and age of the crop. An N:P:K ratio of 1:0.4:0.6 is recommended for the first cut, while for the second and the third cut, the amount of the potash needs to be increased by 40%. Ramie compost can be used as a fertilizer supplement, which improves fiber yield.

(f) Weed management– Needs 2–3 weedings for the first cut, while subsequent cuts require 2 weedings. Mechanical weeding saves time and cost of cultivation.

(g) Irrigation – Depending on the rainfall 2–3 irrigations are needed. A good drainage system is essential in heavy rainfall areas.

(h) Stage back – After the winter season, the standing canes are cut and soil is loosened lightly to allow good crop growth in the rainy season.

(i) Harvesting – The harvesting stage can be identified by inspecting the base of the cane which turns coppery or brown. Harvesting can be done manually or mechanically.

(j) Decortication – After harvesting, the fiber needs to be separated from the core by mechanical decortication using a machine which has scrapping blade attached. Various machines have been designed for fiber separation that cost around USD 2000–5000. The high cost of the decorticator is a major limiting factor for popularizing ramie cultivation.

(k) Degumming – The decorticated fibers contain gum that needs to be removed to obtaining textile quality fiber. The commercial method is boiling with strong alkali, such as sodium hydroxide (1–2%), for 1–2 h. Another method is microbial degumming using pectinolytic bacteria for 4–7 days followed by washing with mild alkali. At ICAR-CRIJAF, India, Mitra et al. (2013) developed a microbial degumming method that eliminates the requirement for chemical degumming and reduces the risk of pollution.

5.2.2 Current Agricultural Problems and Challenges

Despite being a profitable high-value crop, ramie cultivation is limited to certain traditional belts in China. Ramie is a perennial; therefore, the land under its cultivation remains occupied for at least 5–8 years, during which the farmers cannot cultivate any other crop. Most of the cultivated lands under intensive agricultural systems are under multiple cropping, where the integration of ramie into the existing

cropping system would not be profitable. Areal expansion of ramie is thus a major constraint to increasing fiber production. Furthermore, ramie does not grow well under stressed conditions or in soils having poor fertility. As a fast growing crop species, ramie has high nutritional and water requirements; it requires over 150 kg N/year for the first 2 years and about 250 kg N/year from third year onwards. The crop is at the mercy of environmental conditions throughout the year. While annual crops are only exposed to a particular environment depending on the season of cultivation; ramie withstands all the seasonal fluctuations in a year. Ramie is highly sensitive to water stagnation, drought and frost. Consequently it cannot be grown in locations with a frosty winter or in flood-prone areas.

Once the plant is harvested, the fiber has to be extracted mechanically by decortication that requires electrical or fuel-powered decorticator machines. The traditional process of scraping and beating results in low fiber recovery and poor quality fiber. Ramie degumming is expensive, requires strong alkali and produces toxic by-products that are usually dumped in ponds and rivers leading to environmental pollution. Since it is an industrial crop, established linkage to industry for marketing is essential to sustain cultivation. Hence, the extent of cultivation fluctuates greatly with demand and market price.

5.2.3 Genetic Improvement Limitations

As a cross-pollinated species with a complex population structure, ramie has several limitations that create difficulties in genetic analysis and cultivar development. However, once a cultivar is developed, it can be perpetuated through clonal propagation by rhizomes or vegetative stalks. The following barriers are major limiting factors for genetic improvement in ramie.

(a) Ramie produces sexual progeny, but the seedling is extremely small and weak, requiring about 6–12 months for establishment. This slows the breeding progress.
(b) Inbreeding depression in the crop is very high. Development of a pure inbred line is practically impossible due to vigor loss. This limits estimation of combining ability and heterotic potential.
(c) The mode of reproduction is highly complex. This increases the genetic complexity and uncertainty of the source breeding material. Since the plant itself is heterozygous, the selfed F_1 progeny of a single ramie plant resembles a F_2 population generated by crossing two purelines, which contain both homozygous and heterozygous lines. Until now, the major method of ramie breeding is selection of a superior clone from the segregating F_1 population and maintaining it by clonal propagation. The proportion of heterozygosity increases when hybridization is performed between two lines. Theoretically, a much larger F_1 population is needed to identify suitable heterotic cross combinations if the population results from hybridization of two heterozygous parents rather than from selfing.

(d) Pollination control is extremely difficult. The flowers are microscopic and borne in clusters. The pollen is very light and wind borne. Flowering is asynchronous, which makes it very difficult to determine if a particular seed is set from fertilization by self-pollen or non-self-pollen. Targeted hybridization can only be ensured if one of the two parents is either gynoecious or male sterile.
(e) The induction of male and female flowers depends on environmental conditions, which may reverse the sexual status of a plant. Ramie breeders, however, can turn this to their advantage by manipulating environmental conditions.
(f) Heritability and genetics of economically-important traits are poorly understood. In the absence of reliable information on genetics and population structure, ramie genetic improvement remains more of an art than a science.

5.2.4 Traditional Breeding Methodologies and Limitations

Although ramie cultivation can be traced back 5000 years, concerted breeding efforts for genetic improvement were initiated only about 30–40 years ago. The complex nature of its genetic constitution, sexual dimorphism, open pollination and the presence of clonal propagation have made ramie breeding very challenging, which may be a major reason for delayed initiatives in its breeding. Often, the genetic status of the initial breeding material is unknown, which may be a single heterozygous genotype or combination of several heterozygous genotypes. For this, clonal selection from germplasm or landraces, selection of clones from selfed progeny and hybridization of two parents followed by clonal selection are the principal breeding methodologies for genetic improvement of ramie (Table 5.4). The primary breeding objectives are improvements in fiber yield and fiber quality, enhanced biomass as a feed crop, development of resistance to pests and diseases and tolerance to abiotic stresses, development of photoperiod insensitive cultivars and hybrid cultivar development. Germplasm characterization has led to identification and selection of many cultivars with high fiber yield and quality, resistance to mosaic disease, drought tolerance and resistance to breakage against wind (Chen et al. 1993).Even today, a number of successful cultivars are local selections or landraces. For example, Luzhuqing, Xiyelu and Heipidou are leading local cultivars of white ramie, while Zantianhui, Daqinggan, Erfuhan and Anxin are important local cultivars of green ramie in China (Ministry of Agriculture, China 2008).

The primary target traits for ramie genetic improvement are fiber yield, fiber strength, luster, plant biomass, plant height and stem diameter. Selection of clonal progenies is often based on plant height and stem diameter, as both traits show a high correlation with fiber yield. It has been observed that plant height shows very high correlation with fiber filament length ($r = 0.90$) and fiber fineness ($r = 0.95$) (Banerjee et al. 2016). No significant association is present between vegetative traits such as number of nodes/plant, internode length, plant height, stem diameters or root length with reproductive traits (seed size, inflorescence size or number of flowers). On the other hand, the number of nodes, plant height and stem diameter are

Table 5.4 Major breeding methodologies adopted for genetic improvement of ramie

Breeding method	Example
Direct adoption of landrace/germplasm as cultivar or clonal selection from landrace/germplasm	Formosa, Miyasaki, Luzhuqing, Xiyelu, Ningdu Ramie
Clonal selection from exotic collection	R 1411, R 67–34, PI London, PI 87521
Selection from selfed progeny	Chuanzhu 4
Selection from progeny derived from open pollination	Huazhou 1, Huazhu 4,
Selection from progeny derived from hybridization	Hakuhi, Seikeiseshin, Huazhu 3, Zhongzhu no. 3
Selection from induced mutations	Xiangzhu 2, 74–69, Minsizhu no. 1
Hybrid development using male sterility	Chuanzhu-11

positively associated with fiber yield. In addition, net photosynthetic rate was found to be correlated with fiber yield (Liang et al. 1999), which can also serve as an important selection indicator.

Selection can be successfully performed in self- or open-pollinated progeny of a ramie genotype, since the genotype itself is heterozygous. Taking advantage of this feature, a number of cultivars have been developed using several breeding techniques (Table 5.4). For example, cv. Chuanzhu 4 was developed from the selfed progeny of cv. Dahongpi, showed over 70% increase in fiber yield in the first cut compared to control cv. Hongpixiaoma (Zhu et al. 1993). Similarly, selection from the open-pollinated progeny of cv. Luzhuping resulted in the development of cv. Huazhou 1, with yield potential of 2800 kg/ha. Intervarietal crossing followed by clonal selection has also resulted in development of a number of cultivars, such as Huazhu 3, which was developed by crossing Xinyuma and Xijieba. Presently, the leading ramie cultivar in China is Zhongzhu No.1 or Zhongzhu-1. In addition to high yield, this variety has good resistance to insectpests, nematodes and diseases. The green biomass and fiber yield of Zhongzhu-1 were recorded to be 90 mt/ha/year and 2000–2200 kg/ha/year, respectively. The leaves of this cultivar are also rich in protein (>20%).Besides Zhongzhu-1, a number of other ramie cultivars have been developed in China, such as Zhongzhu-2, Xiangzhu-2/Yuan Yeqing, Xiangzhu-3, Hongpixiaoma, Tri-1, Huazhu-3, Huazhu-4, Huazhu-5, Chuanzhu-8 and Chuanzhu-9. Recently, a new cultivar Zhongzhu No. 3 has been developed from the progeny of Houpizhong S2 × Yushanma S2, which is early maturing with 6.33% higher fiber yield potential than Zhongzhu No. 1.

In India, two ramie cultivars have been developed and released for cultivation to date. The first, R-67-34 (Kanai) is a seedling selection from E-56-76, while the second, R-1411 (Hazarika) is a selection from Florida Commercial. R-1411 exhibits 6.8% higher fiber yield (1800–2000 kg/ha/year) than R 67–34 and has low gum content (22%) in the fiber along with better resistance to Indian red admiral caterpillar and leaf folder (Sharma et al. 2017). There are some other ramie cultivars like Formosa, Miyasaki, PI London, Murakami, Baxi series, Hakuhi sporadically grown around the world.

Along with cultivar development, breeding programs have also targeted introduction of specific characters, such as photoperiod insensitivity and development of cultivars suitable for animal nutrition. Initiation of flowering during the short-day condition has a negative impact on the fiber yield of the plant. Particularly during the third cut, fiber yield decreases considerably as the plant moves from vegetative to reproductive phase. Development of photoperiod insensitivity is thus another major breeding objective to provide a continuous supply of fiber as well as biomass throughout the year. A number of photoperiod insensitive genetic stocks have been identified. Most of these lines, however, have reduced plant height and thinner fiber cells, impairing fiber yield and quality (Zhou 1993). The molecular basis of the photoperiod response pathway is well characterized in the plant, which involves the interplay of major genes like *FLOWERING LOCUS T* (*FLT*) and *CONSTANS* (*CO*) for promotion of flowering in short-day plants. Liu et al. (2015a, b) identified six homologs of *CO* (*BnCOL1-BnCOL6*) in ramie. Of these, *BnCOL2*, a homolog of rice flowering gene *HEADING DATE 1* (*HD1*) exhibited distinct diurnal expression variation.

Breeding for ramie as a feed crop has resulted in the development of cultivars like Zhongsizhu No.1.,containing 22% protein in the leaves. Analysis of nutritional quality also revealed that the cultivar contains 16.74% fiber, 15.44% ash, 4.07% calcium, 13.36 mg/kg Vitamin B2 and 1.02% lysine (Xiong et al. 2005). Further improvements have resulted in development of new forage cv. Minsizhu No. 1 that exhibits 32% higher forage yield than cv. Zhongsizhu No. 1 (Yao et al. 2017).

5.3 Genetic Resources, Biodiversity and Conservation

5.3.1 Germplasm Collection, Conservation and Morphological Characterization

Since ramie is one of the principal fiber crops of China, most explorations for germplasm collection have been conducted within this country. A national field gene bank for ramie, affiliated with the Institute of Bast Fiber Crops, Chinese Academy of Agricultural Science (CAAS), China was established in 2001. Through various collection efforts, a total of 2052 accessions of 19 species have been documented and preserved in the Chinese National Ramie Germplasm Nursery of this institute as a field gene bank. Among these, 1901 are cultivar accessions and 151 are wild species accessions. There are some other ramie germplasm repositories in China, such as the Huazhong Agricultural University, Wuhan, but the presence of duplications in these repositories cannot be ruled out. Luan et al. (2010) and Chen et al. (2011) have listed 790 ramie accessions, which were used to construct a core collection. A good number of forage ramie germplasm accessions(120) is also reportedly in the germplasm repository of Hunan Agricultural University. Apart from China, India has collected 181 accessions of ramie and its wild relatives, most of which are

maintained at the field gene bank of the Ramie Research Station, Sorbhog, Assam, under the Indian Council of Agricultural Research (ICAR) – Central Research Institute for Jute and Allied Fibers (Sharma et al. 2014). The accessions were further classified as tall (cultivated), creeping (wild) and bushy (wild) types.

Chinese ramie germplasm is well characterized, revealing variability in plant height, leaf type, stem shape, inflorescence color and phyllotaxy. The germplasm collection has been screened against biotic and abiotic stresses to identify suitable tolerant genotypes. For example, screening of 933 accessions against leaf mosaic disease revealed that 3.1% of accessions are highly resistant to this disease (Zhang et al. 1992). Similarly, genotypes with high breaking strain and fine fiber have been identified by large-scale screening of germplasm for fiber quality. Ramie germplasm has also been screened for waterlogging tolerance, drought tolerance and resistance to insect pests, and a number of desirable lines have been identified (Zheng et al. 1996).

Efforts have been undertaken to characterize the Indian germplasm collections. Deka and Talukdar (1997) evaluated 40 ramie genotypes and found that 4 genotypes, namely SC-12, R-1420, SC-7 and R-67-34, exhibited high fiber yield. Sharma et al. (2014) identified some genotypes showing fairly high resistance to the non-selective herbicide, glyphosate. They suggested that due to the presence of rhizomes, the ramie plant has considerable tolerance to glyphosate. Recently, Gawande and Sharma (2016) screened 129 accessions and identified 11 cultivated and 19 wild accessions to be resistant against anthracnose leaf spot.

When a germplasm collection is large, it often becomes difficult for the end-user to evaluate the total collection. Since a germplasm collection is built up by multiple explorations, duplicate and redundant accessions are common problems. To manage the germplasm more effectively, a core collection is constituted from the germplasm which is a subset, usually 5–20% of the whole collection, capturing the maximum genetic variability of the germplasm. Such collections may be trait-specific, or may be a general core collection representing most of the economic traits. The first core collection of ramie was developed by Luan et al. (2010) based on 25 agronomic characters. A general core collection of 158 ramie genotypes was developed from 790 accessions maintained in the National Ramie Germplasm Nursery, China. The same was also reported by Chen et al. (2011). Furthermore, from this set, a mini-core collection comprised of 22 accessions was constructed from 108 accessions using 21 SSR markers (Luan et al. 2014). The mini-core collection was reliable, but exhibited considerably less variability than the primary core.

5.3.2 Phylogeny and Characterization of Genetic Resources

DNA markers including random amplified polymorphic DNA (RAPD), inter-simple sequence repeats (ISSR), simple sequence repeats (SSR), random amplified microsatellite polymorphism (RAMP) and sequence related amplification polymorphism (SRAP) have been used to investigate genetic diversity in ramie cultivars and wild

genetic resources (Satya and Chakraborty 2015). Most studies indicate lower diversity within cultivars compared to wild genetic resources. These studies helped to formulate an indication of the genetic variability of ramie present in different provinces of China, including Hubei, Jiangxi, Hunan, Sichuan, Guizhou, Chongqing and Guangxi. However, in many of these studies, few genotypes (< 35) have been used for genetic diversity studies, which may not be adequate to obtain a reliable representation of ramie genetic diversity in China. Later, Liao et al. (2014) studied 50 ramie populations sampled all over the ramie-growing regions of China and established a reliable relationship among these populations using 21 SSR markers. Their study identified that over 80% of allelic diversity is present in the wild population, suggesting a population bottleneck event during ramie domestication.

To assess the diversity of ramie in India, both Indian and exotic ramie germplasm were characterized using start codon targeted (SCoT) markers. Twenty SCoT markers were used to characterize ramie germplasm, trace the breeding history of Indian ramie cultivars and to understand the population structure of ramie in India. Results indicate that the exotic ramie accessions exhibited close genetic association with the domesticated breeding material, but not with the Indian natural ramie populations. Initiation of ramie breeding in India has relied primarily on the introduction of new cultivars from other countries. But the Indian ramie populations exhibit high genetic polymorphism, genetic differentiation and gene flow. This suggests that Indian ramie has a different gene pool than exotic ramie and hybridization between Indian and exotic genotypes to increase heterosis is possible. The Indian native ramie has higher adaptability; thus this gene pool is a valuable reservoir for enriching genetic improvement programs targeting higher yield, better quality, adaptability and resistance to pests and diseases. Our study also indicated the presence of a geographical barrier for gene flow among Indian populations. Indian populations were collected from the Upper Assam region, Lower Assam region and Meghalaya. The population from Upper Assam exhibited higher values of Shanon's information index and Nei's expected heterozygosity, but lower genetic polymorphism than the other two populations. Our studies showed high gene flow among these three populations, which may be due to the endemic perennial nature of the species, or due to a high amount of pollen exchange. Close genetic association between ramie populations of lower Assam with both Meghalaya and Upper Assam indicates a gradient of pollen and gene flow from Meghalaya to Lower Assam and Upper Assam (Satya et al. 2015).

Phylogenetic analysis using DNA markers is also a reliable approach for tracing the breeding history of cultivars and breeding lines, identify closely-related genetic stocks in a germplasm collection or to resolve ancestry of cultivars. For example, Liu et al. (2008) compared the genetic association of ramie cultivars from China and Brazil using SRAP markers and suggested that the Brazilian cultivars may have originated from Chinese landraces. In our diversity analysis, we also observed that the Indian cultivars are exclusively developed from the exotic gene pool.

5.3.3 Genetic Resources Conservation Approaches

The perennial nature of ramie allows germplasm to be conserved in field gene banks, as seeds produce heterozygous progeny. In addition, seeds quickly lose viability, so they are not suitable for long-term storage. The major challenges of ramie germplasm management are the following:

(a) Ramie germplasm cannot be maintained through seed gene bank establishment, as the seeds will not breed true. Thus, the field gene bank is the only option for maintaining ramie germplasm through clonal propagation.
(b) The crop needs care throughout the year, adding cost to germplasm management. Field gene banks require more space, management and cost than seed gene banks and are affected by environmental conditions.
(c) Mutations accumulating in vegetative tissues over the year are often inherited, leading to a gradual shift in the population.
(d) There is always a risk of losing germplasm from field gene banks due to disease, drought, flood or any other natural calamities. The Chinese ramie germplasm collection suffered such a setback in 1996, when about 900 germplasm accessions were lost due to flooding in Yuanjiang (Xu et al. 2011a, b).

5.4 Tissue Culture and Genetic Engineering

Protocols for in vitro culture and conservation of rhizomes are not well-standardized, which is a bottleneck for long-term in vitro storage of ramie germplasm. In addition, cryopreservation of seeds has been attempted, but the cryopreserved seeds lose viability after a few months. Thus development of suitable in vitro conservation protocol for ramie rhizomes and seeds are major research needs for long-term conservation of genetic resources. In-vitro whole plant regeneration methodologies for ramie were developed in the 1980s in China. Early efforts targeted regenerating ramie from cotyledons and hypocotyls. These protocols were based on callus induction followed by re-differentiation by manipulating the hormonal combination in the culture media. These studies reported that the cotyledonary tissue is most suitable for whole plant regeneration. Wang et al. (2008) regenerated whole plant from seedling derived explants. They observed that Murashige and Skoog salts supplemented with Gamborg's B5 vitamins basal medium containing 2.27 μM thidiazuron (TDZ) and 0.054 μM naphthalene acetic acid (NAA) was most suitable for successful regeneration. It was also found that cotyledons and hypocotyls from young (4 days) have a higher percentage of shoot formation. Explants from leaf, petiole and shoots from 15 days old seedlings exhibited about 57–64% shoot regeneration. However, when these explants were used from 25-day-old seedlings, the shoot regeneration efficiencies were drastically reduced to 11–15%. They also reported that a 16-h/8-h (light/dark) photoperiod increased shoot regeneration frequency compared to 24-h dark treatment.

The first transgenic ramie plant was developed by Dusi et al. (1993) that contained *bar* gene conferring resistance to herbicide Phosphinothricin. They used cotyledons, hypocotyls and leaf discs as explants and obtained better success from cotyledonary tissue using Gamborg's B5 medium supplemented with indole acetic acid (0.2 mg/l) and benzyl aminopurine (2.0 mg/l). Since development of a reproducible regeneration system is an integral component in regeneration of transgenic plants, several experiments were conducted to develop a suitable transformation protocol. Guo et al. (1998) used explants from leaves and reported shoot regeneration, but whole plant regeneration was unpromising. However, a ramie plant is usually highly heterozygous, thus the progenies from a transgenic plants are also expected to be segregating at several loci, which is a major problem for characterization of transgenic plants at subsequent generations. To address this issue, An et al. (2014) used midrib explants from shoot tips grown in vitro and developed a reproducible transformation protocol. However, since the midrib is also somatic tissue, the problem of heterozygosity cannot be resolved using this approach.

Only a few traits have been targeted for genetic transformation in ramie, including herbicide tolerance (Dusi et al. 1993), insect resistance (Fu et al. 2009; Ma et al. 2009) and delayed senescence and high biomass (An et al. 2017). Attempts have been made to develop Bt-ramie by incorporating genes from *Bacillus thuringiensis*. Fu et al. (2009) reported development of stable Bt-transgenic lines of cv. Luzhqing that showed higher field resistance. Two genes, *CryIA* (from *B. thuringiensis*) and *CpTI* (cowpea trypsin inhibitor) were transformed into ramie cvs. Zhongzhu 1, Zhongsizhu No.1 and NC01 using both cotyledon and hypocotyl tissues by *Agrobacterium* transformation. Transformation efficiency was higher in hypocotyls than in cotyledons. However, the study did not report expression level Bt-protein or reaction of transgenic plants against insect-pests, without which it is difficult to assess the potential of these transgenic lines. An et al. (2017), on the other hand, transferred the *isopentenyl transferase* (*ipt*) gene under control of SAG12 promoter with an efficiency of 25.6%. Leaves of the transgenic plants had higher chlorophyll content with delayed senescence. Furthermore, plant height, shoot weight and bast weight of the transgenic plants increased significantly.

5.5 Molecular Genetics and Genomics

In recent years, genomic research in crop plants has progressed at an astounding speed, particularly after development of next-generation sequencing (NGS) tools. Genomic research in ramie has advanced remarkably during the past few years, resulting in new gene identification, transcriptome characterization, genome sequencing, marker development, map construction and functional analysis of genes (Fig. 5.5).

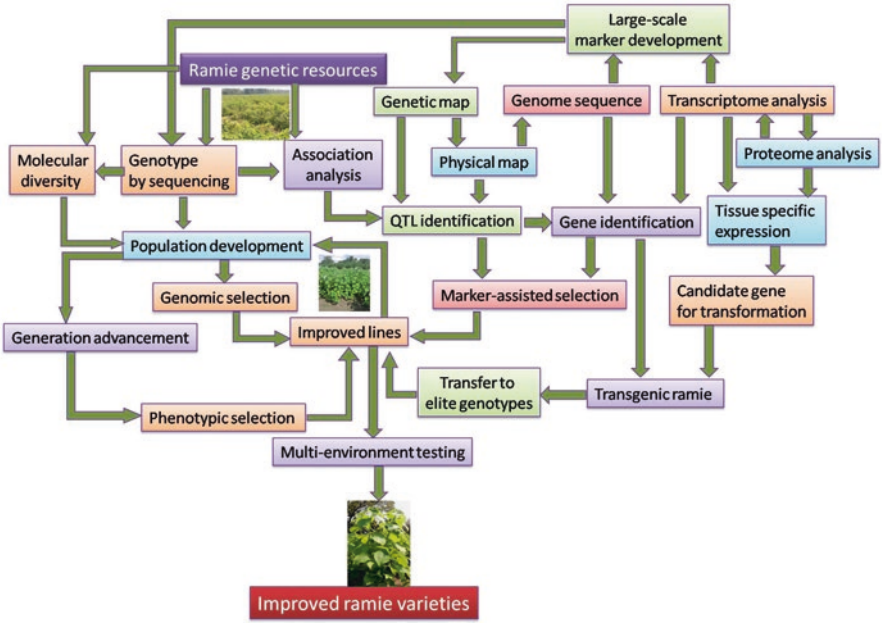

Fig. 5.5 A pipeline for integration of molecular genetics and genomic researches in ramie breeding programs

5.5.1 DNA Marker Development

DNA markers are widely utilized in modern plant breeding programsfor population diversity analysis, identification of heterotic combinations, linkage map construction, QTL identification and marker-assisted selection for economically-important traits. A good number of marker systems have been utilized in ramie, primarily for germplasm characterization, diversity studies and population characterization. Initially, RAPD was employed to study genetic diversity analysis (Jie et al. 1999; Li et al. 2006). Later, other marker systems including SRAP (Liu et al. 2008), ISSR (Liu et al. 2006), SCoT (Satya et al. 2015), SSR (Zhou et al. 2005), random amplified microsatellite polymorphism (RAMP) (Zhou et al. 2004) and restriction site amplification polymorphism (RSAP) (Zou et al. 2012) have been utilized for various purposes including population genetic analysis, core collection development and relatedness of accessions. In addition, SRAP has been utilized for differentiating polyembryonic seedlings of ramie (Wen et al. 2011) and ISSR has been used for differentiating male sterile lines of ramie from male fertile lines (Hou et al. 2006). With the development of genomic research, marker development in ramie has progressed at a rapid pace. Liu et al. (2013a, b, c) developed a set of 1827 EST-SSR markers from the ramie transcriptome. The majority of these SSRs were di-nucleotide and tri-nucleotide repeat motifs. They further utilized 100 SSRs, mostly present within transcription factors, to study genetic diversity within a set of 24 cultivars of

diverse geographical origin. Results revealed that 19 of these cultivars clustered together, which indicates low genetic variability of ramie cultivars. SSR markers have also been utilized for population structure analysis and association mapping for fiber yield and related characters (Luan et al. 2017). Markers showing strong association with plant height, stem diameter and fiber percentage have been identified that can be utilized for marker-assisted selection.

5.5.2 Genetic Map Construction and QTL Identification

Up to now, two genetic maps have been developed in ramie. The first genetic map was based on SSR markers spanning a length of 2265.1 cM harboring 132 loci (Liu et al. 2014). The mapping population was developed by crossing two heterozygous parental lines ZZ1 and QYZM, followed by developing a F_2agamous line (FAL) population. Based on phenotypic and genotypic analyses, a total of 33 QTLs for the 5 fiber yield-related traits (fiber yield, stem number, stem length, bark thickness and stem thickness) could be positioned on it. Later, a SNP based high resolution map was developed from a FAL population derived from cvs. Qingdaye and Zhongzhu1 containing 4338 SNPs distributed over a length of 1942 cM (Liu et al. 2017). Five QTLs were identified for bark thickness with a LOD of 5.2–8.8, while 4 QTLs were mapped for fiber yield trait (LOD: 4.7–6.3). One of the bark thickness QTLs, *qBT4a*was further characterized and a MYB transcription factor was found to be the candidate gene for this locus.

5.5.3 Transcriptome Analysis

Transcriptome sequencing using NGS tools is increasingly being used for gene discovery and identification of tissue or condition-specific gene expression. In ramie, this tool has been extensively utilized during the past few years and led to the discovery of a large number of genes. NGS-based whole transcriptome sequencing has been used in ramie for various purposes, including discovery of new genes, understand phloem, xylem and fiber development, to identify genes favored under domestication, study of the response of biotic and abiotic stresses, understanding physiological processes like hormone signaling and generation of genomic resources like transcription factors, simple sequence repeats and single nucleotide polymorphisms (Table 5.5). A total of 17,322–47,565 unigenes have been functionally annotated from these studies, which serve as valuable biological sequence resources for molecular genetic analysis in ramie.

Many of these studies have identified and demonstrated the role of specific genes or gene families in developmental and stress response processes. By comparison of xylem- and phloem-expressed genes, Chen et al. (2014a, b) observed that more genes (10,870) were up-regulated in phloem than in xylem (4440 genes), which

Table 5.5 Transcriptome sequencing associated studies in ramie

Purpose	Tissue/organ/ condition	No. of unigenes identified	Average unigene length (bp)	Annotated unigenes	References
Gene discovery related to cellulose synthesis	Leaves, root, stem bast, stem xylem and stem shoot	43,990	824	34,192	Liu et al. (2013a, b, c)
Response to drought stress	Drought stress (DS) / control (CO)	328,806 (CO) and 340,187 (DS) tags	Not reported	23,912	Liu et al. (2013a, b, c)
Study of bast fiber development	Shoot and bark	58,369	1343	18,006	Chen et al. (2014a, b)
Study of vascular development	Phloem and xylem tissue	50,434 (phloem)/36,710 (xylem)	635	15,219 (phloem)/ 17,322 (xylem)	Chen et al. (2014a, b)
Study of hormone signaling	In vitro regenerated tissue	43,222	Not reported	19,275	Huang et al. (2014)
Study of domestication	Whole plant of wild and cultivated spp.	56,932 (wild)/ 59,246 (cultivated)	871.4 (wild)/ 984.7 (cultivated)	26,686 (wild)/ 35,216 (cultivated)	Liu et al. (2014)
Study response to nematode infection	Infected plant	50,486	853.3	24,820	Zhu et al. (2014)
Study response to cadmium stress	Control and cd-stressed plants	56,932	817.4	26,686	Liu et al. (2015a, b)
Study effect of PEG treatment	Leaf samples at different time period	138,381	730.6	47,565	An et al. (2015)
Study response to insect damage	Leaf samples at different time period	58,093 (control)/ 52,165 (damaged)	1278 (control) / 1278 (damaged)	26,759 (control)/ 19,988 (damaged)	Zeng et al. (2016)
Adaptation to aquatic environment	Root	43,541	966	31,066	Gao et al. (2017)
Gene discovery	Shoot apex	70,271 scaffolds	Not reported	20,835	Al-Ani and Deyholos (2018)

suggests an enhanced phloem-specific activity during vegetative growth of ramie. Many of these genes were related to RNA biosynthesis, which indicates higher transcriptional activity in phloem tissue. Similarly 26 genes related to the auxin signaling pathway and 11 cytokinin receptors have been identified that are differentially expressed during organogenesis (Huang et al. 2014). Manipulation of expression of key genes like *AUX1*, *GH3*, *PIN* or *WUS* may increase in vitro regeneration response and facilitate transgenic cultivar development.

Transcriptome sequencing studies have also helped to identify a large number of transcription factors (TFs) differentially expressed under various stress conditions. Transcriptome analysis of ramie plants treated with polyethylene glycol (PEG) has identified 25 TFs of 5 different TF families, which were further validated through qRT-PCR expression under drought stress (An et al. 2015). Some of these TFs like NAC, bZIP and MYB are known to be involved in the drought response pathway in rice and *Arabidopsis*, thus these genes may be targeted for inducing drought tolerance in ramie.

5.5.4 Ramie Genome Sequence

The first draft genome sequence of ramie (cv. Zhongzhu 1) was recently deciphered; it has a size of 335.6 Mbp carrying 42,463 genes (Liu et al. 2017). The assembled genome covers 75% of the estimated genome size, with a GC content of 36.9%. Of the predicted genes, 40,922 were annotated by different functional databases, which were distributed in 161 cellular and metabolic pathways. The genome sequence data was anchored to a genetic map, mapping 4304 markers to the genome. Comparative genome analysis with close relatives identified over 4000 genes that are unique to ramie. The genome sequence helped to identify genes related to fiber biosynthesis, focusing on cellulose synthase (*CesA*) and *WAT1*. The genome analysis identified 16 *CesA* genes, of which 5 were unique to ramie. These genes also showed high expression in ramie stem bark, which provides strong evidence for their involvement in cellulosic fiber biosynthesis. Cellulose synthase has been shown to be involved in fiber development in other fiber crops, thus it is a potential target for improving fiber content. Phylogenetic relationship of *CesA* from different fiber crops show that the *CesA* of ramie has a close evolutionary relation with *CesA* of jute, another bast fiber crop (Fig. 5.6a).Structural modeling of ramie *CesA* protein also revealed high structural similarity with known cellulose synthases, which further establishes its functionality as a cellulose polymerization enzyme (Fig. 5.6b). *WAT1* was previously shown to be under positive selection during domestication of ramie; a total of 3*WAT1*-related genes with conserved domains were identified from the genome that may also be involved in ramie fiber biosynthesis (Liu et al. 2017).

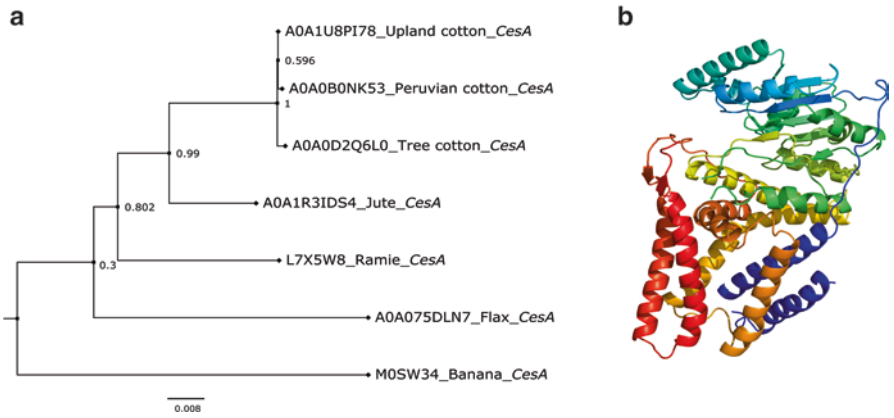

Fig. 5.6 (**a**) Phylogenic relation of cellulose synthase (CesA) present in fiber plants. (**b**) A 3-D model of ramie CesA protein

5.6 Mutation Breeding

In addition to traditional clonal breeding approaches, mutation breeding has also been pursued for ramie genetic improvement. Typically, gamma ray (Co^{60}) has been applied to induce mutation in seeds and rhizomes, followed by evaluation and selection of superior progenies. For treatment with gamma ray, 5.16 C/kg radiation (Co^{60}) for dry seed and 2.06 C/kg radiation (Co^{60}) were found to be semi-lethal (Guo and Yang 1996). The rate of mutation in seed and rhizome treatment were observed to be 3 and 5%, respectively. Only a few cultivars could be developed and recommended for cultivation using the mutation breeding approach. Notable among these are cv. Xiangzhu 2 and cv. 74–69, which were developed by irradiation of seeds of cv. Xiangzhu 1 (Luo et al. 1991). Xiangzhu 2 is a high fiber yielding mutant cultivar. The mutant cv. 74–69 exhibited about 40% increase in yield and better fiber fineness over check cv. Luzhuqing. It was also found to be resistant to root rot nematode and anthracnose (*Colletotrichum boehmeriae*). Another variety, Minsizhu No. 1, has been developed and released in 2012 by gamma ray irradiation of Phinghe ramie (Yao et al. 2017).

5.7 Hybrid Development

A number of hybrids have been developed in ramie by crossing two different parental lines. As the parental lines are clonally propagated and are cross-pollinated, they are expected to be highly heterozygous. Therefore, ramie hybrids resulting from crossing two heterozygous parental lines are expected to be heterozygous and heterogeneous, which often exhibit inbreeding depression. Although some

cross-combinations exhibit over 20% heterosis, most F_1s are of intermediate types (Yang and Zhu 1997). Inbred development is a prerequisite for hybrid development, but due to high heterozygosity, inbreeding depression is very high in ramie. Successive inbreeding for 5–6 generations often affects the survival of the genotypes. Thus a short inbreeding cycle is advantageous for isolation of superior clones. Peng (1993) showed that during selfing, selection based on petiole and pistil color of parental lines for two generations may be advantageous for developing inbred rather than overall evaluation of the progeny lines.

Since ramie is wind pollinated, it is extremely difficult to distinguish a hybrid seed from a self-seed unless some mechanism to prevent of self-pollination is developed. Male sterility ensures seed set only by non-self-pollen, thus it has been targeted for a long time to develop hybrids. As ramie can be easily maintained by clonal propagation, development of a maintainer line is not required for maintenance of male sterility. This makes a hybrid development program practically a two-line system. Furthermore, restoration of fertility is also not mandatory, since seed is not the economic product and the F_1can be maintained by clonal propagation for an indefinite period. Cytoplasmic male sterility was first identified in ramie in the 1960s–1970s from local landraces. Later, fertility restorer lines were identified (Liu et al. 1998) and male sterile lines like C-26 were utilized in ramie breeding programs (Zhang et al. 2005). Morphological characterization of the male sterile lines showed that these lines produce few smaller male flower buds and the anthers are devoid of pollen grains. Moreover, in some lines female flowers start appearing only when male inflorescences start to wither. By investigating microsporogenesis in a male sterile line MS-R29, Pierozzi et al. (2008) identified that despite normal meiotic events, the tetrads of the male sterile lines started degenerating, resulting in failure to develop pollen. The male sterility systems in ramie also exhibited differential sensitivity to both photoperiod and temperature. Liu et al. (2011) recorded the response of several male sterile ramie genotypes under different photoperiod and temperature regimes. They observed that a photoperiod of 9.5–12.5 h (short day) accelerates reproductive development and reduces the duration of the reproductive period. High temperature, on the other hand reduces the vegetative growth period. Therefore, a combination of short photoperiod and high temperature would reduce both the vegetative and the reproductive period. However, a differential response among the male sterile lines to both these environmental stimuli suggests that hybrid breeding programs in ramie using male sterility should be carefully planned considering the season and the environment.

To understand the molecular basis of the cytoplasmic male sterility system in ramie, two studies were undertaken in 2012, led by F. H. Liu of the Yunan University at Kunming, China, using male sterile line SS370 and its restorer Xiyeqing. The first study (Huang et al. 2012) reported cloning of mitochondrial *coxII* and *atpA* genes. The authors observed higher expression of *atpA* but not *coxII* at full bloom stage, suggesting *atpA* might be involved in inducing male sterility. In the second study (Liu et al. 2012) mitochondrial *atp6* and *atp9* genes were cloned. Based on higher expression of *atp9* at full bloom stage and deletion of *atp9* at 3′-end, it was

suggested that *atp9* was responsible for male sterility mechanism. Despite using the same genetic material, these two studies provided contradictory and inconclusive results. At present the molecular basis of male sterility in ramie remains elusive. However, this has not limited the development of hybrid cultivars using male sterile lines.

A number of hybrid ramie cultivars have been developed to date. An example of development of a two-line hybrid cultivar using male sterility is Chuanzhu-11, which was created by crossing male sterile line C-9451 with restorer line R79–20. This hybrid was released in 2007 and has since been widely cultivated in the Yangtze River Basin. However, a study by Huang et al. (2014) showed that the fiber yield of cv. Chuanzhu-11 was inferior to the male sterile parent under both low and high fertility conditions.

5.8 Conclusions and Prospects

At present most of the natural fibers except cotton are fighting for survival against the challenges of man-made fibers. As industrial crops, cultivation depends largely on market demand, which fluctuates considerably. Ramie being a perennial crop requires long-term investment by the farmer. Shifting from an agricultural to a non-agricultural occupation is a general transition observed in the ramie farming areas of China, which has greatly affected the production of this crop. Ramie fiber has high value and high demand in the international market. Despite this, farmers have become reluctant to cultivate the crop in recent years, as profitability has declined due to increased cost of production. Efforts to establish ramie cultivation in Europe and the USA have failed miserably, despite high demand for the fiber in the international market, which was principally due to problems and costs associated with fiber extraction. Despite great advances in science and technology, no effective improvement has been achieved in cost-effective and user-friendly machinery design for separation of the fiber from the bark, which is the principal bottleneck for areal expansion of this crop. The next major problem is associated with removal of the gummy substances from the fiber, which not only adds to the cost of cultivation but also pollutes the environment. Various microbial and enzymatic degumming technologies have been proposed and novel approaches like nanogenerator are being tested (Li et al. 2016); yet most of these technologies are still limited to the research stage. A thermostable alkaline pectate lyase that exhibit highest activity at pH 10.5 and 70 °C has been identified from *Bacillus clausii*, which holds good promise for degumming (Zhou et al. 2017); hopefully, these researches will help to advance cost-effective degumming technologies.

While the first issue is difficult to address by biological means, the genetic improvement of ramie with modern biotechnology can take up the challenge of reducing gum content in ramie fiber. Until now, no mutant line impaired in gum

synthesis has been identified. The natural population has a variation of gum content in the range of 20–32%. Genomic research in ramie has progressed exceptionally well in the last 2–3 years, and the draft genome sequence of ramie is available. Identification of genes that are involved in gum biosynthesis would bring out the crucial loci that can be targeted for manipulating the fiber-associated gum content in ramie. These loci can be tagged using the large pool of available DNA markers to assist breeding programs aimed to reduce gum content, development of mutants having low or minimal gum content, and for alternating gene sequences using genome editing technologies.

Improving the quality of fiber, particularly elasticity, is another challenging issue. Although ramie fiber has several excellent qualities, it suffers from two major drawbacks: low elasticity and higher roughness of the fiber. Unlike the lignocellulosic fibers of jute, flax or hemp, ramie fiber is comprised of pure cellulose. Therefore, biological manipulation of fiber structure would be based on an understanding of cellulose biosynthesis and the mechanism of microfibril orientation. The interplay of physical and biological sciences is absolutely essential to have a proper understanding of fiber formation in ramie and targeting genetic improvement for elasticity and other quality parameters.

Production stagnation is a major concern for ramie, as most of the cultivars developed have similar production potential. Since little improvement could be realized in fiber content of the bark, increasing yield related traits like plant height, stem diameter and lodging resistance are primary target traits for ramie improvement. Increasing fiber content per plant may not be a wise option, as more fiber cells per unit area of phloem will reduce the capacity of phloem translocation, thereby negatively affecting plant growth. Furthermore, the vascular differentiation mechanism in ramie is poorly known, and a number of questions related to xylem and phloem differentiation remains unanswered. It is not yet known whether these two tissues have synchronous or differential rate of development and which genes are involved in triggering these developments. Transcriptome analysis revealed that more genes express in ramie phloem than in xylem; these genes will be crucial to understand vascular development as well as fiber development processes. In essence, much basic research is needed in this crop to understand the key biological processes and their influence on fiber growth and development. Without such an understanding, random biotechnological interventions or plant breeding efforts based on intelligent guesswork may not be helpful to achieve breeding objectives. Finally, cultivar development for alternate use of ramie in addition to fiber would be a key step towards better utilization of the crop. A number of cultivars for feed ramie have already been developed, which is the first step towards diversification of economic use of ramie. More cultivars are expected to be developed for specific end-uses; for example, high flavonoid or alkaloid contents or biofuel production. Thus the future of ramie lies in better utilization of biomass in addition to fiber production under a profitable, industry-supported farming system.

Appendices

Appendix I: Major Research Institutes and Online Information Resources Relevant to Ramie

(A) **Major research institutes**

Institution	Specialization and research activities	Contact information and website
Institute of Bast Fiber Crops (IBFC), Changsha, Hunan, People's Republic of China	Research on bast fiber crops	http://www.caas.cn/en/administration/research_institutes/research_institutes_out_beijing/hunan_changsha/77937.shtml
Indian Council of Agricultural Research-Central Research Institute for Jute and Allied Fibres, Barrackpore, Kolkata, India	Research on improvement of jute and allied fiber crops, monitoring, coordination, dissemination of technologies and capacity building.	http://www.crijaf.org.in/
Indian Council of Agricultural Research-National Institute of Natural Fibre Engineering and Technology, Kolkata, India	Research on processing natural fibers and their agro-residues, development of value added products and quality assessment, skill development and business incubation service on natural fiber technologies.	http://www.nirjaft.res.in/
Huazhong Agricultural University, Wuhan 430,070, People's Republic of China	Multi-disciplinary education and research	http://www.hzau.edu.cn/en/HOME.htm
Hunan University, Changsha 410,082, People's Republic of China	Multi-disciplinary education and research	http://www-en.hnu.edu.cn/
University of Pisa, via S. Michele degliScalzi 2, 56,124 Pisa, Italy	Multi-disciplinary education and research	https://www.unipi.it/
InstitutoAgronômico Campinas (IAC), CPDP RecursosGenéticosVegetais. Av.Barão de Itapura 1481. Caixa Postal 28. Campinas, SP, Brasil.	Multi-disciplinary education and research	www.iac.sp.gov.br
Universidade de Brasilia, 70,919 Brasilia-DF, Brazil	Multi-disciplinary education and research	https://www.unb.br/

(B) **Online resources**

Resource Name	Web address
SRX2843758: Sequence, assembly, and characterization of ramie.	https://www.ncbi.nlm.nih.gov/sra/SRX2843758[accn]
SRX2830696: Sequencing reads for SNP discovery.	https://www.ncbi.nlm.nih.gov/sra/SRX2830696[accn]
SRX1334525: Transcriptomic basis to nitrogen deficiency- T13-C	https://www.ncbi.nlm.nih.gov/sra/SRX1334525[accn]
SRX843088: GSM1585374: CH; *Boehmeria nivea*; RNA-Seq	https://www.ncbi.nlm.nih.gov/sra/SRX843088[accn]
SRX591248: Transcriptome of ramie xylem	https://www.ncbi.nlm.nih.gov/sra/SRX591248[accn]
SRX591247: Transcriptome of ramie phloem	https://www.ncbi.nlm.nih.gov/sra/SRX591247[accn]
SRX523226: Root under the severe drought stress	https://www.ncbi.nlm.nih.gov/sra/SRX523226[accn]
SRX523181: Leaf under moderate drought stress	https://www.ncbi.nlm.nih.gov/sra/SRX523181[accn]
SRX522560: Transcriptomic comparison reveals the patterns of selection in domesticated and wild ramie (*Boehmeria nivea* L. Gaud)	https://www.ncbi.nlm.nih.gov/sra/SRX522560[accn]
SRX516609: Transcriptome of mixed sample from petiole at different culture stage	https://www.ncbi.nlm.nih.gov/sra/SRX516609[accn]
SRX507188: Ramie fiber transcriptome-bottom of bark	https://www.ncbi.nlm.nih.gov/sra/SRX507188[accn]
SRX507187: Ramie fiber transcriptome-middle of bark	https://www.ncbi.nlm.nih.gov/sra/SRX507187[accn]
SRX507186: Ramie fiber transcriptome-top of bark	https://www.ncbi.nlm.nih.gov/sra/SRX507186[accn]
SRX473608: Transcriptome of ramie hairy roots	https://www.ncbi.nlm.nih.gov/sra/SRX473608[accn]
SRX180727: De novo assembly and characterization of transcriptome using Illumina paired-end sequencing and identification of CesA gene in ramie (*Boehmeria nivea* L. Gand)	https://www.ncbi.nlm.nih.gov/sra/SRX180727[accn]

Appendix II: Genetic Resources of Ramie

Cultivar	Important traits	Cultivation location
Huazhou 1	High fiber productivity	China
Huazhou 3	High fiber productivity	China
Huazhou 4	High fiber productivity	China
Huazhou 5	High fiber productivity	China
Chuanzhu 4	High fiber productivity	China
Zhongzhu-1	High fiber productivity, resistance to insect-pests, nematodes and diseases	China
Zhongzhu-2	High fiber productivity	China
Zhongzhu-3	High fiber productivity, early maturity	China
Zhongsizhu no.1	High protein, fiber and vitamin	China
Minsizhu no. 1	High forage yield	China
R 67–34	High fiber productivity	India
R 1411	High fiber productivity, low gum content, resistance to Indian red admiral caterpillar and leaf folder	India
Florida selection	High fiber productivity	USA

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Chapter 6
Genetic Improvement of Guayule (*Parthenium argentatum* A. Gray): An Alternative Rubber Crop

Hussein Abdel-Haleem, Zinan Luo, and Dennis Ray

Abstract Among the more than 2000 plant species known to synthesize rubber, guayule, *Hevea* and *Taraxacum kok-saghyz* produce commercial grade rubber and latex. Guayule (*Parthenium argentatum* A. Gray), originates from the Southern Texas and Northern Mexico deserts, and is receiving wide attention as a natural rubber crop that could successfully grow in arid and semiarid regions. Continued improvement of guayule for higher biomass, rubber production and resistance to biotic and abiotic stresses, as well as maximizing agronomic practices are necessary to meet the increasing demand of the guayule rubber industry. Early domestication and commercialization efforts have all centered on using natural guayule stands and unimproved germplasm as a source of natural rubber. However, limited and sporadic breeding efforts have slowed down guayule's genetic gains compared to other crops. This chapter summarizes the most recent breeding progress, biotechnological advancements, and agronomic practices to increase guayule rubber and other industrial byproducts. This provides plant breeders an insight into the status of guayule improvement and possible directions to speed up the breeding progress.

Keyword Agronomy · Biotechnology · Genetic improvement · Genetic variability · Guayule · Natural rubber · Resin · Sustainability

6.1 Introduction

Guayule (*Parthenium argentatum* A. Gray), family Asteraceae (or Compositae), originates from north-central Mexico and southwest Texas in the USA (Hammond and Polhamus 1965). The potential supply shortage of natural rubber and unstable

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prices in rubber-importing industries make it imperative to exploit rubber production from alternative rubber-producing crops. Guayule is one of these resources, which could generate high molecular-weight and hypoallergenic natural rubber (Cornish 1996).

Most current breeding methods in guayule are restricted to sporadic single-plant selection, mass selection, recurrent selection and interspecific hybridization (Coffelt et al. 2015). No comprehensive and systemic molecular breeding efforts have yet been carried. Undeniably, conventional breeding studies have made some progresses in guayule, such as improved rubber yield, disease resistance and plant vigor; however, the nature of facultative apomixis and polyploidy, the narrow genetic variation in current germplasm, the challenges in agronomic practices, limited resources for sexual reproduction and unsustainable funding resources make it very difficult to continue guayule breeding efforts (Coffelt et al. 2015; Thompson and Ray 1988). To overcome these constraints, along with the conventional breeding, advanced biotechnological or molecular breeding methods need to be adopted. Recent advanced biotechnological tools such as tissue culture, genetic engineering, next generation sequencing (NGS) and flow cytometry have been used in guayule to accelerate the improvements of desired traits.

This chapter summarizes the most recent achievements in breeding progress, agronomic practices, biotechnology, genetics and genomics for guayule, and reports the applications of these advanced tools towards the improvement of rubber production, abiotic stresses and disease resistance. The chapter also discusses the problems and challenges facing guayule breeding, which will provide plant breeders with possible directions to speed up the breeding progress.

6.1.1 Origin and Distribution

Guayule (*Parthenium argentatum* A. Gray) is a perennial semiarid shrub native to the drylands of north-central Mexico in the states of Coahuila, Chihuahua, Durango, Zacatecas, San Luis Potosi and Nuevo Leon, and to the Trans Pecos area of Southwest Texas (Stockton Plateau and Big Bend Region) in the USA, covering some 34 million ha (Hammond and Polhamus 1965). Indigenous US guayule populations are scattered over a wide range of climatic conditions (Foster and Coffelt 2005), on plateaus of 1200–2100 m elevation, with an annual rainfall of 25–38 cm, but restricted to calcareous soils (Hammond and Polhamus 1965). Although guayule grows better in regions with sufficient rainfall, competition from other plants (e.g. grasses and shrubs) makes it hard to survive (Hammond and Polhamus 1965). Temperatures throughout its habitat ranges from 0–49 °C and the height of guayule plants barely exceeds 1 m in their native areas (Downes 1986; Goss 1991).

6.1.2 Botanical Classification

Guayule is a flowering shrub belonging to the Asteraceae, which is a large family with 1911 genera and 32,913 accepted species (List 2013). The Asteraceae are easily distinguished from other plant families mainly due to their characteristic inflorescence, which appears to be a single flower but is actually a cluster (composite) of smaller flowers (Judd et al. 2007). Several taxa in this family, such as *Taraxacum kok-saghyz* (rubber dandelion) and *Solidago speciosa* (goldenrod), can also produce considerable amounts of natural rubber and are potential natural rubber resources (Goss 1991). However, of the 17 species of *Parthenium*, only guayule produces appreciable amounts of rubber (Coffelt et al. 2015).

The genus *Parthenium* belongs to the subtribe Ambrosiinae, which includes the *Ambrosia, Parthenice, Dicoria, Iva, Hymenoclea* and *Xanthium* genera. However, *Parthenium* can be distinguished from the other genera because it is insect pollinated. For instance, the very closely related genus, *Parthenice*, does not produce nectar and can only be pollinated by wind. Other closely related genera such as *Dicoria* and *Iva*, even with a similar capitula, are also wind pollinated. The majority of species in the subtribe Ambrosiinae accumulate large amounts of leaf sesquiterpene lactones, which are also found in *Parthenium* (West et al. 1991).

The 17 species of Parthenium range morphologically from small trees (e.g. *P. tomentosum* var. *stramonium*) to small shrubs (e.g. *P. incanum*) (Rollins 1950) (Fig. 6.1). *Parthenium hysterophorus* is a weedy species in many countries and its

Fig. 6.1 Guayule and related species. *Parthenium tomentosum, P. argentatum* and *P. incanum* grown at the Maricopa Agricultural Center, Maricopa, Arizona. (Photo courtesy of Greg Leake, USDA Agricultural Research Service)

sesquiterpene compounds can cause contact dermatitis (Rodriguez et al. 1976). However, the sesquiterpene generated in guayule has not yet been found to cause contact dermatitis (Downes 1986; Rodriguez et al. 1981). Trichome morphology of the desert species of *Parthenium* is also specialized. Unlike the prominent uniseriate conical trichome observed in tree species such as *P. tomentosum*, *P. schottii* and *P. fruticosum*, and the very long uniseriate, whiplike trichomes in *P. incanum* and *P. rollinsianum*, guayule is covered with a layer of unique T-shaped trichomes (West et al. 1991).

The general morphology of guayule is summarized by Goss (1991) and Hammond and Polhamus (1965). The guayule taproot system may lose its prominence and give way to intricate system of fibrous laterals and their branches, which mainly spread in the upper 15 cm of soil but can extend up to 3 m or more to facilitate soil moisture utilization under conditions of very shallow water penetration (Hammond and Polhamus 1965; Muller 1946). Another factor that helps guayule survive damage caused by meager rainfall, erosion or harvesting is the formation of adventitious shoots (*retoños* in Mexico). On shallow exposed roots the basal portion of the retoños will then develop and extend the root system (Hammond and Polhamus 1965). Guayule leaves are up to 10 cm long, which is three times their width (Downes 1986; Fangmeier et al. 1984). Leaf size and shape vary among guayule accessions and are usually used as a morphological characteristic (Fig. 6.2).

Leaf size and shape are largely affected by water supply (Downes 1986; Fangmeier et al. 1984). Insufficient water supply can lead to shriveling and shedding of older leaves, but younger leaves can survive when water supply is recovered (Downes 1986; Fangmeier et al. 1984). The T-shaped trichomes in the leaf surfaces produce the light silvery gray sheen color characteristic of the guayule plant (Downes 1986; Hammond and Polhamus 1965). The primary stem terminates with the formation of the first inflorescence, but further growth will occur at the uppermost buds by developing new branches. Elongation of the uppermost lateral buds results in a symmetrical and closely-branched shrub (Lloyd 1911). Flowers are borne in heads with 5 fertile ray-florets, each with 2 subjacent sterile disk-florets, which contain an abortive pistil and fertile stamens (Downes 1986; Hammond and Polhamus 1965).

Flowering in guayule is largely stimulated by favorable water conditions. Under irrigation, flowering is possible from early spring to late fall (Backhaus

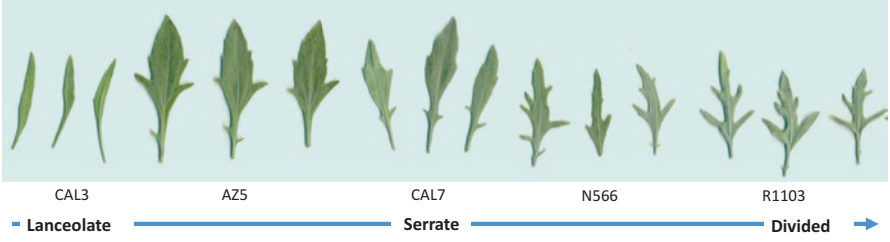

Fig. 6.2 Guayule leaf shape and size from different accessions

et al. 1979; Downes 1986). As mentioned, guayule is both wind and insect pollinated. Ladybird beetles, lygus bugs, cucumber beetles and honeybees are all effective carriers of guayule pollen. The mature fruit contains an embryo enclosed in two seed coats, which inhibits or delays germination (Downes 1986; Hammond and Polhamus 1965).

Rubber particles in guayule are mainly located in the cortical parenchyma cells in the stems and roots (Coffelt et al. 2015). In mature guayule plants, rubber is found in vascular rays of the phloem and xylem while in young plants, rubber is mainly stored in the cortex, pith and vascular rays and resin canals (Benedict et al. 2010; Downes 1986). The rubber content is proportional to the amount of bark parenchyma tissue (Artschwager 1943), and usually reaches its lowest point in July and increases to a peak in January in 2-year-old guayule plants (Benedict et al. 2010). Reportedly, low temperature in fall/winter can stimulate formation of rubber-producing cortical parenchyma and the rubber particles in it (Benedict et al. 2009, 2010).

6.1.3 Domestication, Selection and Early Improvements

Guayule domestication and commercialization has experienced four stages, during which sporadic selection and breeding efforts occurred (Coffelt et al. 2015). The initial commercialization attempt began at the beginning of the twentieth century due to the high price of imported *Hevea* rubber from the Amazon Region (Bonner 1991). At that time, wild guayule stands were harvested in Mexico and several extraction methods were evaluated (Ray et al. 2005). Natural rubber production from guayule in Mexico reached a peak in 1910, during which 24% of the total natural rubber imported into the USA was guayule rubber from Mexico (Bonner 1991). However, production by the Continental Rubber Company ceased in 1912 due to the Mexican Revolution. Commercialization efforts then moved across the border to Arizona and California (Ray 1993), where agronomic and breeding efforts of collecting and selecting plants and their seeds from wild stands continued for 17 years, but halted again in 1929, due to the Great Depression (Bonner 1991; Ray 1993).

The second domestication and commercialization effort occurred with the Emergency Rubber Project (ERP) during World War II. This effort led to the planting of over 13,000 ha of guayule at 13 different locations, but ceased with the end of the war, and the renewed availability of natural rubber from Southeast Asia, as well as the development of synthetic rubber (Huang 1991). The four-year ERP was successful, realizing the production of some 1400 mt of guayule rubber, and provided significant knowledge regarding basic biology, agronomy, and was the origin of the guayule germplasm upon which current breeding programs are based (Hammond and Polhamus 1965; Thompson and Ray 1988).

The third major effort to commercialize guayule started in the late 1970s because of the quadrupling of crude oil price (Ray et al. 2005), which raised the fear of a

potential natural rubber shortage due to either natural disaster or political unrest in Southeast Asia. This fear induced the enactment of two laws by the US Congress: the Native Latex Commercialization and Economic Development Act of 1978 and the Critical Agricultural Materials Act of 1984 (Laws 95–592 & 98–284), providing for the support of guayule projects for about 12 years (Huang 1991; Ray et al. 2005). A tremendous amount of work, although not as concentrated as the second effort, was accomplished during this third effort, resulting in significant yield increases and refined cultural practices to fit modern mechanized agriculture (Foster and Coffelt 2005; Ray 1993; Ray et al. 2005; Whitworth and Whitehead 1991). This third effort also demonstrated that guayule could be planted, cultivated, harvested and processed as an alternative natural rubber resource to replace *Hevea* in high temperature applications, especially tires (Ray et al. 2005). However, as the political climate changed, this effort was also terminated.

With the occurrence of widespread life-threatening latex allergy from *Hevea* rubber products (Ownby et al. 1996), it was realized that guayule latex was a perfect potential replacement due to its hypoallergenic feature and similar rubber quality (Cornish 1996; Siler et al. 1996). This opened the fourth and present commercialization effort in the 1990s and made it closer to reality when Yulex Corporation (www.Yulex.com) was granted the exclusive license to US Patent No. 5580942 (Cornish 1996) and to US Patent No. 5717050 (Cornish 1998) on guayule latex processing and products. During this process, a business institution was established, a financial base developed, seed collections of promising lines increased, a latex extraction processing plant constructed and large areas of plantings were in progress to support industry demand (Coffelt et al. 2015). Recently, the National Institute of Food and Agriculture (NIFA) funded a USD 6.9 million grant to a research consortium led by the Cooper Tires, and an Agriculture and Food Research Initiative (AFRI) grant for USD 15 million to a group led by the University of Arizona. Both grants include collaboration among industry, academic and governmental entities. In addition, other companies, such as Bridgestone Americas (www.bridgestone.com), Cooper Tires (http://us.coopertire.com/), and Versalis S.P.A. (https://versalis.eni.com/irj/portal/anonymous?guest_user=anon_en) are investing in research activities to develop new and/or improved germplasm and production practices, and rubber quality for tire manufacturers. Unlike previous commercialization efforts, this most recent one is mainly driven by commercial companies instead of government institutions. The interest by these commercial companies is not only for guayule natural rubber and/or latex products, but also due to a predicted increase in natural rubber demand and the potential shortage in the *Hevea* rubber supply resulted from plant diseases and/or political instability in the rubber-producing countries in Southeast Asia. Moreover, other byproducts from guayule such as termite-resistant wood products (Nakayama et al. 2001), perfumes (Battistel et al. 2018), and bioenergy products (Kuester 1991), make guayule an attractive crop in arid and semiarid regions.

6.2 Guayule Cultivation Strategies

For guayule, as in any crop, increased yield will come from continuous improvement through genetic enhancement and cultivar development strategies, and, at the same time, optimization of agronomic practices for maximum biomass and rubber production (Foster and Coffelt 2005; Ray et al. 2005). Guayule agronomy and agronomic practices are described below.

6.2.1 Planting Method and Plant Establishment

Transplanting seedlings is a reliable means to establish guayule stands, although direct seeding, using seed conditioning techniques and precision planting, has been successful on an experimental scale in Texas, New Mexico and Arizona (Foster and Coffelt 2005). Conditioning facilitates seed emergence and to overcome dormancy, resulting in stands with a more rapid and uniform manner (Chandra and Bucks 1986; Foster and Coffelt 2005). The survival rate of transplants can reach 95% at El Paso, Texas and in Arizona, when frequent irrigations are applied (Bucks et al. 1984; Foster and Coffelt 2005; Miyamoto and Bucks 1985). Although direct seeding has advantages over transplanting in terms of cost, improvements are needed before applying this technique on a commercial scale. Direct seeding has been successful in establishing the crop, but can be improved by conditioning seeds with polyethylene glycol (PEG), gibberellic acid and light; by precision planting to accurately plant seeds on the soil surface; by precise irrigation strategies and by enhancement of seed viability and germination rate through breeding (Foster and Coffelt 2005).

6.2.2 Irrigation

Even though guayule is desert shrub, it is an inefficient water user, requiring 1000–1300 mm of water per year for maximum rubber production (Nakayama et al. 1991). Rubber yields in transplants are positively correlated to irrigation (Hunsaker and Elshikha 2017). Therefore, the quantity of optimal irrigation depends both on growing conditions and production practices (Foster and Coffelt 2005; Maas et al. 1988; Miyamoto et al. 1984a,b; Retzer and Mogen 1946). Water quality and water stress are two limiting factors affecting guayule production and rubber yield. It has been reported that water salinity exceeding 1.0 ds/m results in the failure of plant establishment and that above 4.5 ds/m leads to mortality during early plant growth (Maas et al. 1988; Miyamoto et al. 1984a,b; Retzer and Mogen 1946). However, salt accumulation can be minimized when off-centered or double-row planting is coupled with alternate row watering (Foster and Coffelt 2005). Water stress can increase rubber content (percent rubber), but decreases shrub biomass (Hunter and Kelley

1946). In order to clearly define the interaction between water stress and rubber yield, the crop water stress index (CWSI) was applied to guayule (Nakayama and Bucks 1983). This index uses shrub canopy temperature and atmospheric vapor pressure deficits as they relate to plant water and soil water stresses. Based on this, several studies have reported that an inverse correlation was found between rubber yield and CWSI (Nakayama and Bucks 1984). Although the relationship varied during different guayule growing stages (Bucks et al. 1985a) and in varieties with different levels of drought tolerance, supplemental water is still needed to increase rubber yield and to shorten the growth cycle (Foster and Coffelt 2005).

6.2.3 Clipping

Guayule harvest includes digging whole plants or clipping the above ground biomass, as first suggested by Lloyd (1911). Initially clipping above ground, followed by digging the entire plant, can increase rubber productivity per unit area by allowing for early investment return to growers and distribute the cost of stand establishment across several years, as well as reduce the labor load of digging (Ray et al. 1986). Foster and Coffelt (2005) summarized clipping as the preferred harvesting method because rubber yield was greater in clipped plants than whole plants. Sequential clipping could also increase rubber yield compared to whole plant harvesting over the lifespan of a field. When clipping 2-year-old plants there were no yield differences between direct-seeded plants and transplants.

6.2.4 Fertilization

Guayule is reportedly a low user of soil nutrients; fertilizer requirements depend on soil fertility and plant-growing conditions (Foster and Coffelt 2005). The application of nitrogen fertilizer can improve plant growth and rubber productivity in both transplants and direct-seeded plants, more than other major nutrients. For example, plant height and width in transplants were significantly increased under nitrogen treatments compared to non-fertilized controls (Cannell and Youngner 1983). Seed biomass was also increased by applying nitrogen (112 kg/ha) in guayule production fields at Marana, AZ (Rubis 1983). However, to achieve maximum production, both sufficient irrigation and nitrogen applications were required in 2-year-old shrubs (Bucks et al. 1985b). Likewise, in direct-seeded guayule, nitrogen fertilizer application of 280 kg/ha (Hammond and Polhamus 1965) was shown to be beneficial in California, provided that heavy seeding rates were applied (Kelley et al. 1946). Briefly, the combination of sufficient irrigation, fertilizer supplements, sowing density, soil and climate conditions, all potentially affect the ultimate plant growth and rubber yield in guayule.

6.2.5 Weed Control

Hand weeding and post-emergence oil sprays were two major weed control strategies used in the 1950s. More recent weed control has focused on exploring different modern herbicides. A number of common herbicides and preplant incorporated herbicides were tested in the USA in California, Arizona, New Mexico and Texas, and in Australia (Foster and Coffelt 2005). Trifluralin was reported to efficiently control broadleaf and grass weeds in Arizona and California during the pre-emergence stage in guayule transplants (Elder et al. 1983; Siddiqui et al. 1982) and fluridone is a broad-spectrum herbicide that gave 91% weed control without injuring guayule plants (Foster and Coffelt 2005). DCPA (9.0 kg a.i./ha), bensulide (3.4 kg a.i./ha) and pendimethalin (0.6–1.1 kg a.i./ha) were shown to be effective in direct-seeded plants in Maricopa, AZ (Foster et al. 2002). Pendimethalin obtained a Special Local Needs registration for pre-emergence control of most annual grasses and broadleaf in Arizona (Agriculture 2003). However, no post-emergence treatments have been found to successfully control weeds in both transplants and direct-seeded plants, except during dormant periods (Ferraris 1986; Foster and Coffelt 2005; Siddiqui et al. 1982).

6.3 Germplasm Biodiversity and Conservation

Native people in Mexico discovered rubber in guayule centuries before its first commercial use, chewing guayule bark to produce rubber balls used in sports games. Commercial use began at the end of nineteenth century, when companies collected large quantities of guayule shrubs from natural stands and extracted rubber at industrial plants in Mexico and Texas. This led to a rapid depletion of natural stands and the guayule germplasm resources in these areas. With an interest in increasing the genetic diversity in the available guayule germplasm, several germplasm collections were made during the twentieth century, collecting guayule and related *Parthenium* species from their native habitats (Ray et al. 2005). Unfortunately, much of this recently-collected genetic material has been lost due to a reduction in research funds, the termination of guayule breeding programs, and/or reduced seed viability and low germination rates of collected seeds. The result being that few improved germplasm lines have come from these collections. Nevertheless, there has been improvement for rubber and resin concentrations, faster growth, higher biomass and disease resistance (Estilai 1985,1986; Ray et al. 1999; Tysdal et al. 1983), and these lines have been preserved.

Guayule and *Parthenium* accessions are curated at the USDA-ARS National Arid Land Plant Genetics Resources Unit (NALPGRU), Parlier, CA. The current USDA guayule collection includes 110 accessions of guayule (*P. argentatum*), 1 of *P. confertum*, 15 of *P. incanum*, 2 of *P. schottii*, 2 of *P. tomentosum var. stramonium* and 5 accessions of an unknown *Parthenium* hybrid. Not all of these accessions are

available upon public request due to low seed numbers and/or poor seed viability. To address the reduction in guayule genetic resources two points were addressed by the guayule research community; first, to explore and collect more guayule genetic material from wild populations and, second, to characterize the present USDA guayule collections. In 2005 the National Plant Germplasm System (NPGS) sponsored an exploration trip made by Terry Coffelt, Michael Foster and David Stout to collect new genetic materials from areas in Texas where guayule was previously reported. They were able to collect 16 accessions including 3 of guayule, 1 of *P. confertum*, 1 of *P. hysterophorus* and 10 of *P. incanum*. The NPGS is also sponsoring Hussein Abdel-Haleem and Claire Heinitz (USDA, guayule curator) to conduct an exploratory field trip around the Bend National Park in Texas during 2019, with the hope of collecting new genetic material.

There are efforts to characterize the present guayule collection both genetically and phenotypically. Recent studies indicate that the USDA guayule accessions have a wide range of ploidy levels including diploid (2n = 36), triploid (3n = 54), tetraploid (4n = 72) and pentaploid (5n = 90) (Gore et al. 2011; Ilut et al. 2015, 2017), with the improved germplasm mostly tetraploid. Using high throughput genotyping technology, genotyping by sequencing (GBS), Ilut et al. (2015, 2017) developed 50 K single-nucleotide polymorphism (SNP) genetic markers and used them to genotype 69 accessions of the USDA guayule collection and *P. incanum* to study genetic diversity and correlations within the collection.

As the available USDA guayule collection is now genotypically characterized, we initiated efforts to phenotypically characterize them under field conditions (Fig. 6.3), including variations in plant height (Fig. 6.4), biomass, and other agronomic traits. These populations will also be evaluated for rubber and resin production under field and stress conditions. These data will serve as a phenotypic atlas to better understand the collection and to help select parental material for future breeding programs (Fig. 6.5).

6.4 Traditional Breeding

Guayule has experienced intermittent research efforts since the first large-scale rubber extraction in 1888, but thus far no commercial fields have been developed anywhere in the world. Guayule yields were first improved by large-scale cultivation techniques instead of breeding. This is normal in new crops since plant breeding programs usually take many years to achieve genetic gains and many new crops lack sustainable funding support to continue plant breeding programs.

In addition, guayule is a perennial shrub, which usually takes more than 2 years for the first harvest. Other factors also hampered its breeding progress, including: the lack of nondestructive rubber/latex quantification techniques, narrow genetic variation among the currently available germplasm and asexual reproduction by facultative apomixis. In general, there are three continuous steps for guayule genetic

Fig. 6.3 An aerial image of USDA guayule collections planted at fields at Maricopa agricultural Center, Maricopa, AZ (Photo by Hussein Abdel-Haleem, USDA Agricultural Research Service)

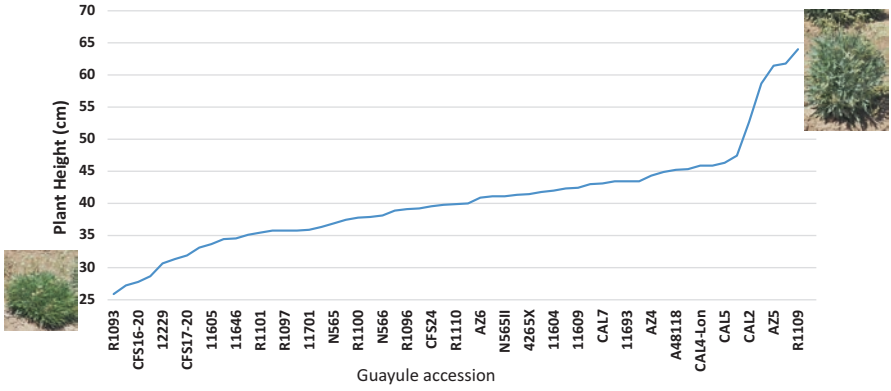

Fig. 6.4 Variations in plant heights of transplanted 1-year old guayule accessions planted under field growth conditions

improvement that begin with creating new genetic materials from different resources including commercial guayule varieties, improved breeding lines, old varieties, wild landraces and related wild species. This step is followed by crossing and selection, then testing the selected germplasm with desired trait(s) in common variety trials (Fig. 6.5).

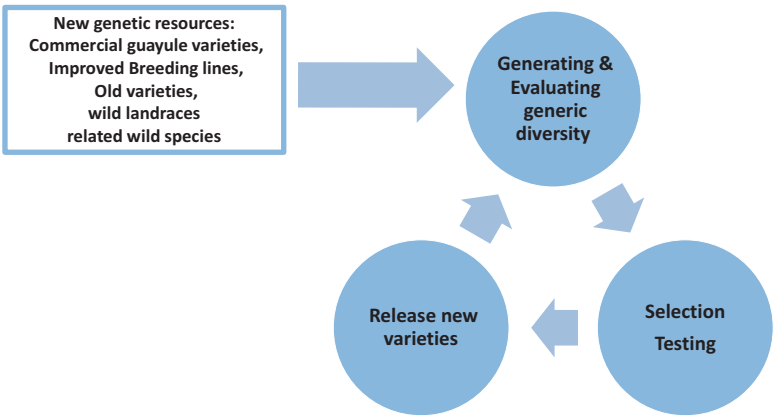

Fig. 6.5 Genetic improvement steps in a guayule breeding program

6.4.1 Guayule Genome Size and Reproduction System

6.4.1.1 Polyploidy

Due to the superiority of diploids in terms of biomass, vigor and disease resistance, it is not surprising that most of today's guayule germplasm collections consists of polyploids ranging from triploids to pentaploids (Estilai and Ray 1991; Gore et al. 2011) with the majority being apomictically reproducing triploids and tetraploids. Previous research suggested that apomictic polyploids are self-compatible, but do not always produce viable seeds. This is not a problem if a selected variety is grown just for rubber production and there is no need to maintain the plants as a seed source (Coffelt et al. 2015). However, it will become a problem if the breeding programs aim to maintain the selected variety by producing seeds.

6.4.1.2 Sexual Reproduction

Diploid guayule possesses a sporophytic self-incompatibility (SSI) system, in which the success or failure of generating viable seeds depends not only on the genotype of the stigma in the maternal genotype, but also on the genotype of the paternal sporophyte (pollen-producing plant). Although the self-incompatibility feature makes diploid guayule an obligate outcrosser and cannot generate homozygous progenies, the resulting high degree of heterozygosity in the populations facilitates long-term evolutionary adaptation in this species (Estilai and Ray 1991).

6.4.1.3 Apomixis

Most currently available guayule germplasm consists of triploid and tetraploid accessions, which are facultative apomicts (Coffelt et al. 2015; Estilai and Ray 1991). The meiotic division in apomictic guayule is blocked in the megaspore mother cells (MMCs), resulting in the unreduced embryos with the same chromosome complement as the maternal parent. However, unlike common obligate apomixis, facultative apomixis requires fertilization of male gametes to realize endosperm development and thus potentially results in four types of seeds: (1) asexual seeds derived from non-reduced female gametes without fertilization, (2) sexual seeds derived from non-reduced female gametes and fertilized by meotically reduced male gametes, (3) polyhaploid seeds from reduced female gametes without fertilization and (4) sexual seeds derived from reduced female gametes and fertilized by reduced male gametes (Cruz et al. 2017; Esau 1946; Estilai and Ray 1991; Gore et al. 2011; Hammond and Polhamus 1965). This complexity in apomictic progenies results in the presence of various ploidy levels and genotypes among progeny, leading to new genetic variation for further use in plant selection and breeding programs. Previous researchers hypothesized that at least three loci are involved in guayule apomixis: one inhibiting meiosis in MMCs, a second controlling fertilization and the third controlling seed development (Hammond and Polhamus 1965; Kupzow 1969; Powers 1945; Powers and Rollins 1945).

6.4.2 Breeding Objectives

Among all guayule breeding objectives, improving rubber yield has been paramount. Other breeding objectives such as rubber quality, resin yield, plant vigor and regrowth ability after clipping, disease resistance, drought tolerance and salinity tolerance are all important traits of interest, and should also be improved (Coffelt et al. 2015; Estilai and Ray 1991; Ray et al. 2010; Thompson and Ray 1988).

In order to breed for traits of interest, breeders need to first determine the heritability of these traits, whether they are controlled by single or multigene families, the environmental effects and the number of generations required to fix a trait. Rubber yield is equal to plant biomass multiplied by rubber concentration (%), meaning that either increasing plant biomass or rubber concentration can improve rubber yield. However, selecting for large biomass involves additional costs in harvesting, transportation and handling, resulting in reduced efficiency in the entire processing chain. Therefore, selecting normal-size plants with high rubber concentration seems to be a solution. This may be difficult because previous researchers have found that there was a negative correlation between rubber concentration and plant biomass in

guayule (Coffelt et al. 2015; Dierig et al. 1989; Thompson et al. 1988). However, fresh and dry weights, and plant height and width were found to be highly positively correlated with rubber yield (Dierig et al. 1989; Ray et al. 1993; Thompson et al. 1988), suggesting that plant biomass can be used as a primary selection index for rubber yield (Coffelt et al. 2015). The heritability of eight components of rubber yield was estimated in a study using parent-offspring regression in an open-pollinated population derived from single-plant selections (Ray et al. 1993). None of the parent-offspring regressions were significantly different from zero among all these components, indicating a low heritability for all these traits. In addition, a larger phenotypic variation was found among parents than progenies (Ray et al. 1993), which was probably compounded by the environmental effects since parents were 1 year older than the progenies (Coffelt et al. 2015; Dierig et al. 1989). Based on these results, family selections, either full-sibs or half-sibs, should be considered in future breeding programs rather than single-plant selections as described in this study (Ray et al. 1993).

Other than heritability, an accurate estimation of genotypic, environment effects and genotype × environment interactions is another aspect in improving rubber yield. A 2-year field trial in Maricopa, AZ showed that the main effects of location, line and plant age were significant, but the interactions were not significant for plant growth, biomass and latex content (Majeau et al. 2003). Moreover, the impact of environment on guayule was very significant, which accounted for over 50% of the phenotypic variation (Majeau et al. 2003). However, since only four lines were used in this field trial, the results might be overestimated. Some of the nonsignificant interactions may become significant with a larger or wider germplasm base (Majeau et al. 2003). Dierig et al. (1989) also observed significant environment effects within a single field trial. Small or slight genotype × environment interactions are desired in breeding programs since this suggests that variety selection can be done in one location without considering the impact of different environments on the lines and that selected superior lines could maintain their superiority in different locations (Coffelt et al. 2015). This has happened with several AZ lines, which gave similar results when evaluated under different locations such as in the USA, Spain, Australia, South Africa and China (Coffelt et al. 2005, 2015; Ray et al. 2010).

Other traits related to resin and latex have also attracted research interest. Thompson et al. (1988) found that rubber concentration was significantly correlated with resin concentration, meaning that simultaneously breeding for new lines with both high rubber and resin concentration is possible. This feasibility was confirmed by Ray et al. (1999). Improving both traits is important in determining the value of guayule end products for successful commercialization. Studies to explore the relationship between latex and rubber concentration, however, have not always been consistent (Coffelt et al. 2009a, b). A recent field trial conducted in six environments in Arizona and Texas showed moderate to high heritability and positive correlations among dry biomass, and rubber and resin content (Abdel-Haleem et al. 2018). The phenotypic variation, genetic variation and genotype by environment interactions were found to be significant in both

2-year- and 3-year-old plants, meaning that multiple-location field trials are required (Abdel-Haleem et al. 2018).

Cold tolerance identified in accessions 11,591 and N6–5 in a 3-year field trial on the Texas High Plains suggested that sufficient variation does exist in response to cold stress among contemporary germplasm and can be used to expand guayule planting areas (Foster et al. 2011). Other desirable characteristics to facilitate guayule commercialization include: improvements of drought tolerance, salt tolerance, seed quality, reduced postharvest degradation and the elimination of wild characteristics such as intermediate flowering, seed shattering, seed dormancy and complicated reproduction systems (Coffelt et al. 2015; Estilai and Ray 1991; Ray et al. 2010).

To conclude, although several field trials (Dierig et al. 1989; Estilai and Ray 1991; Majeau et al. 2003; Thompson et al. 1988) listed above were attempted in the recent decades, few studies have been consistently continued due to the lack of funding resources. Therefore, continuous multiple-year yield trials under various environmental conditions need to be implemented in order to accurately evaluate the phenotypic effects, genotypic effects, environmental effects and genotype × environment interactions for traits of interest in future breeding programs. Accurate phenotyping is of great significance but not easy, especially for rubber/resin/latex traits, because the quantification analyses are often time consuming, labor intensive and cost-inefficient, which largely limits the sample size that can be processed. In addition, moisture content, deterioration of latex and rubber during the processing procedures can obscure results in rubber and latex quantification (Teetor et al. 2009). Therefore, rapid and accurate phenotyping strategies such as near infrared (NIR) spectroscopy techniques could be used to speed up the quantification processes (Kopicky 2014). However, establishing a reliable NIR model with high R^2 is another important issue to be addressed.

6.4.3 Traditional Breeding Methodologies

6.4.3.1 Single-Plant Selection

Single-plant selection among apomictic polyploids has been the most widely used approach in guayule breeding. This could be the simplest and most efficient way when the heritability of desired traits is high because genetic gains can be increased rapidly in a relatively short time, but in the long run, only modest genetic gains can be achieved due to the lack of new genetic combinations. Therefore, whether this method succeeds or not depends on several factors, including (1) the heterogeneity of the population, (2) the degree of genetic effects and (3) population size that can be effectively screened (Coffelt et al. 2015; Thompson et al. 1988). Previous studies successfully utilized this method to increase annual rubber yields from 300 to 1000 kg/ha by simultaneously selecting individuals with both high rubber concentration and high biomass production (Coffelt et al. 2015; Estilai and Ray 1991; Ray

et al. 1999). However, when heritability is low, as mentioned above, family selection, either by full-sibs or half-sibs, might be more efficient to evaluate parent plants. In this case, parents are selected based on the performance of their progenies instead of on their own generation (Coffelt et al. 2015). This method, although possibly lengthening generational intervals, could be successfully conducted in guayule, a perennial shrub with continuous flowering and seed production from a single parent plant.

6.4.3.2 Recurrent Selection and Mass Selection

Although apomictic polyploids ensure the uniformity of genetic background in selected varieties, new desirable genes from diverse resources need to be introduced into long-term breeding programs. Sexual diploids are potentially useful to introduce new genetic combinations because cross-pollination is the predominate mode of reproduction in guayule. Mass selection is a simple, common and established breeding method, in which large numbers of varieties are selected by collecting seeds from selected plants to propagate the next generation (Romero and Frey 1966). Recurrent selection, as an extension of mass selection, is the intermating of selected plants/varieties for generation after generation (Hayes and Garber 1919). In diploid guayule, problems such as reduced biomass, lower rubber content and increased susceptibility to root diseases have occurred; however, previous researchers have successfully used modified recurrent selection breeding schemes to increase yield (Estilai and Ray 1991) and mass selection to develop *Verticillium*-resistant lines (Ray et al. 1995). Newly-released varieties were exploited as follows (Estilai and Ray 1991): (1) if biomass production and rubber concentration are both economically acceptable in the population, the open-pollinated seeds can be released or they can be continuously crossed to other diploids to generate more genetic variation for later selections; (2) if rubber concentration is high but biomass is low, they are crossed to selected apomictic polyploids to improve biomass; (3) after the improvement is done, the improved diploids may have their chromosomes doubled chemically to produce autotetraploid cultivars. Following this scheme, Cal-3, a diploid germplasm, was released (Hashemi et al. 1989). These breeding efforts have resulted in only limited advancements because most financial support grants are for a period of 3 years, but plant breeding programs need continuous funding. This is compounded in guayule because it is a perennial plant, requiring several years to mature before phenotyping, and a large number of samples must be collected requiring corresponding labor, time and costs. Finally, since guayule is grown several years before harvest, genetic effects can become masked by the compounded environmental effects; therefore, the ability to make early selections is very important.

6.4.3.3 Hybridization

Intraspecific Hybridization In the past, intraspecific hybridization was sparingly used in guayule due to the lack of genetic markers to separate progenies from asexual apomixis and from sexually reproduced hybrids. Keys et al. (2002) identified a method to distinguish plants expressing high levels of sexuality from asexual apomicts. By this method, seeds with predominately apomictic features can be released as a new variety, and seeds with predominately sexual features used for self-pollination, cross-pollination and backcrossing (Coffelt et al. 2015).

Interspecific Hybridization Only limited efforts have been made to generate interspecific hybrids in guayule due to the dramatic decrease in rubber content, which needs many backcrossing generations to restore as well as to keep the new desirable traits such as increased vigor, resin content, biomass, disease and insect resistance, regrowth capability after clipping and cold tolerance (Coffelt et al. 2015; Estilai and Ray 1991; Ray et al. 2010). Despite these difficulties, three germplasm lines (Cal-1, Cal-2, Cal-5) derived from crossing guayule and three different *Parthenium* species were released by the University of California Riverside (Estilai 1985, 1986). These three germplasm lines have improved vigor, biomass production and *Verticillium* resistance. AZ-101 is another interspecific hybrid derived from natural crossing in a guayule field, between guayule and *Parthenium tomentosum*, and maintains both high vigor and biomass production (Estilai and Ray 1991).

6.5 Functional Genomics, Tissue Culture, Genetic Engineering and Molecular Characterization

Limited studies of guayule have been conducted utilizing advanced biotechnology techniques. These are limited to a few transgenic, functional genomic, physiological and biochemical studies, and polyploidy analyses using flow cytometry. Most of these investigations were designed to understand the rubber biosynthesis pathway. No comprehensive molecular breeding studies have been carried out so far in guayule. Despite that, a 1.6 Gb guayule genome of a diploid guayule accession was sequenced, assembled and around 40,000 transcribed, protein encoding genes were annotated (Valdes Franco et al. 2018). This study describes the first partial draft genome of guayule and is expected to enhance research topics important to guayule genetic improvement and the future application of modern biotechnology, molecular breeding, functional genetics and comparative genomics.

6.5.1 Tissue Culture

Only a few tissue culture studies have been carried out on guayule. Guayule micropropagation has been achieved from callus and shoots. Although there have been several reports that somatic embryogenesis (callus culture) could realize regeneration of shoots, or even whole plants (Dhar et al. 1989; Finnie et al. 1989; Zavala et al. 1982), induction of callus requires high concentrations of growth regulators and leads to somaclonal variation. Therefore, since the 1990s, researchers have used direct organogenesis through shoot culture, followed by rooting and subsequent acclimation of plantlets to simplify and improve the efficiency of guayule tissue culture (Castillon and Cornish 2000; Dong et al. 2006; Pan et al. 1996; Staba and Nygaard 1983; Trautmann and Visser 1990). These studies laid the foundation for subsequent creation of genetically-engineered guayule plants by mediating their metabolic pathways to produce rubber.

6.5.2 Genetic Engineering

Two studies have been conducted to evaluate genetically-engineered guayule lines. Veatch et al. (2005) overexpressed three allylic pyrophosphate initiators including farnesyl pyrophosphate (FPP), geranylgeranyl pyrophosphate synthase (GGPP) and hexa-heptaprenyl pyrophosphate synthase (H-HPP) in three transgenic guayule lines (AZ101, AZ-2, N6-5). Even though the rubber concentration was not significantly altered in transgenic lines, resin production was enhanced.

Dong et al. (2013) evaluated transgenic guayule lines with the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) overexpressed. HMGR is a key regulatory enzyme of mevolanate (MEV) carbon flux in mammals, microbial systems and possibly in plants since it converts 3-hydroxy-3-methylglutaryl-CoA to mevalonate, a precursor of isopentenyl pyrophosphate (IPP) (Dong et al. 2013; Kirby and Keasling 2009). In this study, overexpression of the HMGR gene produced a 65% rubber increase in one out of five genetically-modified guayule lines (HMGR6), but there was no significant differentiation in rubber production in the other lines in this lab-based experiment. Although differences were observed in size, biomass and plant morphology of HMGR6 in the field, rubber and resin content were not significantly different from the control lines. However, the survival rate during regrowth was significantly improved for these transgenic lines, indicating that the overexpression of HMGR enhanced carbon flux to produce important secondary isoprenoid metabolites (e.g. growth phytohormones).

6.5.3 Functional Genomics

Rubber biosynthesis in guayule increases as night temperatures fall and accumulate to the highest degree in the cold winter months (Benedict et al. 2008; Cornish and Backhaus 2003). It is important to examine the effect of cold temperatures on this biosynthetic pathway, to understand the regulation of rubber biosynthesis in guayule, if the goal of improving guayule as a domestic rubber crop is to be realized, by means of breeding or genetic engineering. Ponciano et al. (2012) conducted a transcriptome analysis using 11,748 expressed sequence tags (ESTs) to evaluate expression of genes potentially related to rubber biosynthesis. Contrary to a previous study by Cornish and Backhaus (2003), Ponciano et al. (2012) found that none of the genes encoding 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS) and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), farnesyl pyrophosphate synthase (FPP), squalene synthase, small rubber particle protein (SRPP), *cis*-prenyltransferase (CPT) and allene oxide synthase (AOS) were positively correlated with air temperatures, except for CPT, which reached a peak in gene expression right after a sudden increase in night temperature 10 days before harvest. They concluded that either the critical members of the rubber transferase complex are yet to be identified, or the enzymatic activity of the rubber transferase complex is more likely controlled by posttranslational modifications rather than gene expression. Moreover, it is possible that the low abundance of proteins (~1%) present in rubber particles or participating in rubber biosynthesis accounted for only a very small part of the entire guayule transcriptome, which might be masked by the high threshold established by other differentially expressed genes. Similar results were also found in transcriptome studies of other rubber-producing plants (Ko et al. 2003; Luo et al. 2017; Priya et al. 2007).

Research by Valdes Franco et al. (2018) revealed 15 families of SaTar (satellite targeted) elements with unique chromosomal distribution profiles. These transposable elements (TEs) are frequently observed in multimeric linear arrays of unrelated individual elements.

6.5.4 Flow Cytometry Analyses

Flow cytometry has been used fairly often in guayule genetic studies, compared to other modern genotyping techniques, to characterize ploidy levels of guayule accessions (Gore et al. 2011; Ilut et al. 2015; Sanchez et al. 2014). These studies provide a foundation for interspecific hybridization and genome sequencing studies in guayule breeding programs. Cruz et al. (2017) utilized flow cytometry to estimate the rate of apomixis and validate the reproduction mode in guayule polyhaploids, they found that seed embryo and endosperm DNA content varied during seed development and maturity stages, and that the nuclear DNA content is more consistent when seeds are collected during middle to late development stages.

6.6 Conclusions and Prospects

Among more than 2000 plant species known to produce rubber, based on its origin in Southern Texas and Northern Mexico, guayule is a good candidate for arid and semiarid sustainable agricultural systems as an alternative rubber crop to *Hevea*. Commercialization of guayule as a new/alternative crop is possible and advisable due to the disadvantage of being totally dependent upon *Hevea*. To establish guayule as a new crop, we must continue genetic improvement and establish best agronomic practices. Farming strategies have been developed for guayule, but there are still agronomic challenges to be met. For example, in order to replace transplanting with the more cost-efficient direct-seeding strategy, problems of seed quality, seedling vigor and salt tolerance remain to be addressed. Even though guayule is a semiarid, drought-tolerant shrub requiring only low nutrient inputs, suitable irrigation and fertilization applications still need to be adjusted and optimized according to different growing conditions and maximum sustained production targets. The control of water quality is also very important because salt tolerance of guayule at emergence and seedling stages is reportedly very low, even lower than carrots, one of the most salt-sensitive crops grown in the southwestern USA. Clipping can improve input/output efficiency, but lines vary in their capability to regrow after clipping. Therefore, selecting lines with high regrowth potential should be considered by breeders and agronomists in future studies prior to releasing new germplasm. As a perennial crop, one of the major challenges in guayule is to shorten the plant growth cycle and maximize rubber production, which can be complicated by the strong influence of environmental effects and changing climate.

While previous attempts to improve rubber yield achieved limited success, a better understanding of the genetics of rubber production will help by providing an estimate of the number of genes controlling rubber production. Traditional breeding methods including the recurrent or mass selection in sexual diploids, the selection and hybridization of apomictic polyploids and backcrossing of interspecific hybrids have produced new and improved germplasm. As result, since 1970, improved germplasm has been released with higher rubber and resin concentrations, faster growth with high biomass and disease resistance. Fortunately, with the development of advanced biotechnology and molecular markers, these activities can be accelerated. For example, GBS technology has provided fast and precise discovery of SNP markers in guayule; this technology could be used to identify interspecific hybrids or sexual intraspecific hybrids at an early stage of selection and recurrent or mass selections could be accelerated by marker-assisted recurrent selection (MARS). Molecular breeding integrated with marker-assisted selection (MAS) may be more effective for the quantitative traits controlled by a small number of genes. Both MAS and MARS could be used to select for simple and complex traits in early generations and stages of a breeding program. One of the major steps toward using

MAS and/or MARS in guayule breeding is to identify markers associated with the trait(s) of interest. The key steps in identification of molecular markers linked to trait are: identifying parents differing in specific traits, developing mapping populations segregating for these traits, constructing a linkage map of the population to locate QTLs associated with the traits of interest, identifying molecular markers that cosegregate with the trait of interest and validating the associated markers in different backgrounds. Due to the complexity of apomictic reproduction resulting in a mixture of various ploidy levels among guayule progeny, there are significant hurdles for a population to reach homogeneity. Using sexual diploids is the way to create populations and linkage maps to identify associated markers. The current USDA guayule collection has very few, and narrowly diverse, diploid accessions. Therefore, more collecting trips are needed to discover more wild diploids. Genotyping such large mapping populations will be facilitated by advancements in high-throughput sequencing technologies, making it feasible to create linkage maps for a large population using GBS technology. With the increased ability to discover and map thousands of molecular markers, phenotyping is emerging as the bottleneck for genomics-based approaches, not only in guayule but in many other crops. Complementary, precise and robust technologies of high-throughput phenotyping (HTP) are needed to increase the statistical power and identify marker/trait associations in large populations. HTP technology is a rapidly expanding field, allowing the characterization of complex traits using proximal sensors and imaging systems. We are starting to develop HTP protocols, collect and analyze traits related to guayule growth performance, responses to environments including normalized difference vegetation index (NDVI), canopy height and plant canopy temperature.

Taking into account that guayule is a perennial, and a not a food or feed plant, genetic engineering techniques may be effective for potential simple traits such as herbicide tolerance and insect resistance. Herbicide and insect tolerance are traits that are important during early establishment or regrowth stages in guayule, since growth is usually slow and weed competition is high during this period. Improvements of these two traits will be even more important if researchers want to replace transplanting cultivation systems with a direct-seeding system. In addition, with the development of gene editing technologies such as CRISPR-Cas9, genetic engineering strategies can be more targeted and effective in future applications. Gene editing technology such as CRISPR-Cas9, coupled with functional genomics, could help reveal the genetic architecture behind apomixis in guayule.

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Appendices

Appendix I: Research Institutes Relevant to Guayule

Institution	Specialization and research activities	Contact information and website
USDA-ARS	Agronomy and breeding	Hussein Abdel-Haleem US Arid-Land Agricultural Research Center 21881 North Cardon Lane, Maricopa, Arizona, 85138, USA Tel: 520-316-6355; Fax: 520-316-6330 Hussein.Abdel-Haleem@ars.usda.gov
University of Arizona	Agronomy and breeding	Dennis Ray School of Plant Sciences, Arid Lands Studies 1140 E. South Campus Drive, P.O. Box 210036 303 Forbes Building, Tucson, Arizona 85,721, USA Tel: 520-621-7612 dtray@email.arizona.edu
USDA-ARS	Natural rubber biosynthesis and production	Colleen McMahan 800 Buchanan st, Albany, California 94,710, USA Tel: 510-559-5816; Fax: 510-559-5818 Colleen.McMahan@ars.usda.gov
USDA-ARS	Genetic resources conservation	Claire Heinitz Nat'l Clonal Germplasm Repository (NCGR) 9611 S. Riverbend Ave, Parlier, California 93,648, USA Tel: 559-596-2980 claire.heinitz@ars.usda.gov
Bridgestone Americas, Inc.	Agronomy and breeding	David Dierig Guayule Research Farm, Eloy, Arizona, USA DierigDavid@bfusa.com

(continued)

Institution	Specialization and research activities	Contact information and website
The Ohio State University	Natural rubber biosynthesis and production	Katrina Cornish 108A Williams Hall, 1680 Madison Avenue, Wooster, Ohio 44691, USA Tel: 330-263-3982 cornish.19@osu.edu
Centre de coopération internationale en recherche agronomique pour le développement (CIRAD)	Natural rubber biosynthesis and production	Serge Palu 34398 Montpellier Cedex 5, France Tel: 33-04 67 61 58 99; Fax: 33 04 67 61 65 47 Serge.palu@cirad.fr

Appendix II: Guayule Genetic Resources Worldwide

The guayule genetic resources can be accessed through the Germplasm Resources Information Network: https://npgsweb.ars-grin.gov/gringlobal/taxonomydetail.aspx?id=26802

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Chapter 7
Biotechnological Advances in Rubber Tree (*Hevea brasiliensis* Muell. Arg.) Breeding

Sankaran Sobha, Karumamkandathil Rekha, and Thomas K. Uthup

Abstract The aim of *Hevea* breeding is to provide new varieties/clones which are genetically superior in terms of yield, disease tolerance, better adaptability to climatic fluctuations and good timber quality. Although traditional breeding strategies could achieve a substantial increase in yield, breaking the current yield plateau is possible only with the aid of nonconventional breeding strategies. In addition to large-scale propagation, tissue culture holds unique advantages for crop improvement and this has been utilized successfully in many crops for specific purposes. Various tissue-culture techniques like somatic embryogenesis, embryo rescue, culture of protoplast, anther, pollen and embryo sac are practiced in *Hevea*. Interventions were also made in the area of molecular breeding through the development of molecular markers and through *Agrobacterium*-mediated genetic manipulation. The present chapter gives an overview on the constraints in *Hevea* breeding and reviews the progress of in vitro techniques comprehensively towards complementing conventional breeding. A road map to effectively combine the traditional and non-traditional methods for future *Hevea* breeding is presented. This takes on importance in the present scenario of unprecedented climatic vagaries and resource constraints. Progress made in the advancement of biotechnological applications in the natural rubber-producing tree *Hevea brasiliensis* Muell. Arg. worldwide and its implications in breeding are described in detail.

Keywords *Hevea* · In vitro culture · Somatic embryogenesis · Anther culture · Cryopreservation · Embryo rescue · Genetic manipulation · Haploids

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7.1 Introduction

In the modern world, there is hardly any segment of life that does not make use of natural rubber (NR) in thousands of rubber-based products. Chemically, natural rubber is cis-1,4-polyisoprene with a molecular weight of 500,000–800,000 daltons. Natural rubber is produced by 7500 plant species confined to 300 genera of 7 families; *Hevea brasiliensis* Muell. Arg. is the principal source of natural rubber (Priyadarshan et al. 2009).

The industrial revolution led to a boom in rubber prices and an escalating demand for natural rubber worldwide and which eventually resulted in large-scale cultivation of the tree crop by the end of nineteenth century. Natural rubber is harvested from the tree in the form of latex, which has 30–45 % dry rubber content. The global projected gap between the demand and supply of natural rubber necessitates production enhancement. However, adverse environmental conditions such as drought, high and low temperatures; high solar radiation, low atmospheric humidity, poor soils, etc. limit the expansion of cultivation in several rubber-producing countries (Priyadarshan 2003). Rubber breeders are aiming to produce high-yielding clones with vigorous growth and resistance to biotic and abiotic stresses. Conventional breeding efforts have resulted in substantial yield improvement by developing high-yielding clones through hybridization and selection. However, constraints faced by breeders such as high heterozygosity, narrow genetic base, perennial nature, long breeding cycle, brief and seasonal nature of flowering and low seed set retard the progress of breeding programs (Kavitha et al. 1989). The development of in vitro culture techniques and gene transfer technology opens up new vistas in *Hevea* breeding.

This chapter gives an overview of the *Hevea* rubber and breeding constraints in conventional crop improvement and describes the possible in vitro techniques to overcome these constraints, the progress already made in this direction and concludes with a roadmap for the future.

7.1.1 Botany and Distribution

The genus *Hevea* belongs to the large, mostly tropical, Euphorbiaceae family and consists of ten species. Among them, *H. brasiliensis* is the only one commercially cultivated. Commonly known as the Pará rubber tree, named after the Brazilian port of Pará, *H. brasiliensis* is the major source of NR. The tree is traditionally grown in tropical moist climatic areas between 10° north and south latitudes.

H. brasiliensis trees attain a height of 40 m in the wild, whereas in commercial plantations they rarely exceed 25–30 m due to tapping stress and are usually replanted after 25–30 years when yield levels start declining and become unprof-

itable. Most modern plantations are raised by bud grafting. The tree is deciduous with annual wintering followed by refoliation and flowering. Rubber trees exhibit more than 60 % natural, and intraspecific outcrossing (de Pavia et al. 1993, 1994). Fruit set, both under open pollination and hand pollination is very low (5–6 %). Normally the trees are tapped for latex on attaining a stipulated girth of 45–50 cm at a height of 125 cm from the bud union, which takes about 6–7 years (Fig. 7.1). Harvesting is continued for 20–25 years after which the plantations are clear felled. Due to diminishing sources of forest products, the demand for rubber wood is also on the increase and research on wood treatment technologies have been intensified by several countries to expand the utilization of rubber wood.

Latex is a colloidal suspension of rubber particles along with other cytoplasmic organelles. Although latex is present in all parts of the plant, only the bark of the trunk is usually exploited for rubber because of the high density of latex containing vessels or laticifers. The latex vessels are spirally oriented along the phloem in a right inclination about 7° and hence in order to extract latex, the bark is cut deep from top left to bottom right which is enough to sever most of the vessels avoiding damage to the cambium.

Fig. 7.1 A mature rubber plantation under tapping. Inset: Latex being harvested in collection cups

7.1.2 World Natural Rubber Scenario

According to the International Rubber Study Group Report (IRSG 2017), world NR production during 2016 (provisional) was 12.40 million mt whereas consumption was 12.58 million mt. Thailand, Indonesia and Vietnam are the major producers and countries like China, India and USA are the major consumers. The demand for NR increased with the rapid growth of the automobile industry in the twentieth century. Over the past 50 years the demand and supply of natural rubber saw a tight balance between production and consumption of rubber (Hayashi 2009). The world supply-demand balance of NR during 2016 showed a deficit of 1.88 million mt (IRSG 2017). In order to meet the escalating demand for the raw material, rubber cultivation has been extended into marginal non-traditional areas for its growth by all major producing countries.

7.1.3 History and Domestication

H.brasiliensis is native to the Amazon River basin of South America. Although *H. brasiliensis* is commercially cultivated in Asia, Africa and Latin America, more than 90 % of NR comes from Southeast Asian countries. The present rubber plantation industry in tropical Asia had its origin from seeds collected from the Amazon in 1876 by Sir Henry Wickham. He collected 70,000 seeds of *H. brasiliensis* from Boim on the Tapajós River, a major Amazon tributary, and transported them to the Royal Botanic Garden, Kew. About 2700 seeds successfully germinated and 1900 seedlings were dispatched, mainly to Ceylon (now Sri Lanka) and to Malaysia, Singapore and Indonesia, during the period 1876–1877 (Baulkwill 1989; Dean 1987). Only 22 seedlings survived from this collection and they formed the base material of the entire rubber plantation industry in Southeast Asia (Schultes 1984; Wycherley 1968). Thus, the genetic base of *Hevea* in the east is very narrow, and is referred to as the *Wickham base* (Simmonds 1989). Within a few decades, the crop spread to many Southeast Asian and African tropical areas. The original narrow base has been further narrowed through the unidirectional selection for yield, coupled with vegetative propagation by bud grafting. Moreover, the system of breeding with the best genotypes in one breeding cycle being the parents for the next resulted in the development of clones with genetic similarity. This is evident from the highly conserved nature of mitochondrial DNA in the modern clones (Priyadarshan and Goncalves 2003). This indicates that there are only a few maternal clones.

7.2 Genetic Diversity and Germplasm Resources

The genus *Hevea* consists of 10 species: *H. brasiliensis, H. guianensis, H. benthamiana, H. pauciflora, H. spruceana, H. microphylla, H. rigidifolia, H. nitida, H. camporum* and *H. camargoana* (Schultes 1990; Webster and

Paardekooper 1989; Wycherley 1992). As noted in section 7.1.3, the genetic base of *Hevea* in the Far East is very narrow, representing only a miniscule of the vast areas where the tree occurs in the wild (Schultes 1977). Considering the urgent need for broadening the genetic base, a series of wild germplasm collections from the Amazonian forest have been undertaken by different groups since 1980 (Gonçalves et al. 1983; Tan 1987), referred to as the *Amazonian* population. Wild germplasm accessions in general displayed an average yield of 12 % of the latex yield of Wickham clones (Clement-Demange et al. 2001) with a fairly high tolerance to leaf diseases caused by *Microcyclus ulei* or *Corynespora cassiicola*. A more elaborate joint expedition for wild *Hevea* germplasm collection was undertaken to Brazil by the International Rubber Research and Development Board (IRRDB) and the Brazilian Government, in 1981. As many as 64,736 seeds and budwood accessions from 194 presumably high-yielding mother trees (ortets), which were not affected by *Microcyclus* and *Phytophthora*, were collected from the states of Acre, Mato Grosso and Rondônia (IRRDB 1982). The seeds and budwood were dispatched to the primary nursery at Manaus, Brazil, where 50 % of the entire seed collection was retained; 75 % of the remaining seeds were sent to the Asian multiplication and distribution center in Malaysia and 25 % to the African Center in Ivory Coast. Varying proportions of this fresh germplasm were distributed as per requests from IRRDB member countries.

In India, the new germplasm was imported from Malaysia during the period 1984–1990. A total of 4967 genotypes have been established in field gene banks in the traditional and non-traditional rubber growing areas of the country (George 2000). These are conserved, documented and evaluated in a phased manner with the objective of utilization for various breeding priorities. Wide variability has been observed in various secondary traits contributing to rubber yield, including timber traits, physiological and biochemical parameters, growth habits, biotic/abiotic stress resistance, etc. The potential accessions have been utilized as parents for W (Wickham) x A (Amazonian) hybridization programs. There are also indications of the possibility of obtaining direct selections for yield from this collection (Mydin 2011).

7.3 Propagation

7.3.1 Seeds

Seedlings were used as planting material during earlier days. There are monoclonal and polyclonal seeds. Monoclonal seeds are collected from plantations of a single clone. Due to their inferior performance compared to modern clones they are not recommended for raising commercial plantations. Polyclonal seeds, which are hybrid seeds, are produced from polyclonal seed gardens. Clones with desirable

characters are planted together in a single plantation so as to ensure maximum cross pollination. Seeds collected from these polyclonal gardens exhibit better performance as compared to other seedlings and occasionally are used for direct planting, especially for marginal areas.

7.3.2 Bud Grafting/Budding

Rubber is currently propagated by bud grafting and planted in polybags. The buds are collected from budwood nurseries of elite clones. Rootstocks are developed by sowing assorted seeds either in the polybags or in the nurseries. For budding, a patch of the bark is removed from the seedling plant (stock) and replaced by the bud patch taken from the elite clone to be multiplied (scion). A thin film of polythene is wound over the bud patch. It takes 15–20 days for the union of the scion and stock. After 21 days the polythene is removed. The stock is then cut off above the graft union and the bud from the scion will develop into a new shoot. The stock seedling will absorb water and nutrients for the growing scion. In a monoclonal plantations, the scion part will be genetically uniform in all the plants, whereas the stock seedlings will be assorted and genetically different. There are different types of bud grafting based on the age of the buds and seedlings viz. brown budding, green budding, young budding and also crown budding, under special circumstances when it becomes necessary to replace an undesirable crown with a new one.

7.3.3 Root Trainer plants

As an alternative to polybag plants, the root trainer planting technique has been standardized for *Hevea* in India (Soman and Saraswathyamma 1999; Soman et al. 2013). Plants raised in root trainers will have better growth uniformity than polybag plants and also have a greater number of lateral roots. The problem of tap-root coiling and related root malformations can be avoided through root trainers. The typical root trainer containers used are of 26 cm in length, with a capacity of 600 cc, a tapering shape with vertical ridges on the inside wall and a drainage hole. Well cured coir pith mixed with neem cake, bone meal, pesticides and single super phosphate is used as potting medium. The curing of coir pith is done by immersing it in a water tank for a minimum period of 2 months, changing the water fortnightly to leach out phenolic toxic compounds. The potting medium should be packed well inside the root trainer cup before planting germinated seeds. The root trainers are stacked by pushing them into raised beds of soil taking care that the drainage hole is not clogged. The developing tap root grows into the soil through the drainage hole. When the plants exhibit healthy growth, the root trainer is lifted from the bed, the root pruned close to the drainage hole and stacked on stands made of iron or split bamboo so that the plants in the containers remain suspended in air without

touching the soil. Although the tap root resumes growth, it undergoes natural air pruning at the drainage hole. This stress induces emergence of large number of lateral roots into the potting medium. The ridges on the sidewall of container direct these lateral roots downward which in their turn also undergo air pruning. Thus the hardened root trainer plants will have a tap root and well oriented lateral roots forming a dense root plug.

7.4 Classical Breeding Approaches

Rubber generally takes about 4–5 years to produce sufficient number of flowers for hand pollination and has a long immaturity period of 7–8 years before the initiation of tapping. A further period of 4–15 years is required after maturity, for a reliable assessment of yield performance and secondary characters. The development and release of a clone in rubber, starting from nursery selection, passes through various stages of field evaluation and takes about 10–15 years to complete one breeding cycle and more than 3 decades to complete the entire cycle before release of the clone to the farmers. The major problems encountered by rubber breeders are: 1) long breeding and selection cycle, 2) narrow genetic base, 3) non-synchronization of flowering, 4) low fruit set, 5) susceptibility to biotic and abiotic stresses, 6) seasonality in yielding pattern, 7) lack of reliable early prediction methods and 8) high genotype x environment (G x E) interaction (Tan 1987). The long duration of field testing continues to be the major challenge for a breakthrough in conventional breeding.

Rubber cultivation is predominantly done by small farmers in all the major rubber-growing countries (Priyadarshan and Clément-Demange 2004). The aim of rubber breeding is to provide genetically superior planting materials such as bud grafted clones with high latex/dry rubber yield. Desirable attributes of an ideal clone, apart from high yield, include high initial vigor leading to early tappability, smooth and thick bark with a good laticiferous system, precocious high yield, good bark renewal, high growth rate under tapping stress, tolerance to major diseases, wind and tapping panel dryness and good response to low frequency tapping. Specific objectives towards achieving these goals are gaining research priorities in the wake of global warming and climate change issues on the one hand and increasing cost of cultivation on the other.

7.4.1 Plus Tree (Ortet) Selection

Hevea breeding was initiated with mass selection among trees during the early twentieth century. Use of unselected seedlings soon gave way to selected seeds from healthy trees. Ortets refer to *plus trees* and ortet selection or mother tree selection, is the oldest breeding method in *Hevea*. It involves systematic

screening of vast seedling plantations, resulting from natural genetic recombination and multiplication of elite trees, followed by field evaluation and selection of outstanding genotypes. Clones developed through ortet selection are called primary clones. A good number of early primary clones like Tjir1, PR 107, GT 1, BD 10, AVROS 255, Gl 1, PB 28/59, Mil 3/2 and Hil 28 were developed in Indonesia, Malaysia and Sri Lanka by screening extensive seedling plantations. Many of these primary clones registered 150 % yield improvement over the original unselected population (Khoo et al. 1982). Some of the primary clones like PB 56, Tjir 1, Pil B84, Pil D65, Gl 1, PB 6/9 and PB 86 were used as parents for developing elite clones (Saha and Priyadarshan 2012). A few early primary clones like GT1, Tjir1, PB 86 and GL1 were used as parents in developing some of the very high yielding hybrids cultivated worldwide (Jacob et al. 2013).

7.4.2 Hybridization and Clonal Selection

Hybridization followed by clonal selection are the most important conventional breeding method in *Hevea.* Once desirable recombinants are selected they can be fixed easily through vegetative multiplication. A good number of high-yielding hybrid clones have been evolved in all the major rubber growing countries, some of which are performing very well across diverse locations. The early primary clones were used as parents in the first phase of breeding. Rubber breeders followed a cyclical generation-wise assortative mating (GAM), wherein the best clones in each series were used as parents in the subsequent series (Simmonds 1989). In Malaysia, some of the early hybrids of commercial significance belonged to the RRIM 500 and 600 series, the yield levels of which were much superior to those of the parent clones. Clones of the RRIM 500 to 1000 series were developed by the Rubber Research Institute, Malaysia, whereas a series of PB clones with commercial importance were identified by the Prang Besar Institute in the private sector in Malaysia. The Indonesian Research Institute evolved the PR, AVROS, BPM and LCB clones. The RRIC clones originate from Sri Lanka, KRS clones from Thailand and Haiken, and the YRITC and SCATC clones from China. In India crop improvement programs were initiated in 1954 and the early hybrid clones developed by the RRII were designated as RRII 100, 200 and 300 series clones (Annamma et al. 1990) among which RRII 105 is a clone highly adapted to the traditional rubber belt in India and until recently occupied more than 90 % of the cultivated area. Very recent hybrid selections include five clones of the RRII 400 series among which RRII 430 is an outstanding high yielder with high growth and yield stability, and a high level of tolerance to biotic (*Corynespora* leaf disease and ALF) and abiotic stresses (drought) (Mydin 2014; Mydin et al. 2011).

7.4.3 Advances in Yield Improvement over the Past Century

With the introduction of bud grafting, seedlings and grafted clones were concurrently used as planting materials (Dijkman 1951). Gradually, realizing the yield potential of bud grafted clones compared to selected seedlings, development of high-yielding hybrid clones became the global mandate of *Hevea* breeding to increase productivity. The average yield of 300 kg/ha/year from unselected seedlings during the early 1900s, improved to 1600 kg/ha/year for the best clones during the 1950s. Further yield improvement up to 2500 kg/ha/year was achieved by the development of clones like RRIM 501, RRIM 600, RRIM 712, PB 217, PB 235, PB 260, RRII 105, RRIC 100, IRCA 18, IRCA 230, IRCA 331 and BPM 24, by the respective institutes, through 70 years of rigorous breeding and selection (Tan 1987; Simmonds 1989; Clément-Demange et al. 2001; Priyadarshan 2003; Saha and Priyadarshan 2012;). Productivity improvement through the advances in planting material from seedlings to new generation hybrids (3000 kg/ha/year) was tenfold, which is unique in the history of any perennial tree species (Varghese and Mydin 2000).

7.4.4 Conventional Breeding Constraints

Hybridization is laborious and time consuming. Low average fruit set (< 5 %) limit the recovery of hybrids and seeds are highly recalcitrant. The brief and periodic nature of flowering in *Hevea* impedes the progress of pollination programs. Heterozygosity of parents necessitates screening of large families to achieve tangible results. Very often, it becomes impossible to carry out sufficient number of hand pollinations to obtain adequate family size for effective selection. Although *Hevea* trees produce flowers in abundance, barely 3 % of female flowers develop into fruits. Under artificial pollination as well, fruit set and recovery of hybrid fruits is equally low. The low rate of recovery of fruits at the mature stage poses serious hindrance to *Hevea* breeding efforts, necessitating considerably large number of hand pollinations each year (Kavitha et al. 1989).

The lack of early selection parameters is another hindrance for selecting the promising genotypes from the hybrid population. The modified Hamaker Morris Mann method (Tan and Subramaniam 1976), test incision (Varghese et al. 1989), and estimation of biochemical components of yield like sucrose, total solid content, thiols and inorganic phosphorus at immature phase (Licy et al. 1998) have been tried as early-selection parameters. However, nursery yield can be considered as only a fair indicator of mature yield (Varghese et al. 1993). Juvenile-mature correlation was moderate to low for most of the component traits; juvenile mature correlations between seedlings and their bud-grafted counterparts showed that only around 25 % of selections from seedling nurseries produce high yield on bud grafting (Mydin 2012; Mydin and Gireesh 2016). In the current scenario of unprecedented

climatic change, breeders resort to broadening the genetic base by incorporation of wild germplasm accessions, choice of divergent parents for hand pollination and breeding, and selection for desirable secondary characters like biotic/abiotic stresses apart from yield *per se*. However, the land, labor and time consumed for this great achievement has been demanding and is no longer feasible due to pressure on resources and the need for long-term investment. It is in this context that the exploitation of in vitro culture techniques and molecular interventions to supplement conventional breeding assumes significance in *Hevea*.

A bud-grafted population is expected to have high homogeneity and minimum intraclonal variation, except for the influence of soil heterogeneity and environmental factors. However, the final performance of a selection is influenced by the seedling stock, among others factors to a larger extent. A difference of 5–325 ml in the total volume of latex per tap and 1.8–144 g dry rubber content was reported in RRII 105 plantations (Chandrasekhar et al. 1997). Similar reports on significant intraclonal variations are also available from Malaysia, Sri Lanka and Indonesia (Priyadarshan 2017). Although soil heterogeneity and difference in juvenility of buds were attributed to this, the effect of rootstock is considered the most intriguing factor. Developing self-rooted clones is a serious challenge in *Hevea* where in vitro techniques can contribute substantially to conventional breeding.

7.5 In vitro Approaches to Complement Conventional Breeding

7.5.1 Organogenesis

Intraclonal variability is a major constraint affecting the productivity of rubber plantations. Propagation through bud grafting using divergent rootstocks is one of the major factors responsible for this phenomenon (Nayanakantha and Seneviratne 2007; Senanayake and Wijewantha 1968). Later researchers (Koepke and Dhingra 2013) established the influence of seedling stocks on the performance of scions and related changes in the overall performance of the plant. The genetic distance between the stock and scion determines the extent of intraclonal variation. Raising of plants with their own roots and thus avoiding bud grafting gained importance in *Hevea* in the early years of introduction of tissue culture. Rooting of microcuttings, air layering and tissue culture were attempted by different researchers (RRIM 1959, 1962; Sobhana et al. 1995). Tissue culture in rubber was first attempted by Bouychou (1953). Subsequently, Paranjothi and Ghandimathi (1976) induced rooting of shoot apices taken from axenic seedlings using tissue culture techniques. Later shoot sprouting from stem nodes of greenhouse plants was achieved by Carron and Enjalric (1982). Researchers including Gunatilleke and Samaranayake (1988), Carron et al. (1989) and Te-Chato and Muangkaewngam (1992) developed plantlets successfully by culturing shoot tips of in vitro germinated seedlings. Despite these achievements, the propagation of clonal material remained an uphill struggle due to

the recalcitrant nature of this tree (Carron and Enjalric 1983), and high rate of bacterial and fungal contamination (Seneviratne 1991). Asokan et al. (1988) successfully induced rooting from clonal shoot tips, and plants were established in the field. Induction of multiple shoots from nodal explants of juvenile and clonal origin was also reported (Seneviratne and Flegmann 1996; Sushamakumari et al. 1999). Plants were also developed from axillary buds by culturing on MS medium supplemented with plant growth hormones (Mendanha et al. 1998). However, the scale-up of the technique to a commercial level could not be achieved; further advancement in this field was slowed down for almost a decade, until the early twenty-first century. Micropropagation through tissue culture was again attempted (Hui et al. 2009; Ighere Dickson et al. 2011; Sirisom and Te-Chato 2012) and recently shoot growth from apical and lateral meristem was achieved from the axillary buds of clone RRIM 2020 by Malaysian group (Nor Mayati and Jamnah 2014) (Table 7.1). Since these techniques could not be commercialized, bud grafting is still practiced worldwide as the common propagation technique.

Table 7.1 Progress of in vitro culture techniques in *Hevea* during the last decade[a]

Explant/Experiment	Results	Reference	Country
Micropropagation			
Culture of stem cuttings from mature tree	Culture initiation	Hui et al. (2009)	China
In vitro shoot multiplication of *ex vitro* raised seedlings	Multiplication rate of 5 shoots per explant	Sirisom and Te-Chato (2012)	Thailand
Lateral meristem of mature budded stumps	79–93% shoot formation, rooted embryoids	Nor Mayati and Jamnah (2014)	Malaysia
Embryo culture			
Zygotic embryo	Tap root induction	Ighere Dickson et al. (2011)	Nigeria
Immature zygotic embryo	Plant regeneration	Rekha et al. (2010)	India
Half ovulo embryo culture	Multiple seedlings of single zygotic origin	Rekha et al. (2015)	India
Somatic embryogenesis			
Comparison of somatic and mature explants.	Embryogenic potential was much higher for the explant derived from somatic plants compared to that of explant excised from mature tree	Lardet et al. (2008b)	France
Hypocotyl region of leaf derived embryos	Secondary embryogenesis and plant regeneration	Kala et al. (2008)	India
Secondary somatic embryogenesis	85% plant regeneration frequency	Hua et al. (2010)	China
Root explants	Callus induction	Zhou et al. (2010)	China
Laticifer cells	Laticifer number has an inverse relation with embryogenic capacity in two clones Reyan 8-79 and Haiken -2	Tan et al. (2011)	China

(continued)

Table 7.1 (continued)

Explant/Experiment	Results	Reference	Country
Characterization of embryogenic callus through histochemical localization of storage reserves	Accumulation of considerable amount of storage starch, lipid and protein in embryogenic callus	Kumari Jayasree et al. (2012a)	India
Compared changes in protein profile during the sequential stages of somatic embryogenesis	More proteins were accumulated at embryo induction stage	Kumari Jayasree et al. (2012b)	India
Anther	Plant regeneration with confirmation of diploid nature	Srichuay et al. (2014)	Thailand
Different sources of leaves	In vitro derived leaves gave maximum embryogenic competence	Kala et al. (2009)	India
Relationship of explant juvenility on in vitro culture responses	Higher expression of chlorophyll A/B binding protein (*Cab*) gene with juvenile plants	Kala et al. (2012)	India
Embryo desiccation	Improved embryo vigor	Kala et al. (2015)	India
Somatic embryogenesis from root explants	Plant regeneration	Sushamakumari et al. (2014)	India
Association of ethylene production and embryogenesis	Ethylene production and signalling genes plays an important role during *Hevea* somatic embryogenesis	Piyatrakul et al. (2012)	Thailand
Protoplast culture			
Young leaves	Isolated protoplasts from mesophyll cells	Das and Dey (2009)	India
Intact pollen grains	Microcolonies	Sushamakumari et al. (2012)	India
Cell suspension derived from anther	Regenerated plantlets from protoplast derived callus	Dai et al. (2014)	China
Ploidy manipulation			
Embryo sac culture	Embryogenesis from haploid tissue	Divya (2016)	India
Endosperm culture	Direct embryogenesis	Divya (2016)	India
	Callus induction from endosperm		
Tetraploid induction through colchicine treatment	Embryos and plantlets with ploidy confirmation	Divya (2016)	India

[a]See: Venkatachalam et al. (2007) and Thulaseedharan et al. (2009) for earlier reviews

7.5.2 Somatic Embryogenesis

Since the abovementioned micropropagation methods did not produce tangible results, somatic embryogenesis from different *Hevea* tissues was attempted. The earliest attempt of somatic embryogenesis was the induction of callus for studying laticiferous system by Bouychou (1953). Later, Chua (1966) initiated callus from

plumule tissue of germinating seedlings and Wilson and Street (1975) induced callus from stem explants. Paranjothy and Rohani (1978) succeeded in the development of somatic embryos and shoots from callus derived from anther walls. Wang et al. (1980, 1984) were successful in somatic embryogenesis and plant regeneration from anther walls and successfully established plantlets in soil. By culturing inner integument tissue of immature fruits of clone PB 260, somatic embryos were induced and plantlets were regenerated (Carron 1981; Carron and Enjalric 1985). Later plantlets were developed from stamen culture through somatic embryogenesis and optimized temperature conditions for callus induction, embryo induction and plant regeneration (Wang and Chen 1995; Wang et al. 1998). Kumari Jayasree et al. (1999) developed a system for somatic embryogenesis and plant regeneration with immature anthers as the explant for Indian clone RRII 105. Kala et al. (2007) developed a plant regeneration system from leaf explants. Zhou et al. (2010) established a system from in vitro root explants of anthers derived somatic plants. Recently, Srichuay et al. (2014) induced callus and embryogenesis from anthers and confirmed the diploid nature of regenerated plants.

Other than plant regeneration, intensive research have been carried out on various aspects of somatic embryogenesis viz. media components, culture method, culture vessel, ontogeny and anatomy in different countries to further improve the system (Auboiron et al. 1990; Blanc et al. 1999, 2002; Cailloux et al. 1996; Carron and Enjalric 1982; EI Hadrami et al. 1989; EI Hadrami and d'Auzac 1992; Etienne et al. 1991a, b, 1993a, b, 1997; Lardet et al. 1999, 2008b; Linossier et al. 1997; Martre et al. 2001; Montoro et al. 1993, 1995; Nayanakantha and Seneviratne 2007; Sushamakumari et al. 2000a; Veisseire et al. 1994a, b). Studies on secondary embryogenesis were carried out by different researchers (Cailloux et al. 1996; Kala et al. 2008; Kumari Jayasree and Thulaseedharan 2004; Lardet et al. 2008a). The root architecture of tissue-cultured plants derived by somatic embryogenesis and microcuttings were investigated by Carron et al. (2000) and revealed that the tap root and lateral root systems of in vitro plants are similar to that of seedling roots. So far, somatic embryogenesis and plant regeneration have been reported from many clones and their evaluation for field performance is ongoing in different countries. Increased growth, vigor and yield was reported for plants propagated through tissue culture (Carron et al. 1995, Dibi et al. 2010) than bud-grafted plants. However, the occurrence of abnormalities compared to the control was also reported in plants derived from long-term cultures (Montoro et al. 2012).

Somatic embryogenesis pathways from different explants for different clones had been standardized in different laboratories in *Hevea* worldwide. However, several factors have hampered the exploitation of the technique to its full potential. The seasonal variation observed in the quality and culture response of the explants is one of them. This affects the repeatability of the results and represents a major hurdle for the exploitation of this technique at a commercial scale. There is no standardized method available for the long-term storage of the material without quality deterioration. The only method now available for maintenance is repeated subculture, which is laborious, time-consuming and involves the risk of contamination. Deterioration of the regeneration potential due to repeated subcultures of the calli was also

observed frequently. Repeated subculture also leads to somaclonal variations which are difficult to detect. Other limitations are the difficulty in embryogenic calli induction from primary callus (Englemann et al. 1997; Kumari Jayasree et al. 2012a) and low germination rate and plant conversion (Cailloux et al. 1996). Above all, hardening and acclimatization still remain a major hurdle and prevents the exploitation of this technique at a commercial scale. Although the major objective of commercialization could not be realized due to failure in raising planting materials on a large scale through somatic embryogenesis, the system is extensively being used for developing transgenic plants in different countries.

7.5.3 Embryo Culture

Embryo culture/embryo rescue is generally practiced to develop a viable plant from an immature or weak embryo obtained from wide hybridizations or incompatible crosses, in which poorly-developed endosperm causes embryo abortion. Embryo rescue techniques are among the oldest and most successful in vitro procedures.

As mentioned, a major problem in conventional *Hevea* breeding is the low recovery of fruits after hand pollination and lack of sufficient family size for effective selection of desirable recombinants. Changing the hand-pollination method (Kavitha et al. 1989), addition of boric acid to the pollen germination medium (Majumder 1964) and use of growth regulators (Leconte et al. 1984) were suggested to improve fruit set. However, fruit set could not be increased by more than 5 % (Kavitha et al. 1989), except in a unique cross between PB 330 x RRII 414, reported by Chandrasekhar et al. (2004), where a 19 % fruit set was obtained, which is the highest rate ever reported in *Hevea*. In this context, any attempt to increase fruit set will be of great value to support classical breeding.

Embryo culture has been attempted in *Hevea* with a view to address the problem of low recovery of recombinants. In their early experiments, Muzik (1956) concluded that at least one third of the cotyledons are essential for the embryo to develop into a healthy plant. Paranjothy and Gandhimathi (1976) developed complete seedlings from embryonic axes with a portion of a cotyledon by culturing in MS liquid medium. Chen (1984) also reported that the presence of a small portion of cotyledon is necessary for proper germination of the embryo, but the growth of seedlings was relatively poor. Toruan and Suryatmana (1977) used decotyledonized embryos, regenerated seedlings in MS medium and planted in soil. Paranjothy et al. (1979) also developed seedlings by culturing freshly fallen fruits and after 3 weeks of maintenance in culture, seedlings were established in soil. According to Normah et al. (1986), the addition of activated charcoal was highly essential for normal seedling development from the culture of embryonic axis. Das et al. (2003) reported in vitro culture of mature embryos and subsequent plant regeneration in *Hevea*. They observed that dark incubation of embryos promoted plant regeneration. Culturing of mature seeds or embryos alone was reported until 2010.

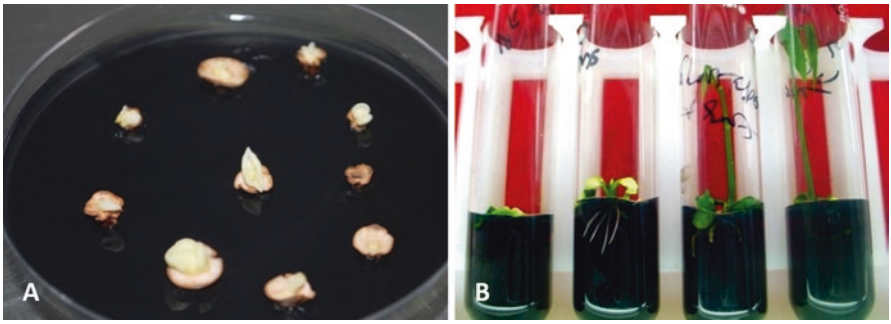


Fig. 7.2 Embryo rescue from immature fruits through half-ovulo embryo culture (**a**) and subsequent plant regeneration (**b**)

Hevea fruits normally mature within 22–24 weeks of pollination. Extensive withering of pollinated flowers and loss of developing fruits occur during the first 10–12 weeks after pollination (Chandrasekhar et al. 2004); the reason for fruit drop is unknown, except because of fungal attack. Experiments have focused on standardizing the embryo culture protocol for the rescue of embryos collected from immature fruits before withering to improve the recovery of hybrid seeds. Fruits of different maturity (1–8 weeks) were collected from the field-grown trees and cultured. The age of the fruit, culture media and growth regulators were standardized. Embryos could be rescued from 5 weeks of age, immature fruits onwards and the highest frequency of embryo recovery (42%) was obtained for 8-week-old fruits (Fig. 7.2). The plantlets developed were hardened and established in the field (Rekha et al. 2010). One important observation of this work was the absence of endosperm in many of the fertilized ovules cultured. Either the failure of triple fusion during fertilization or the degeneration of triploid nuclei after triple fusion, due to unknown factors, may be the reason for the absence of endosperm development and thereby the reason for the withering of immature fruits. Embryo rescue at early stages provides an artificial nutrient medium which serves as a substitute for the endosperm, thereby allowing the embryo to continue its development. A team from Nigeria also attempted in vitro culture of embryos and induced rooting (Ighere Dickson et al. 2011). Culturing of immature fruits from artificial pollinations and the study of growth characters revealed that the age of *Hevea* embryo is a significant factor for consideration to ensure better growth performance in vitro; older embryos had better performance than those from younger embryos (Akpobome et al. 2017).

7.5.4 Induction of Zygotic Polyembryony

Refinements in embryo culture techniques have led to the development of half-ovulo embryo culture which has been used for the induction of zygotic polyembryony (Fig. 7.3). Multiple, uniform seedlings of single zygotic origin could be

Fig. 7.3 Induction of multiple embryos and development of uniform seedlings**.** (**a,b,c**) Different stages of developing multiple embryos inside the ovule. (**d**) Four weeks old germinating individual embryos, isolated from the proliferated embryonic mass. (**e**) One year old hardened, uniform polyembryony derived seedlings in polybags. (**f**) Three year old established polyembryony derived plants in the field

developed through this approach. The genetic and epigenetic uniformity of seedlings derived through polyembryony were confirmed by SSR, RAPD and methylation sensitive AFLP analysis (Rekha et al. 2015). Development of continuously growing calli was obtained from very young embryos by the manipulation of media components (Rekha et al. 2011). This achievement in *Hevea* tissue culture opens up a way for large-scale multiplication of uniform rootstocks, thereby reducing

intraclonal variation to a large extent. Another protocol has been developed for somatic embryogenesis and plant regeneration from the zygotic embryo derived callus of the rubber tree from Malaysia (Rahman et al. 2017). This also offers the possibility of developing uniform rootstocks. Identification and multiplication of ideal rootstock is a major challenge in *Hevea* breeding. A portion of the embryogenic calli induced through this technique can be cryopreserved, until the evaluation of lines for utilization as rootstocks is completed and the selected lines can be retrived, revived and multiplied.

7.5.5 In Vitro Fertilization

The technique of in vitro pollination and fertilization is a breeding tool which has been used in numerous plants to overcome pre-fertilization barriers (Rangaswamy and Shivanna 1967; Valsala et al. 1996). Production of interspecific and intergeneric hybrids (Chin et al. 1997; Zentkler 1980); haploid production (Hess and Wagner 1974; Musial and Przywara 1998); pollen tube-female gametophyte interactions and cellular events surrounding fertilization (Dupuis and Dumas 1989; Fernando et al. 1998) have been explored. The in vitro fertilization (IVF) system has been accomplished in 57 species representing 14 families.

In *Hevea*, IVF was attempted with a view to circumvent seasonal and asynchronous flowering and inaccessibility of flowers for pollination. It was envisaged that in combination with pollen storage, large numbers of crosses could be conducted in the lab and hybrid seeds could be developed in vitro from any desired cross. Flowers, 1 day before anthesis, were identified as the right stage for pollination. The preliminary attempt of IVF in *Hevea* has resulted in successful fertilization and embryo formation. The ovules were grown up to 90 days after fertilization and embryo growth up to the cotyledonary stage was achieved. This is the first ever report of IVF in *Hevea* (Rekha et al. 2002). The results suggest the possibility of recovering seedlings after IVF by improving the nutritional status and culture conditions. Refinement of the technique at all stages is warranted so as to recover a considerable number of progenies and to utilize the system in *Hevea* breeding. Coupled with pollen storage, it is possible to bypass the asynchrony in flowering and also the scarcity of labor; there is immense potential to recover recombinants from rare crosses.

7.5.6 Ploidy Manipulation by Tissue Culture

Cytogenetic studies on *Hevea* species carried out have shown that the 2n chromosome number of somatic cells of *Hevea brasiliensis* is 36 with a base number of n = 18 (Majumder 1964: Ramaer 1935; Saraswathyamma et al. 1984). Although a few attempts at ploidy manipulations through conventional methods have been reported, the possibilities of in vitro techniques has not been explored fully (Nazeer and

Saraswathyamma 1987; Saraswathyamma and Panikkar 1988). Recent initiatives and progress in this line are discussed below.

7.5.6.1 Development of Haploids through Androgenesis/Gynogenesis

For developing androgenic haploids in *Hevea*, in vitro culture using different explants like anthers, pollen and dehisced anthers, were attempted. Sachuthananthavale and Irugalbandra (1972) were the first to culture anther. Subsequently, microcalli formation and callus induction from different anther tissue were reported by various groups (Das et al. 1994; Gandhimathi and Paranjothy 1975; Jayashree et al. 2005; Sachuthananthavale 1973). Successful plant development from pollen and soil establishment were also reported (Chen et al. 1979, 1981, 1982; Chen 1984). Attempts were also made to culture pollen protoplasts for the induction of haploid callus (Sushamakumari et al. 2012).

As an alternative approach for developing haploids, where anther/pollen culture fails or is difficult, gynogenic haploids such as unfertilized ovaries, ovules or female gametophytes are used. Several attempts have been made to develop plantlets by culturing unpollinated ovules (Guo et al. 1982; Kouassi et al. 2008). Recently Divya (2016) reported callus and embryo induction from the culture of embryo sacs. The haploid nature of the callus (n=18) was proved by cytological and flow cytometry analysis, a remarkable achievement in a tree crop like *Hevea.* A novel attempt to isolate female germ units enzymatically was also reported by Divya (2016).

7.5.6.2 Development of Triploids through Endosperm Culture

The totipotency of triploid endosperm tissue is being exploited in many vegetatively-propagated crops and this technique has much relevance in *Hevea* for many reasons. The conventional method of triploid production by crossing a diploid with an artificially-induced tetraploid is a lengthy and laborious process. In vitro regeneration of plants from endosperm tissue provides an easy and direct approach for the production of a large number of triploids. Many triploid lines have proved to be superior to their diploid and tetraploid counterparts, in terms of yield, nutritional qualities and biotic and abiotic tolerance in other crops (Bhojwani and Johr 1971; Thomas et al. 2000). High vigor and increased biomass is expected in triploids and is highly desirable in *Hevea* since it may lead to a reduction in the immaturity period as well as an increase in yield. In vitro regeneration of plants from endosperm, the naturally occurring triploid plant tissue, offers the opportunity to develop a large number of triploids in a single step. Rekha et al. (2007) in *Hevea* reported successful induction of callus, embryos and plantlets from endosperm culture for the first time. Recently Divya (2016) reported the successful induction of direct embryogenesis from endosperm tissue. This is an area, which is underexploited and needs attention. Once plant regeneration is standardized, it is easy to obtain triploid plants with different gene combinations and the lines can be identified and utilized. It may be noted that in triploids developed in vitro, the two

maternal sets of chromosomes will be homozygous, since the primary endosperm nuclei (2n) is formed from the fusion of two identical nuclei in the embryo sac. Hence there is the chance of partial fertility and recovery of anueploids among the progenies which can be further utilized.

7.5.6.3 Tetraploids

Tetraploid cells are much larger than those of diploids, as tetraploids have twice the number of sets of chromosomes. Usually tetraploids exhibit more vigor, faster growth and increased biomass compared to their diploid counterparts. With the advent of in vitro techniques for chromosome doubling using antimitotic agents, in vitro induction of tetraploids through colchicine treatment has been achieved in many plants such as *Pyrus communis* (Sun et al. 2009), *Morus alba* (Chaicharoen et al. 1995) and *Cinchona ledgeriana* (Nair 2010). Colchicine ($C_{22}H_{25}NO_5$), is an alkaloid extracted from *Colchicum autumnale* and inhibits spindle fiber formation during cell division. In vitro induction of polyploids by treating the diploids with colchicine has also been successful in tree crops, such as African oil palm (Atichart 2013).

In *Hevea*, a reduction in gestation period is a major breeding objective and any attempt to achieve this goal is significant. The yield per tree per tap is also expected to increase with increase in tree girth due to an increase in the length of the tapping cut. High-girth trees are also suitable for cultivation as latex-timber clones. Polyploids showed faster growth in *Acacia senegal* and their superiority was estimated to be 17 % in trunk diameter and 9 % in hight over diploids (Adja M. Diallo et al. 2016). Attempts to induce polyploidy in *Hevea* have been initiated by the various rubber research institutes since 1969 (Shepherd 1969). Mendes and Mendes (1963) reported that tetraploid clones of *Hevea* showed an increase in yield compared to the diploid in the immature stage, based on a microtapping test. Other ex vitro attempts reported include those by Markose (1975) and Saraswathyamma et al. (1984). After the development of in vitro plant regeneration systems, the scope of developing polyploids through chromosome doubling by colchicine treatment in embryogenic cell lines was exploited. Compared to seeds and shoot apexes, use of callus for diploidization maintains clonal integrity, except for somaclonal variations. As it arises from a single cell, complete tetraploidy is ensured in the regenerants and the chance of chimeras is remote. Divya (2016) first reported chromosome doubling through colchicine treatment and the successful regeneration of tetraploid plants. The ploidy of regenerants was confirmed by cytological and flow cytometric analyses.

7.5.7 Protoplast Technology

Protoplasts are plant cells from which the cell wall have been removed with the help of cell wall digestion enzymes. Even without cell wall, plant cells retain totipotency and can dedifferentiate, re-enter the cell cycle, go through repeated mitotic divisions and regenerate into plants. Thus there is a possibility of using this technology as a

powerful breeding tool by fusing protoplasts from different species and regenerating plants from the protoplasmic hybrids (Johnson and Veilleux 2001). Creation of homokaryon, heterokaryon as well as cybrids is possible by this technique (Xia 2009). During the last decade, interest in protoplast research was renewed, partly due to public antagonism towards genetically-modified organisms (Eeckhaut et al. 2013). The possibility of utilizing this technique was exploited and reported in *Hevea* by various research groups (Cailloux and Lleras 1979; Haris Ndarussamin and Dodd 1993; Rohani and Paranjothi 1980; Wilson and Power 1989). The use of tobacco nurse culture to maintain viability, promoted division of protoplasts and formation of microcalli for the first time was reported by Cazaux and d'Auzac (1994, 1995). Sushamakumari et al. (2000b) experimented with different explants like leaf, young stem, friable callus and fine suspension cells of inflorescence-derived callus and observed that among various tissues, cell suspension was the best source for obtaining high yield of viable protoplasts. Sushamakumari et al. (2000b) also reported plant regeneration in *Hevea* for the first time by using *Lolium multiflorum* as nurse cells. Later, Das and Dey (2009) found young leaves as a potential source for protoplast isolation with 95 % healthy and viable protoplast. Recently, Sushamakumari et al. (2012) succeeded in isolating viable protoplasts from intact pollen grains and cultured them in presence of *Hevea* nurse culture, leading to the formation of microcolonies. Plantlets were regenerated from protoplast derived callus recently by Dai et al. (2014). Isolation of protoplasts from mature and immature endosperm has also been attempted in *Hevea* (Divya 2016). Contrary to the expectations, very few successful events came out of this technique, mainly due to the requirement for sophisticated instrumentation and laboratory facilities, in addition to the common issues associated with in vitro regeneration of *Hevea.*

7.6 Molecular Breeding

Molecular breeding is the application of molecular biology tools like molecular genetic markers and genetic engineering manipulation, for the improvement of existing varieties of plants or animals.

7.6.1 Development of Molecular Markers

Molecular markers have a great impact on various aspects of crop improvement, from diversity studies to genome mapping, gene tagging, map-based cloning and physical mapping of genes, and ultimately in the precise selection of promising genotypes. After proper validation, molecular markers have immense potential to circumvent lengthy field-evaluation procedures. Molecular biology techniques have become very useful in providing information regarding time scales on how related species diverged from each other, based on the analysis of genetic variations in

different species (Ratnaparkhe et al. 1998). They are also valuable in biodiversity studies of gene pools of crops for cultivar development and in the classification of germplasm collections. Molecular markers have been used in rubber extensively for the last three decades for diversity studies, characterization of clones, linkage map construction and QTL analysis. In rubber, isozymes were utilized for clonal identification from the 1980s (Chevallier 1988). Over the last two decades, several tools like minisatellites (Besse et al. 1993), RFLPs (Besse et al. 1994), RAPDs (Varghese et al. 1997; Venkatachalam et al. 2001, 2002), AFLPs (Lespinasse et al. 2000) and SSRs (Atan et al. 1996; Low et al. 1996; Mantello et al. 2012; Roy et al. 2004; Saha et al. 2005) were developed in *Hevea brasiliensis*.

In *Hevea*, AFLP and RFLP markers have been widely used for clone identification (Low et al. 1996). The genetic diversity of the Amazonian and Wickham clones were also assessed by Besse et al. (1994), based on RFLP profiles, and they concluded that the Amazonian population brought genetic enrichment to *Hevea* germplasm. Genetic diversity analysis using microsatellite markers also confirmed that wild accessions are more polymorphic than cultivated Wickham clones (Lekawipat et al. 2003). RAPD markers were also successfully used to estimate genetic distances and relationships in cultivated *Hevea* clones and to distinguish them based on their origin (Varghese et al. 1997; Venkatachalam et al. 2002). The presence of highly polymorphic DNA in the Amazonian accessions was also reported by Luo and Boutry (1995), using mitochondrial DNA (mtDNA) analysis.

Development of genetic maps is one of the important applications of molecular markers in any species. Genetic maps indicate the position and relative distances between markers along the chromosomes. A linkage map enhances the probability of finding markers linked to quantitative trait loci (QTLs) by helping in the selection of molecular markers which are evenly distributed over the entire genome. Such QTL-linked molecular markers will co-segregate with the genes involved in the traits of interest and could be used efficiently to study introgression and accumulation of favorable traits during recombination events. In rubber, several research groups have constructed genetic linkage maps from both intraspecific and interspecific crosses with the intention of linking markers with QTLs. The first rubber tree genetic map was developed by Lespinasse et al. (2000) from an interspecific cross between *Hevea brasiliensis* and *H. benthamiana*. Another map based on SSR markers was constructed by Feng et al. (2010). A linkage map exclusively based on RAPD markers was constructed by Novalina and Sagala (2013). A linkage map making use of RAPD, AFLP, SSR and SNP markers was constructed by Bini (2013). Another map was constructed by Souza et al. (2013) using SSR markers to identify QTLs associated with growth-related traits in a full-sib family. In order to generate high-density linkage maps, researchers shifted from anonymous markers like AFLPs and microsatellites to SNPs by direct analyses of sequence variations. Advances in high-throughput next generation sequencing technologies have enabled large scale SNP discovery in rubber tree and aided in the development of high-density linkage maps (Mantello et al. 2014; Pootakham et al. 2011, 2015; Shearman et al. 2015). The above studies on sequence diversities have shown that SNP frequencies in rubber are comparable to those in other crops. Molecular markers also aid in locating

genes governing agronomically-important characters through such genetic linkage maps. Major genes can be mapped by establishing association between molecular markers and inherited traits. Mapping of genes can also be done by anchoring large contigs and scaffolds obtained from RNA sequencing experiments into linkage groups. Adopting this strategy, Shearman et al. (2015) mapped 20,143 predicted genes from rubber tree which will be useful for further mapping studies.

Recent molecular genetic investigations in rubber indicate that SNPs, including insertion/deletions (indels), can provide a rich source of useful molecular markers in the genetic analysis of *Hevea*. However, there are only limited reports regarding the identification and utilization of SNP markers in rubber. The first report on large scale SNP identification and validation of SNP in *Hevea* was by Pootakham et al. (2011). They identified 5883 biallelic SNPs by transcriptome sequencing and 50 of them were validated in *Hevea* clones. Later, Mantello et al. (2014) reported 404,114 SNPs by de novo assembly of transcriptome data of rubber tree generated using NGS. They also validated 78 SNPs in 36 genotypes. By an allele-specific amplification technology, Salgado et al. (2014) characterized 23 *Hevea* genotypes using 191 SNPs screened out of 2191 single nucleotide variation (SNVs), discovered by the RNA sequencing method. In the following year, Shearman et al. (2015) identified more than 300,000 SNPs from 6 popular clones and used these SNPs to construct a linkage map for rubber. They reported a frequency of approximately 1 SNP in every 270 nucleotides in the *Hevea* genome. Recently SNP/indels were also used to investigate yield heterosis of rubber tree (Li et al. 2016).

Thus far, several molecular markers and significant genomic resources have been developed from *Hevea*; however, their direct utilization in crop improvement programs is hindered, mainly due to factors such as the polygenic nature of traits of interest (yield, disease resistance), difficulties in maintenance of a population for marker validation, accurate phenotyping of desirable characters, etc. It is anticipated that a comprehensive approach incorporating conventional as well as molecular breeding techniques may hasten the ongoing crop improvement programs in *Hevea*, rather than a unilateral approach.

7.6.2 Genetic Manipulation and Development of Transgenic Plants

Over the past decades, the value of introducing alien genes into plants using recombinant DNA technology has been well documented. This technology is expected to reduce the constraints associated with conventional breeding methods and accelerate breeding programs in woody species like *Hevea.* The ability to obtain the expression of specific foreign or native genes by genetic manipulation in *Hevea* opens up the possibility of improving it commercially. The identification and fixation of a particular gene through conventional methods of breeding requires several generations of crosses, and field trials carried out over many years in *Hevea*. The

emergence of new functional genomic strategies for the identification and characterization of genes have enormous potential to augment plant breeding and to genetically engineer plants for specific purposes. Recent developments in recombinant DNA technology allow gene or genes for a particular character to be isolated from any organism and introduced into any crop species by genetic transformation. A wide range of traits can be introduced without compromising the genetic background of the elite clones, provided a reliable method of plant regeneration for the transformed cell is available.

For the introduction of a transgene into a cell, several methods have been developed. Among them, *Agrobacterium*-mediated gene transfer is the most widely used and powerful technique in the recent past. The ability of *Agrobacterium* to genetically transform a wide variety of plant species, has contributed much in modern biotechnology. *Agrobacterium tumefaciens*, the causative agent of crown gall disease in nature, called a natural genetic engineer, was discovered at the turn of the last century. Transformation results from the production of a single-stranded copy of a T-DNA molecule by the bacterial virulence machinery, its transfer into the host cells, followed by the integration into the host genome.

The availability of an efficient in vitro plant regeneration system is an essential prerequisite for developing transgenics. In *Hevea brasiliensis,* protocols for plant regeneration from different explants such as integument, immature anthers, immature inflorescences and leaves through somatic embryogenesis is already reported (Thulaseedharan et al. 2009). *Agrobacterium* and biolistic-mediated genetic transformation systems have been successfully employed in the production of transgenics. Since the major harvested products such as latex, wood and rubber seed oil are not used as food material, biosafety concerns are less for the genetically-modified rubber plants compared to food crops (Rekha et al. 2014). Therefore, genetic engineering will play an important role in rubber breeding in the years to come.

7.6.2.1 Progress in Genetic Manipulation

Efforts to genetically modify *Hevea* species have been made over the past three decades. Successful induction of tumors in the stem of in vitro and in vivo propagated seedlings, using *Agrobacterium tumefaciens* was demonstrated in *Hevea* in 1994. For optimizing various transformation systems in rubber tree, different investigators employed GUS as the reporter gene and *npt*II as the marker gene under the control of cauliflower mosaic virus (*CaMV*) 35S promoter (Arokiaraj et al. 1994, 1996, 1998; Blanc et al. 2006; Montoro et al. 2000, 2003). The first successful report of *Hevea* transgenic plants was from Malaysia in 1994 with the introduction of the β-glucuronidase (*GUS*) gene (Arokiaraj et al. 1994). Later, different laboratories were successful in developing transgenic plants for increased tolerance to abiotic stresses and TPD, enhanced rubber yield and recombinant protein production (Thulaseedharan et al. 2017). Transgenic plants were also produced using particle bombardment (Arokiaraj et al. 1998), but with low efficiency.

7.6.2.2 Transgenics for Stress Tolerance

Unprecedented changes in temperature, rainfall distribution, the occurrence of extreme weather events and global warming experienced by rubber growing regions have serious consequences in the growth and yield of natural rubber. Excessive rain leads to higher disease incidence which necessitates use of chemical fungicides which is not advisable from an economic and environmental perspective. Developing climate resilient clones is the best option at this juncture. Classical breeding and selection cannot yield tangible results within a reasonable period and so the GM approach is again the viable option.

Transgenic rubber plants were developed at Rubber Research Institute of India using the gene coding for manganese superoxide dismutase (*HbMnSOD*), which is the first enzyme involved in the detoxifying process of reactive oxygen species, through *Agrobacterium*-mediated genetic transformation using immature anther-derived calli as the target tissue. The incorporation of the *uidA*, *npt*II and *HbMnSOD* genes in the hardened transgenic plants were confirmed by polymerase chain reaction and genomic Southern blot hybridization analysis (Jayashree et al. 2003). Transgenic plants integrated with *MnSOD* gene were also developed with a different gene construct with Figwort mosaic virus 34S (FMV 34S) promoter for the expression of *MnSOD* along with the selectable marker gene *npt*II and the reporter gene *GUS* (Sobha et al. 2003b). Overexpression of SOD, peroxidase and catalase enzymes in response to abiotic stresses was also reported in these transgenics (Sobha et al. 2003a). Jayashree et al. (2011) studied the transgene expression and other drought-tolerant traits in MnSOD transgenic plants. Six-month-old polybag plants were subjected to water stress and drought tolerance was evaluated through molecular, physiological and biochemical tools. A higher SOD transcript level and SOD enzyme activity, were found in the transgenic plant compared to the control. The physiological performance of 1-year-old MnSOD transgenic bud grafted plants was evaluated in a dry sub-humid environment by withholding irrigation to assess the recovery after re-watering. The parameters for drought tolerance studied viz. the pre-dawn leaf water potential, relative water content, net photosynthesis rate, stomatal conductance and antioxidant enzyme levels were better in the transgenic plants compared to their controls (Sumesh et al. 2014). Leclercq et al. (2012) regenerated transgenic *Hevea* plants overexpressing a *H. brasiliensis* cytosolic *CuZnSOD* gene. They studied the physiological parameters related to drought tolerant traits after the plants were subjected to water deficit treatments. Efficient ROS scavenging by overexpression of *HbCuZnSOD* and activation of all ROS-scavenging enzymes were observed in the transgenic line. Since tapping panel dryness (TPD) is a major problem, especially in high-yielding clones and reduced levels of cytokinins were reported in the TPD-affected trees (Das et al. 1998), an attempt was made to overproduce cytokinin in rubber tissues by incorporating the gene coding isopentenyl transferase (*ipt*) to combat TPD. The putative transformed calli showed higher levels of cytokinins and were capable of survival without external supply of hormones. Occurrence of embryos with developmental abnormalities was also observed (Kala et al. 2003). In order to achieve tolerance to a variety of stresses, introduction of a

stress responsive multifunctional protein, osmotin, was successfully carried out in *Hevea*. Transgenic cell lines and embryos were developed, germinated, regenerated plantlets were hardened and established in polybags (Fig. 7.4). The molecular confirmation of gene integration and expression was done by PCR, Southern and RT-PCR (Fig. 7.5) (Rekha 2013; Rekha et al. 2013, 2014). The stress tolerance studies conducted using transgenic calli, integrated with the osmotin gene, showed a higher accumulation of proline under stress, indicating stress tolerance ability. Similarly, the transgenic calli could survive and proliferate in culture media containing 150 mM NaCl, whereas the non-transgenic calli showed retarded growth even at 50 mM NaCl (Rekha et al. 2016). Recently, efforts were made to introduce the sorbitol-6-phosphate dehydrogenase gene, isolated from apple, into the *Hevea* genome to impart drought tolerance. Sorbitol serves as a compatible solute, under conditions of decreasing water, an antifreezing agent for chilling or as a scavenger of free radicals. Embryogenic calli from immature anthers were used as the target tissue for *Agrobacterium*-mediated transformation and plantlets were regenerated (Kumari Jayasree et al. 2015). In rubber-growing areas, high wind can seriously damage rubber trees; pruning is carried out to minimize such damage. In order to develop dwarf phenotypes, for imparting tolerance to strong wind, Wang et al. (2013) attempted the introduction of an *Arabidopsis* gibberellic acid insensitive

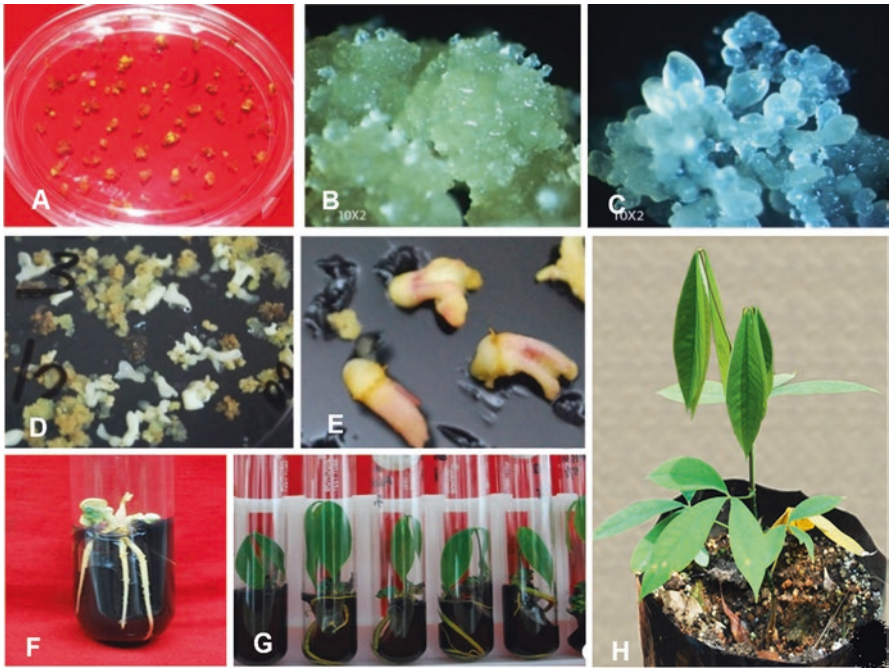
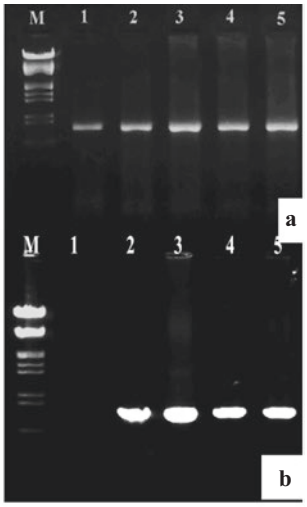


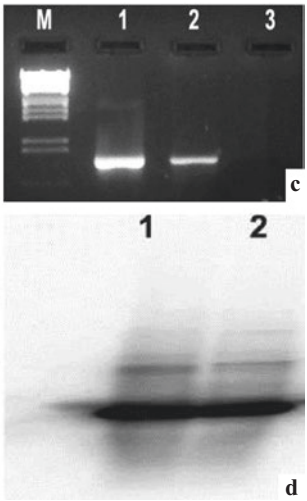
Fig. 7.4 Development of transgenic plants integrated with osmotin gene. (**A**) Selection of transgenic lines, (**B**) Embryo induction, (**C**) Globular embryos, (**D, E**) Embryo maturation, (**F**) Germination, (**G**) Plant regeneration, (**H**) Acclimatized plant



a. Amplification of osmotin gene (750bp)
Lane 1-4- Transgenic cell lines
Lane 5 - Plasmid

b. Amplification of *npt* II gene (700 bp)

Lane 1 - Non transgenic control
Lane 2-4 - Transgenic lines
Lane 5 - Plasmid



c. RT PCR

Lane-M -Marker
Lane -1- Positive control
Lane- 2- Transgenic sample
Lane -3- Non-transgenic sample

d. Southern blot
Lane -1&2 →*Eco* RI digest of the DNA from transgenic plants

Fig. 7.5 Molecular confirmation of transgene integration

(*GAI*) gene into *Hevea.* The genetic transformation was carried out through microparticle bombardment using 40-day-old calli and plantlets were regenerated. To improve the agronomical trait for glyphosate-resistance gene transfer through *Agrobacterium* was conducted in Thailand by Kalawong et al. (2014). The bacteria carrying plasmid pCAMBIA 1304, harboring *GUS* as a reporter and EPEPS (5-enolpyruvylshikimate-3-phosphate synthase (*CP4 EPSPS*) gene for herbicide tolerance, was used. Shoots and nodal segments were used as target tissue and integration and expression of the gene was confirmed through *GUS* histochemical assay, PCR and dot blot hybridization.

7.6.2.3 Transgenic Rubber Plants for Enhanced Rubber Yield

From the Wickham base, an almost a ten-fold increase in productivity has been achieved through classical breeding (Mydin and Gireesh 2016; Varghese and Mydin 2000). However, the elucidation of the fundamentals of gene regulation the biosynthetic machinery and limiting steps in key biosynthetic processes needs to be understood for future breeding (Sethuraj and Jacob 2012). Manipulating the factors influencing the rate of rubber biosynthesis is expected to provide yield enhancement at a required level in a shorter period. Similarly, an increase in the latex vessel number can be accomplished by manipulating the jasmonic acid biosynthesis, since jasmonic acid is the signaling molecule involved in the laticifer differentiation (Hao and Wu 2000). The introduction of genes encoding enzymes involved in the jasmonic acid biosynthesis can bring about latex vessel differentiation in *Hevea*. A key enzyme in this pathway, allene oxide synthase (AOS), has been cloned and further studies are in progress (Arokiaraj et al. 2002). Rubber biosynthesis occurs in the latex, mainly by the mevalonate pathway (MVA) where acetyl Co A is converted to polyisoprene.

One of the key regulatory points in the isoprenoid pathway is the irreversible conversion of HMG-CoA to mevalonate, catalyzed by 3-hydroxy-3-methylglutaryl coenzyme A reductase (*hmgr*1), which is considered as a rate-limiting enzyme in the pathway (Lynen 1969). The association between *hmgr*1 enzyme activity and rubber biosynthesis and its regulatory role is also well proven (Nair and Kurup 1990; Nuntanuwat 2006; Suwanmanee et al. 2007). Initial attempts to transform *Hevea* with the *hmgr*1 gene were made by Arokiaraj et al. (1995) and succeeded in obtaining a higher *hmgr*1activity in the transformed callus. Transgenic *Hevea* plants integrated with laticifer specific *hmgr*1 gene were developed and established with good transformation frequency (Jayashree et al. 2014, 2018). Recently two *Hevea brasiliensis* ethylene responsive factors, *HbERF-IXc4* and *HbERF-IXc5*, key regulators of ethylene and jasmonate signalling pathways, were used for transformation and transgenic lines, overexpressing the genes were developed (Lestari et al. 2017).

7.6.2.4 Rubber Trees as Living Factories

Advancements in recombinant DNA technology have opened up a promising area of research, *molecular pharming*, during the past two decades, for the conversion of plants into living factories to synthesize products of high commercial value. The use of plants to produce recombinant proteins of pharmaceutical importance has gained much attention recently, since these green bioreactors offer considerable advantages. Factors like the capacity to carry out eukaryotic post-translational modifications (Ma et al. 2005), simplicity, reduced risk of contamination, cost effectiveness and simplified purification system enabling easy scaling-up of the process, has attracted researchers to this technology (Tremblay et al. 2010).

The use of plants as bioreactors will be effective only when stable high-level production over the lifetime and in subsequent generations is assured at minimum cost (Sunderasan et al. 2010). In this context the rubber tree has many unique advantages over other plants for biopharming. For example, the latex consisting of rubber

particles, lutoids and double-membrane organelle rich in carotenoids and Frey-Wysling particles, makes it a perfect *machine* for protein synthesis, if the desired gene is inserted (Pardekooper 1989). Moreover, the latex is harvested by a nondestructive method, which is a continuous process for a period of over 25 years. The vegetative propagation of this crop is another factor that favors the maintenance of transgenics for biofarming. Considering the above advantages, the rubber tree is the most suitable candidate tree for biopharming (Yeang et al. 1998).

Utilization of transgenic plants for the production of a variety of recombinant proteins, including edible vaccines, therapeutic proteins and antibodies for immunotherapy, was documented by Thulaseedharan et al. (2017). The heterologous expression of proteins in the latex vessels was first explored by Arokiaraj (2000); and Arokiaraj et al. (2002). They developed transgenic rubber plants that secrete human serum albumin (HSA) in the serum fraction of rubber latex, through *Agrobacterium*-mediated genetic transformation. Similarly Yeang et al. (2002) reported the expression of a functional recombinant single-chain variable fragment (ScFv) antibody in the latex of transgenic rubber. Attempts were also made to transform *Hevea* using TB antigen protein isolated from *Mycobacterium tuberculosis* (Kala et al. 2006). Transgenic *Hevea brasiliensis* was also developed for the expression of a gene encoding human atrial natriuretic factor (hANF), a peptide hormone involved in regulating cardiac blood pressure (Sunderasan et al. 2012). The major gene transfer attempts reported so far in *H. brasiliensis* are listed in Table 7.2.

7.6.2.5 Gene Stacking

Gene stacking is a viable approach for introducing multiple genes which involves the integration of two or more genes simultaneously or sequentially into the target tissue by standard delivery systems such as *Agrobacterium*-mediated transformation. In *Hevea brasiliensis*, genes coding for different agronomic traits viz. manganese superoxide dismutase (*MnSOD*) for enhanced environmental stress tolerance and 3-hydroxy-3-methyl-glutaryl-CoA reductase (*hmgr1*) for improved latex yield were integrated in *Hevea* callus by *Agrobacterium*-mediated repeated transformation (Sobha et al. 2014).

Intensive research was also carried out for protocol improvements, explant sources additive for increasing transformation efficiency, bacterial strains, promoters and media components for regenerating transgenic plants; these are reviewed by Thulaseedharan et al. (2017).

7.6.3 Constraints for Developing Transgenic Plants in Hevea

7.6.3.1 Varying Response of Explants

Similar to somatic embryogenesis, seasonal variation observed in the quality and culture response of explants is a major difficulty in genetic transformation, often affecting the reproducibility of the process. In a single transformation experiment,

Table 7.2 Gene transfer attempts in *Hevea brasiliensis*

Gene	Genotype	Method	Vector	Target tissue	Reference	Highlights
Initial attempts with reporter genes						
GUS, (*nptII*) (*cat*) gene	Gl 1	Particle gun	pCAMBIA2301	Anther callus	Arokiaraj et al. (1994)	Embryos with GUS expression
	PB5/51	*Agrobacterium* strain 541	No vector	Seedlings	Arokiaraj and Rahaman (1991)	First attempt on transformation through *Agrobacterium* and developed tumors on seedlings
	Gl 1	*Agrobacterium* LBA 4404	P35GUSINT	Anther callus	Arokiaraj et al. (1996)	First transgenic *Hevea* plant through *Agrobacterium* mediated transformation
		Agrobacterium GV2260		Anther callus	Arokiaraj et al. (1998)	Transgenic plant
*GUS, npt*II	PB260	C58pMP90, C58pGV2260, AGL1, LBA4404, EHA105	pCAMBIA230	Integument callus	Montoro et al. (2000)	Enhanced transformation efficiency
	PB 260	*Agrobacterium*, EHA105	pCAMBIA230	Integument callus	Montoro et al. (2003)	Transgenic cell lines
	PB 260	*Agrobacterium* EHA105	pCAMBIA230	Integument callus	Blanc et al. (2006)	Transgenic plants developed
Functional genes						
MnSOD	RRII 105	*Agrobacterium* EHA 101	pDU 96.2144	Anther derived callus	Jayashree et al. (2003)	Transgenic plants with over expression of MnSOD.
MnSOD	RRII 105	*Agrobacterium* EHA 101	pDU 96. 2412	Anther derived callus	Sobha et al. (2003b)	Transgenic plants

(continued)

Table 7.2 (continued)

Gene	Genotype	Method	Vector	Target tissue	Reference	Highlights
MnSOD	RRII 105	*Agrobacterium* EHA101	pDU96.2144,pDU.96.2111	Anther callus	Rekha et al. (2006)	High frequency transformation in the callus
HbCuZnSOD	Callus line CI05519 of *Hevea* clone PB 260	*Agrobacterium* EHA 105	pCAMBIA2301 pCAMBIA2300	Anther callus	Leclercq et al. (2012)	Regenerated plants and proved overexpression and better stress tolerance in transgenics
IPT	RRII 105	*Agrobacterium*	pDU 97.0612.	Anther callus	Kala et al. (2003)	Transgenic embryos
Osmotin	RRII 105	*Agrobacterium* GV2260	pBin19	Anther callus, zygote derived callus	Rekha et al. (2010, 2013, 2014)	Transgenic plants
Sorbitol 6 phosphate dehydrogenase	RRII 105	*Agrobacterium* EHA101	pDU 93.03.05	Anther callus	Kumari Jayasree et al. (2015)	Transgenic embryos
Yield enhancement						
Hmgr	Gl 1	*Agrobacterium*	Not available	Anther callus	Arokiaraj et al. (1996)	Transgenic plantlets
*Hmgr*1	RRII 105	*Agrobacterium* LBA4404, EHA105,PGV	pBIB	Zygote derived callus	Jayashree et al. (2014, 2018)	Transgenic plants
Pharmaceutical products/Rubber plants as living factories						
TB antigen	RRII105	*Agrobacterium* EHA101	Kanamycin	Anther callus	Kala et al. (2006)	Transgenic embryos
ScFv	Gl 1	*Agrobacterium* GV 2260 and GV 3850)	Kanamycin	Anther callus	Yeang et al. (2002)	Expression of ScFv protein in latex of T plants

Human atrial natriuretic factor (HANF)	Gl1	*Agrobacterium* GV2260	The pGPTV-Kan-HANF	Anther callus	Sunderasan et al. (2012)	Transgenic plants expressing the protein
Human serum albumin (HSA)	Gl1	*Agrobacterium* GV2260	pLGMR.HSA:pToK47	Anther callus	Arokiaraj et al. (2002)	Transgenic Plants
Other recent developments						
Dwarfing (*GA1*)*GUS*, *Npt*II		Particle bombardment	pBI121	Anther callus	Wang et al. (2013)	Gene insertion confirmed in transgenic callus through PCR
HbERF-IXc4 and HbERF-IXc5	PB260	EHA105	pCamway 2300	Integument calli	Lestari et al. (2017)	Transgenic plants with overexpression of ERF genes
EPEPS	Native clone (Thailand)	*Agrobacterium*	pCAMBIA 1304	Seeds	Kalawong et al. (2014)	Transformed shoots
Multiple gene *MnSOD* & *Hmgr*1	RRII 105	EHA 101, PGV	pDU96.2144,pBIB	Anther callus	Sobha et al. (2014)	Transgenic embryo with both genes

it is possible to recover a large number of transgenic cell lines at a time, provided all the conditions are favorable. However, simultaneous maintenance and management of these lines may be difficult.

7.6.3.2 Constraints in Selection, Proliferation and Regeneration

Slow proliferation of transgenic lines is experienced after the initial emergence of numerous lines from the infected cultures. Media manipulations may be considered as a solution to this problem to a large extent. A major problem observed at the plant regeneration stage is the identification of the best line with embryogenic potential. During plant regeneration, although embryo induction is easy, a major percentage of embryos are found to carry abnormalities. Culture-induced or transgene-induced variation may be the reason for the aforementioned abnormalities. In the case of normal looking embryos also, germination is poor, sometimes with strong thick roots but without shoots or vice versa. Therefore identification of the normal lines and media manipulations seems to be necessary. Acclimatization and hardening is the most difficult step in developing transgenics. In *Hevea,* reports show that the hardening percentage is as low as 0.4 %. Even normal, healthy plantlets derived from clonal explants show difficulty in hardening. Even after successful initial hardening, plants show retarded growth leading to mortality at a later stage. Infestation of pests and diseases after transferring from the hardening facility is also a problem.

7.6.3.3 Transgene Silencing and Associated Instabilities

Undesired transgene silencing is a common phenomenon and a major concern in transgenic plants developed for crop improvement. Stability and segregation is a major prerequisite for plants expressing a transgene. But there are several reports which show that a transgenic plant deviates from the Mendelian segregation ratios (Shrawat et al. 2007; Weinhold et al. 2013). These deviations indicate the existence of hitherto unknown cellular mechanisms, which regulate the expression of transgenes. Instabilities associated with transgenes and the reasons behind them were reported by Charrier et al. (2000), Graham et al. (2011) and Stroud et al. (2013). The two different molecular routes proposed for this phenomenon are transcriptional gene silencing and post-transcriptional gene silencing (PTGS), which are associated with blocking the transcription of transgenes by heavy methylation of promoter regions and mRNA degradation of transgenes or endogenous genes (Fagard and Vaucheret 2000). After the cumbersome process of developing transgenics, the lack of transgene expression is highly undesirable. PTGS can silence transgenes as well as endogenous genes depending on their homology, an endogenous gene could be switched off, when a plant is transformed with another copy of the same gene. For example when a pigmentation gene like *Chalcone synthase A* in *Petunia*, was overexpressed, activity of both the transgene and endogenous gene were lost in many transgenic lines, either partially or completely

(Napoli et al. 1990). Another class of small RNAs called siRNAs are also involved in epigenetic modification (Miki and Shimamoto 2008). They silence genes by inducing DNA methylation at CpG nucleotides leading to chromatin modification. Based on the above studies, it is evident that transgenes are prone to silencing in transgenic lines and the rate of silencing may vary from line to line. In the case of transgenic rubber also, high rates of abnormalities were observed among the lines which may be due to epigenetic modifications in the genome. Although experimental evidence is not currently available on transgene silencing in *Hevea*, it is expected that transgene silencing by DNA methylation may be one of the factors responsible for the observed abnormalities.

7.6.3.4 Biosafety and Environmental Issues

Despite its advantages, transgenic technology has generated serious public concerns, particularly about human health and the natural environment. The main risks suspected are toxicity, allergenicity and gene flow. Toxicity depends upon the specific transgene product expressed in the GM crop. Even though there is no evidence for these threats, the introduction of specific proteins leading to the development of allergens is a major concern. The use of antibiotic resistance genes as selectable markers is another issue. In genetic transformation experiments, selectable marker genes are widely used for differentiating transformed cell lines from untransformed ones. However, they serve no purpose once transgenic plants have been regenerated. The presence of these marker genes, especially antibiotic marker genes in the genetically modified (GM) plants has raised considerable public concern regarding their potential transfer to other organisms and their safety (Thomson et al. 2001), the environmental implications of growing GM crops (Ho 2001) and the health aspects of consuming GM foods.

Concerns about environmental risks due to the introduction of transgenic crops include the impact of the transgenic trait on other related species, the potential build-up of resistance in specific insect populations affecting the biodiversity and unintended effects on non-targeted organisms. Contamination of existing varieties through crossbreeding between transgenic crops and traditional varieties through pollen transfer is another point raised by environmentalists. Because of these concerns, commercial cultivation of GM crops is under stringent control and getting permission to conduct field trials has become a herculean task in many countries.

Since *Hevea brasiliensis* is a native of South America and rubber cultivation is predominantly in South Asian countries, where the presence of sexually compatible wild relatives are rare, the risk of gene flow is also very remote. Since *Hevea* is not a food crop there is no threat of contaminating foods. Due to these factors, GM rubber is considered as safer for cultivation than other crops.

7.6.4 Overcoming Constraints: Possibilities and Progress

7.6.4.1 Cryopreservation

For the long-term conservation of genetic resources, preservation in liquid nitrogen at ultra-low temperature (-196 ºC) is an effective alternative to in situ conservation methods. This cryopreservation arrests biochemical and physical processes completely and plant material, can be stored for unlimited periods without genotypic or phenotypic change. This technology is extremely useful for the safe long-term storage of medicinal and alkaloid producing cell lines, hairy-root cultures and genetically-transformed (Elleuch et al. 1998) and transformation-competent culture lines (Gordon-Kamm et al. 1990). Cryopreservation can be employed for the preservation of stock cell cultures (embryogenic calli derived from different sources) for somatic embryogenesis as well as for transformation. Moreover, transgenic events can also be preserved so that the events can be handled one by one with more care and efficiency. The cross contamination and somaclonal variation due to repeated subculture can be avoided. In addition, reliable cryopreservation methods enable the long-term storage of transgenic lines with a high gene expression and regeneration capacity, during the time required for the verification of transgenic plant characteristics.

The cryopreservation technique of embryogenic callus is reported in many tree crops (cocoa, citrus, chestnut, cherry, avocado). Reports available on *Hevea* cryopreservation are limited. Normah et al. (1986) made a preliminary study on cryopreservation of *Hevea* zygotic embryos. Later, Veisseire et al. (1993) successfully froze embryogenic cell suspensions and studied the effect of pregrowth and preculture conditions on the survival of callus. Subsequently, Engelmann et al. (1997) attempted cryopreservation of embryogenic callus of two clones with two protocols, using either a classical freezing or a simplified freezing process. Lardet et al. (2007) developed a cryopreservation technique for embryogenic callus and found that reduced $CaCl_2$ concentration promoted regrowth of callus. More recently, Zhou et al. (2012), reported a simple vitrification procedure by which the callus was successfully cryopreserved in liquid nitrogen and subsequently regenerated into plantlets. Development of a successful and efficient protocol for the storage of materials will enable proper utilization of in vitro techniques to address the formidable challenges in *Hevea* breeding.

7.6.4.2 Zygote-Derived Explant Use to Exploit Juvenility

The success of somatic embryogenesis, as well as development of GM, generally depends upon the selection of the explant. In-vitro propagation of mature trees is more difficult than seedling explants. Establishment of embryogenic cultures in the shortest possible time is very important for the application of somatic embryogenesis for clonal propagation of trees. In *Hevea*, also the physiological age of the source plant from which the explant is collected determines the embryogenic capacity. Among the different sources of explants collected, the leaves from in vitro

derived plants gave best results (Kala et al. 2014). Embryogenic capacity was found to decrease with maturity. Embryogenic calli derived from immature zygotic embryos proved to be a promising explant in *Hevea*, to obtain high transformation frequency, plant regeneration and hardening (Rekha et al. 2011, 2014).

Zygotic embryos have been used as explants for somatic embryogenesis and for developing transgenic plants in different crop species (Elhiti and Stasolla 2011). The increased efficiency of embryogenic callus formation and regeneration of plantlets was observed in many crops including wheat by using polyembryos derived from the zygote as the explant. The secondary somatic embryos derived from the cotyledonary explants of zygotic embryos were successfully utilized for the development of transgenic plants in walnut (Dandekar et al. 1989). In clonally propagated woody plants, the explant source is an important consideration in maintaining clonal integrity. Recalcitrance of clonal explants prevents the production of transgenic plants on a commercial scale. Maturation and aging seem to be responsible for the decline in regenerative potential in woody plant species. The requirements for somatic embryogenesis or organogenesis is already present in zygotic embryos. They show embryogenic potential with the expression of many of the genes required for the induction process. The redirection towards the developmental pathways is not required and embryogenesis and plant regeneration is easy when using immature or mature zygotic embryos. The degree of response in culture is also related to the developmental stage of the zygotic embryos. As a general rule, immature (early cotyledonary) embryos are more responsive than their fully mature counterparts. In the case of transgenics, which are more difficult to regenerate, all these advantages of zygotic embryos could be well exploited. Regeneration of plants from more events is possible since the system allows easy and rapid plant regeneration and hardening. This in turn enables more effective evaluation and identification of the superior events among the transformants. In *Hevea* there is an added advantage of using these plants as stress tolerant rootstocks, thus bypassing the biosafety concerns related to pollen dispersal.

Acclimatization of plantlets is a major problem in any woody plant species and in *Hevea* the difficulty was observed in plants derived from clonal explants. However for zygotic embryo derived plants, better acclimatization was observed (Rekha et al. 2014). A notable difference in the frequencies of embryo induction, maturation, germination, plant regeneration and hardening was observed in the case of transgenics in *Hevea* with the osmotin gene, using explants from different sources. The use of juvenile explant could also solve many problems associated with regeneration and hardening.

The use of zygotic material often results in loss of genotype integrity, because of the involvement of meiosis and recombination. However, this can be combined with conventional breeding with the help of a cryopreservation protocol (Fig. 7.7). This enables the development of rooted transgenic plants on a large scale, which in turn will avoid the stock scion interaction problem in future. Moreover, functional validation of genes and promoters can be done in *Hevea* itself instead of going for model plants, and that will strengthen the reliability of results. Development of stress-tolerant rootstock is yet another possibility of using zygotic explants.

7.6.4.3 In Vitro And Ex Vitro Grafting

Due to the difficulty of in vitro rooting of clonal shoot tips and further establishment, a micrografting technique was adopted by Perrin et al. (1994), and succeeded in grafting shoot apices of mature trees onto in-vitro grown seedlings. Conditions were also optimized for micrografting at the Rubber Research Institute India and 85 % success was achieved with 30-day-old rootstock and 21-day-old scions (Kala et al. 2002). Although this has little significance from the propagation point of view, the technique is ideal for propagating elite plants developed in vitro, such as transgenics.

Micrografting is a technique which can be used to rescue transgenic shoots developed from clonal explants. This technique has been successfully used in many horticultural plants to develop virus-free clones (Murashige et al. 1972; Roistacher and Kitto 1977) and fruit trees like cherry (Ozambak and Schmidt 1991), kiwifruit (Ke et al. 1993) and apple (Richardson et al. 1996). Since 1953, attempts have been made at micrografting in *Hevea* and a few cases of success were reported (Kala et al. 2002; Muzik and Cruzada 1958; Perrin et al. 1994). Since the scion is grafted onto a seedling with a good root system, absorption of nutrients and water are not adversely affected. Micrografting was attempted for rescuing transgenic shoots integrated with the osmotin gene and successful grafts were obtained and developed with new flushes (Rekha 2013).

7.6.4.4 Antibiotic Marker-Free Transgenic Plants

In genetic engineering, antibiotic resistant or herbicide resistant marker genes are widely used for the selection of transformed cell lines. The transformed tissue has a selective advantage over the untransformed cells due to the presence of the antibiotic marker gene, which detoxifies the antibiotic. Once plant transformation is completed, these marker genes serve no purpose and their elimination is advocated for different reasons. Continuous exposure to the antibiotic may affect the normal growth and development of the transgenic plant. The selective agents (antibiotics) have negative effects on proliferation and differentiation of plant cells and retard differentiation of adventitious shoots during the regeneration process (Ebinuma et al. (1997). Horizontal gene transfer among unrelated species such as between plants and microbes with respect to biosafety issue has been addressed in several studies and a number of potential hazards have received much attention and have been debated intensively in the scientific and popular forum (Ho 2001; Thomson et al. 2001). The presence of antibiotic marker genes in GM plants has raised considerable public concern, mainly by environmentalists and consumer organizations, regarding the environmental implications of growing GM crops. Apart from these, the recent developments in genetic engineering technologies permit the integration of multiple genes for complex traits and re-transformation of the same line is needed for multiple trait modifications. Co-incorporation of different markers with each transgene or set of transgenes increases safety concerns; it is expensive and time consuming. In this context, development of antibiotic marker free transgenics in

Hevea assumes importance. For the removal of the antibiotic marker gene from transgenic *Hevea* plants, a binary vector with the heat-inducible *Cre-loxP* system (HSP: *Cre*: loxP) was developed by Sobha et al. (2015). The vector contained transcription units *npt11* under *CaMV35S* promoter and cre gene with inducible heat shock promoter (*HSP*). Functional validation of the construct was successfully carried out in a model plant and proved that the same construct could be employed for producing marker-free transgenic *Hevea brasiliensis* plants (Sobha et al. 2015). This clean vector technology mediated by the *Cre-loxP* system is a novel approach for producing marker-free transgenic *Hevea* plants. Moreover, multiple gene integration by repeated transformation for gene stacking is possible using the same antibiotic, which is highly desirable in a tree crop like *Hevea*.

7.7 Epigenetic Analysis and its prospective in In Vitro Culture

The regulation of gene expression results from the combined action of genetic and epigenetic factors. Quite often, the external environmental stress the plants encounter at various stages of their development influence this gene regulation. Although genetic factors are stable, epigenetic factors such as DNA methylation of cytosines, histone tail modifications and non-coding small RNAs are sometimes reversible depending on the time, location and nature of the change. It is a highly dynamic process that can be influenced by both internal (developmental, hormonal, nutritional) and external (climatic, pathogenic) signals and can determine the progression of the life cycle of the plant (Boyko and Kovalchuk 2011; Finnegan 2002; Grant-Downton and Dickinson 2005). Phenotypic and genetic variations have been prominently observed during plant tissue culture, which is more frequent than natural variation mainly due to the various stress conditions encountered by the cells during the different stages of their culture, like the influence of media, chemicals, hormones, antibiotics, etc. Among various epigenetic factors, DNA methylation-induced changes during tissue culture has been considered as a key factor responsible for these variations. There are several reports on the induction of heritable variation by DNA methylation during regeneration of plants by tissue culture (Bardini et al. 2003; Steward et al. 2002; Xu et al. 2004). In this context, assessment of transgenics as well as plants generated by in vitro techniques will be complete only if a proper analysis of their epigenome is performed.

7.7.1 DNA Methylation and Somaclonal Variants

DNA methylation in higher eukaryotes is merely the presence of 5-methylcytosine (m5C) nucleotides in the DNA, instead of a normal cytosine. It is an important epigenetic modification frequently seen in higher plants and animals which plays

an important role in regulating development and developmental processes (Feng et al. 2010). DNA cytosine methylation has received more attention recently because they are known to alter gene transcription without altering the DNA sequence, which may lead to morphological changes in the organism concerned (Lee et al. 2010; Meijón et al. 2010). By and large this epigenetic modification has been observed abundantly in repetitive regions, transposon sequences and in GC-rich regions of promoters and end regions of transcribed portions called CpG islands (Lippman et al. 2004). While CpG methylation is the most prominent pattern of methylation (commonly seen in animals) CpNpG sites ("N" any nucleotide) and CpNpN asymmetric sites are found in plants (Baylin et al. 2001; Pradhan and Adams 1995).

In the case of tissue culture plants, in-vitro propagation systems for plant regeneration have a profound effect on the likelihood of producing significant quantities of somaclonal variants. A typical epigenetics-related event inducing somaclonal variation in *in vitro* cultured plants would be gene methylation resulting in phenotypic changes. Single gene or qualitative trait mutations were the first to be detected in tissue-culture plants and the frequency of such mutations was found to be related to the age effect; i.e. the longer the cultures are maintained, the higher the frequency of mutations in regenerated plants. *In vitro* propagation systems that pass through an intermediate callus phase (such as in somatic embryogenesis) are especially prone to evoking genetic and epigenetic changes among the regenerated plants (Miguel and Marum 2011). DNA methylation variations have been reported as the main cause of somaclonal variation in different crops (Gonzalez et al. 2013). For example, DNA methylation changes and associated gene expression variations have been reported in genes involved in organogenesis (De-La-Peña et al. 2012) and other developmental processes in plants (Nic-Can et al. 2013). Another example is the epigenetic origin of the mantled abnormality and the non-Mendelian transmission of the trait by conventional genetic crossing in oil palm (Durand-Gasselin et al. 1990, Rao and Donough 1990). Although no differences between mantled and normal palms have been detected, genomic DNA methylation appears to be altered in mantled palms, as revealed by global HPLC analysis of methylation and methylation-dependent AFLP analysis (Jaligot et al. 2004; Kubis et al. 2003, Matthes et al. 2001). Direct evidence from tissue culture regenerants and their progenies for heritable epigenetic changes based on RFLP and AFLP technologies has come from studies in numerous plant species (Bednarek et al. 2007; Jaligot et al. 2002; Kaeppler and Phillips 1993; Rodriguez Lopez et al. 2010; Schellenbaum et al. 2008).

Previous studies have clearly established that the frequency of variants observed is less in more juvenile pre-formed explants like axillary buds, shoot tips and meristems compared to explants from highly-differentiated tissue such as roots, leaves and stems (Duncan 1997). Since artificial induction of embryogenic competence in cells which are not naturally embryogenic is required in conventional micropropagation techniques using somatic explants, the influence of the above factors and the stress induced by chemicals in the media will be more in the somatic embryogenesis process, thereby increasing the possibility of generating more somaclonal variants

(Dodeman et al. 1997). On the contrary the low rate of somaclonal variation in juvenile tissues can be attributed to their high genetic homogeneity and lack of pre-existing genetic variations. In this context, the utilization of zygotic embryos where the above effects will be negligible because of the intrinsically embryogenic nature of the explant zygote is highly recommended for in vitro propagation. Due to the embryonic potentiality of the zygotic cells, their fate is already committed and does not need to be redirected towards a new developmental path. This phenomenon stands true in the case of *Hevea*, based on a study by Rekha et al. (2015) where multiple plants regenerated by embryo culture from a single zygote in *Hevea brasiliensis* were screened for methylation polymorphisms. Based on epigenetic and genetic profiling, it was shown that the polembryony-derived tissue culture plants developed were genetically and epigenetically true to type (Fig. 7.6).

7.7.2 Epigenetic Screening of Cell Lines and Plantlets

As mentioned above, undesired transgene silencing is a common phenomenon and a major concern in the transgenic plants developed for crop improvement. Development of transgenic *Hevea* plants has been successfully done using several genes of interest such as *MnSOD*, *osmotin* and *hmgr*1 by different research groups (Jayashree et al. 2003; Rekha et al. 2014; Sobha et al. 2003a, b). Although integration and expression of the transgenes has been confirmed in the lines developed, significant variations in the level of gene expression were noted in these lines. Silencing of the gene by any of the above-discussed means is highly suspected as one of the reasons for this problem. In this scenario, screening of the different lines at the initial development stage itself may be a viable strategy to predict their disposition towards transgene silencing in the future. Culling out these lines at an early stage thus saves time, effort and resources for the development of successful transgenic rubber plants with the desired traits. According to López and Wilkinson (2015), the production of plants genetically and epigenetically faithful to the original plant material is very crucial in determining the efficiency of the micropropagation and genetic transformation systems. In the case of *H. brasiliensis, in vitro* techniques are used mostly for the improvement of existing varieties by transgenic approaches, rather than using them for propagation. In addition to yield, such studies are focused on the introduction of biotic as well as abiotic stress tolerant characteristics to known high-yielding clones which were developed by conventional breeding techniques. In this context, identification of somaclonal variants from tissue culture regenerated transgenic plants is essential to ensure that the genome as well as the epigenome of the selected plants remains intact except for the specifically-induced changes by the transgene so that the other traits of interests already present in the target plant are preserved. Since *Hevea* is a perennial tree crop with high heterozygosity, the selection criteria should be stringent, incorporating all the techniques available so that the selected individual can be multiplied vegetatively and the transgene can be fixed permanently.

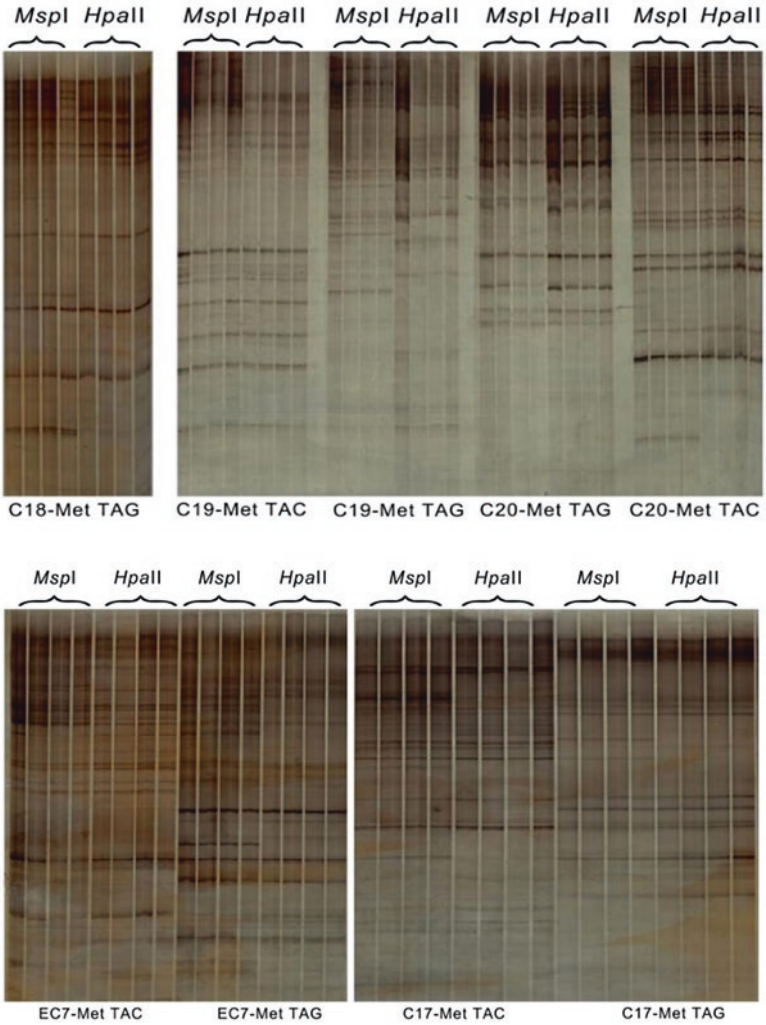


Fig. 7.6 Epigenetic profiling by MSAP analysis: Methylation sensitive AFLP results using nine different primer combinations. Variations in banding pattern between the *Msp*I set and *Hpa*II set indicate the presence of methylated regions in the *Hevea* genome. Lack of variations among the four plants within the same set indicates the absence of genetic and epigenetic (methylation) variation in polyembryony derived plants. (Source: Rekha et al. 2015)

7.8 Conclusion and Prospects

Extensive research carried out in *Hevea* crop improvement over recent decades have led to substantial improvements in yield. However, the increasing demand for natural rubber necessitates further improvement, with more specific characters in addition to high yield. Because of the long breeding cycle and high heterozygosity

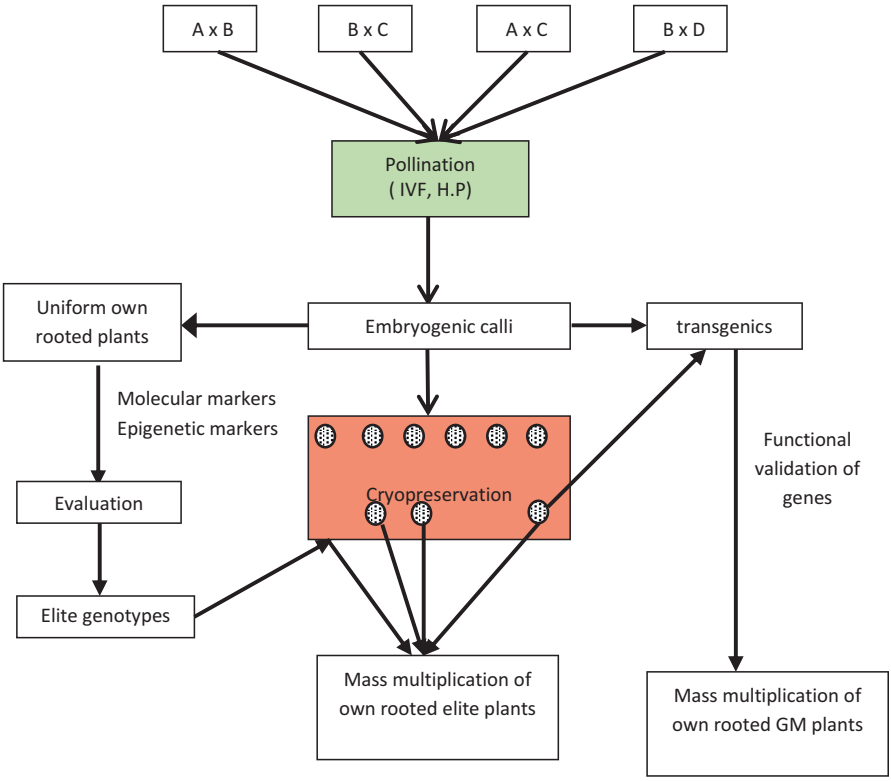


Fig. 7.7 A road map for effective utilization of in vitro techniques for *Hevea* breeding with the aid of an efficient cryopreservation technique

of the crop, the possibility of further improvement through conventional breeding within a short period is rather difficult and therefore non-conventional strategies are to be adopted. Although exciting results are coming out of crop improvement activities, their effective utilization at the expected pace is not happening due to various constraints. Lack of proper concordance between the conventional and non-conventional strategies can be cited as the major reason for this delay. Therefore, it is suggested that a comprehensive approach making use of the modern technologies to hasten conventional breeding will be the most effective strategy towards achieving the goal of developing a *wonder Hevea* clone having all the desired characteristics. Based on the assumptions obtained from a comprehensive analysis of all the crop improvement activities in *Hevea*, a road map for rubber breeders is suggested (Fig. 7.7)

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Appendices

Appendix 1 Research Institutes Actively Involved in Rubber Tree Research

Institutes involved in rubber research	Area of specialization and research activities	Contact information
Rubber Research Institute of India (RRII)	Rubber Research Institute of India (RRII) The major areas of research in the following disciplines: Agronomy and Soils; Biotechnology and Genome Analysis, Botany and Germplasm conservation, Plant Pathology, Plant Physiology and exploitation, Rubber Technology and Agricultural Economics. Involved in the improvement in production and productivity through conventional and molecular approaches. Devising integrated approaches to reduce cost of production and improve quality to combat against diseases. Studies on modification of NR and on polymer blends.	Rubber Board, Kottayam-686009, Kerala, India rrii@rubberboard.org.in
Malaysian Rubber Board (MRB)	Agronomy, exploitation techniques, integrated farming, agroforestry, Crop protection, Biotechnology. To develop technologies to achieve sustainable production of rubber, crop improvement by *Hevea* tissue culture and genetic transformation, molecular markers and genetics, physiology, biochemistry and molecular biology, transcriptomics and latex allergy, develops methods and procedures to manage every economically important disease of rubber.	RRIM Research Station, 47000 Sungai Buloh, Selangor, Malaysia irpecm@lgm.gov.my
The Tun Abdul Razak Research Centre (TARRC) MRB	Genomics of rubber, scientific research into natural rubber, technological developments in the compounding and processing of rubbers, the design of rubber products and the improvement of service lifetime of rubber products. Development of new polymer materials with enhanced properties, genomics and proteomics of natural rubber, development of molecular markers that can be used to enable the rapid identification of rubber tree clones in the field, key areas: i) diagnostics for latex allergens and, ii) comprehensive characterization of foreign proteins in transgenic rubber trees.	Brickendonbury, Hertford, United Kingdom, SG13 8NL, T: + 44 (0)1992 584966 F: +44 (0)1992 554837 general@tarrc.co.uk

(continued)

Institutes involved in rubber research	Area of specialization and research activities	Contact information
CIRAD	Agro-industrial cropping systems; Socioeconomic analyses; assessment of carbon balances and fluxes on a plantation ecosystem scale; micropropagation: cloning of whole plants and rootstocks; soil analyses; analyses of natural rubber structure and quality; analyses of genome and transcroptome of rubber. Development of planting material suited to growing conditions, appropriate crop management sequences; Adapting rubber growing to future climate change; Proposing disease control methods; Determining the qualities of natural rubbers suited to different uses (tires, industrial rubber, health, aeronautics).	Avenue Agropolis, 34398 Montpellier Cedex 5, France jerome.sainte-beuve@cirad.fr
Rubber Research Institute of Sri Lanka	Specializes in rubber research, both in the biology of the crop and the chemistry of the natural rubber and technology of the product. Research and development on all aspects of rubber cultivation and processing for the benefit of the rubber industry.	Dartonfield, Agalawatta, Sri Lanka, 12200 dirrri@sltnet.lk
Rubber Research Institute of Vietnam	Agronomy, exploitation techniques, integrated farming, agroforestry, crop protection, crop improvement. Breeding and selection of elite rubber clones, cultivation practices for rubber planting sectors, studying the characteristics, fertilizer application. Studies on latex diagnosis and tapping systems for mature rubber plantations.	177, Hai ba trung st., District 3, Hochiminh – Viet Nam rriv@hcm.vnn.vn
The Rubber Authority of Thailand (RAOT)	Enhancing the production capacity, from plantation, harvest, primary and secondary processing to produce industrial rubber products. Engaged in breeding for crop improvement.	67/25 Bang Khun Non Road, Bang Khun Non sub-district, Bang Kok Noi district, Bangkok province 10700 orf2008@rubber.mail.go.th
Rubber Research Institute of Cambodia	Breeding for crop improvement.	09, Street 289 , Beung Kak 1, Tuol Kork, P.O. Box 1337, Phnom Penh, Cambodia. RRICAM@Bigpond.com.kh
Rubber Research Institute, CATAS	Research and development on all aspects of rubber cultivation.	No.4, Xueyuan Road, Longhua District, Haikou, Hainan Province, China http://www.catas.cn Email:catasgjhzc@126.com

Appendix 2 A Few Popular Hevea Clones

Clone	Parentage	Country	Characters
RRII105	Tjir1 X Gl 1	India	Branching good with strong unions, canopy dense, foliage dark green, leaflets long and glossy. Wintering and refoliation are early and partial. High yielder.
PB 260	PB 5/51 X PB 49	Malaysia	Trees have tall and straight trunk, branching light and balanced with strong union. Canopy dense, foliage pale green. Vigor before initiation of tapping high and after tapping average. Thickness of virgin bark and renewed bark below average. The clone has moderate tolerance to pink, powdery mildew and abnormal leaf fall. High incidence of tapping panel dryness. Wind damage below average.
RRII414	RRII 105 X RRIC 100	India	Trunk is tall, straight and cylindrical with open, broad canopy of heavy dark green leaves, restricted to the top. Girth, at opening is high and girth increment on tapping, average. Moderately tolerant to Pink disease and abnormal leaf fall. Incidence of *Corynespora leaf* fall is low and powdery mildew is high.
RRII430	RRII 105 X RRIC 100	India	Canopy is open, broad and heavy with broad glossy leaves. Thickness of virgin hark is average and renewed hark is high. Maintains better yield than RRII 105 in the on farm trial also. Tolerant to pink disease, abnormal leaf fall and *Corynespora* leaf fall, but susceptible to powdery mildew.
RRII417	RRII 105 X RRIC 100	India	Canopy is broad, open and heavy with semi glossy leaves. Girth at opening is above average. Thickness of virgin bark is average and of renewed bark is high. This clone has above average tolerance to wind. Susceptible to powdery mildew and moderately tolerant to pink disease, abnormal leaf fall and *Corynespora* leaf fall.
RRII422	RRII 105 X RRIC 100	India	Stem is crooked with high branching. Canopy is open narrow with dark green glossy leaves. Girth at opening is above average. Tolerant to pink disease and abnormal leaf fall, but susceptible to powdery mildew. Moderately tolerant to *Corynespora* leaf fall.
RRIM600	Tjir 1 X PB 86	Malaysia	Tall, straight trunk, moderate to fairly heavy branching and branch unions rather weak. Young plants show spindly growth and late branching with occasional leaning. Narrow, broom shaped crown, foliage sparse with small yellowish green leaves, normal wintering and refoliation. Highly susceptible to diseases caused by *Phytophthora.* Incidence of pink disease mild to severe. Requires effective control measures when planted in areas where these diseases are prevalent. Incidence of powdery mildew is mild.

(continued)

Clone	Parentage	Country	Characters
GT1	Primary clone	Indonesia	Trunk upright hut slightly kinked. Variable branching habit. Main branches long and acute angled, secondary branches light. Narrow globular crown, dense dark green glossy foliage. Wintering and refoliation late and often partial. Girth at opening medium to high. Girth increment on tapping medium. Virgin and renewed bark thickness medium. Fairly wind fast. Occurrence of tapping panel dryness and incidence of pink disease mild. Abnormal leaf fall mild to medium and powdery mildew medium to severe.
RRIM703	RRIM 600 X RRIM 500.	Malaysia	It has an upright hut slightly kinked trunk with a few heavy branches. The canopy is open and narrow. Wintering and refoliation occur early in the season. Girth at opening is high to average and girth increment on tapping low. Virgin bark thickness is high and renewed bark thickness average to high. Wind damage as well as tapping panel dryness high. Abnormal leaf fall is severe in India though reported to be only mild in Malaysia. Occurrence of powdery mildew is mild. The clone is susceptible to pink disease.
RRIl 429	RRII 105 X RRIC 100	India	This clone with tall, straight and cylindrical trunk has a dense, heavy canopy with dark green leaves. Girth at opening is high and girth increment on tapping average. Occurrence of TPD is above average. The incidence of *Corynespora* leaf fall and abnormal leaf fall are low. The clone is highly susceptible to pink disease.
IRCA130	PB5/51X IR22	Côte d'Ivoire	Tall and straight with strong union, Above average vigor, moderately heavy branching, and small canopy with foliage confined to the top, moderate tolerance to powdery mildew, leaf spot and high yielding.
IRCA111	PB 5/51 X RRIM 600	Côte d'Ivoire	This clone shows a very good vegetative vigor in immature period and an average annual increase after opening. It has a sensitivity to tapping panel dryness that increases with the latex harvest period.
RRIM 2020	PB 5/51 X IAN 873	Malaysia	Dense and high set crown. Open and balanced canopy. Persistent main leader but with moderate branchlets on main and secondary branching. Color of latex appeared to be cream in color.

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Chapter 8
Genetics and Breeding of Jojoba [*Simmondsia chinensis* (Link) Schneider]

Mohamed M. A. Khairi

Abstract Jojoba, *Simmondsia chinensis* (Link) Schneider, is a dioecious, extremely heterogeneous perennial shrub, native to the Sonoran Desert of North America where it has long been known as a source of oil, used for medicine and food, as well as a browse plant. Analysis of jojoba oil revealed that it is a unique liquid wax, similar to sperm whale oil, maintaining viscosity at very high temperatures. Jojoba oil gained worldwide interest for industrial, pharmaceutical and cosmetic use, as a lubricant, and an ingredient for medicines, cosmetics, toiletries and livestock feed. Jojoba also gained interest for afforestation, abatement of desert creep, browse and development of marginal lands, as well as a source of income for local inhabitants. Seed propagation is not economical and rooted cuttings from selected elites are the current commercial means of propagation. Cultivars with high yield and oil quality were selected and given mainly coded names. The females Barindji, Wadi-Wadi and Waradgery, and males Dadi and Guyambul were selected in Australia. Equipment has been developed in Argentina for transplanting cuttings, pruning the plants and preparing the soil surface for harvest. Environmental variation has significant effect on jojoba yield. The feasibility is demonstrated of using RAPD-PCR in distinguishing between jojoba sexes and producing wax esters in transgenic plants by expression of genes derived from jojoba. Rutherglen bug, aphids and *Heliothis* caterpillar insects, spider mites, *Fusarium oxysporum* and black scab (*Elsinoë* sp.) fungi cause minor damage to jojoba.

Keywords Diversity · Domestication · Ester · Fatty acid · Jojoba · Pests · Sebum · Utilization

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8.1 Introduction

Jojoba [*Simmondsia chinensis* (Link) Schneider] is a dioecious woody perennial shrub native to the Sonoran Desert of southwestern North America. It is also known by different names such as goat nut, pignut, deer nut and coffee-berry. Jojoba is an evergreen plant producing durable waxy leaves. Seeds are brown, oval-shaped and contain approximately 54% of a unique liquid wax which makes it a desirable shrub (Wisniak 1987). Native Americans collected jojoba seed and each tribe used it for various purposes. Seeds were sometimes ground to powder and brewed into a coffee-like drink. Other tribes ground jojoba seeds to a paste and used it for medicinal purposes such as dermatological aids, and for healing skin and hair conditioning. Other tribes ate jojoba seeds in food emergencies and as a snack. Jojoba seeds, as well as the leaves, are also used as forage for livestock; but jojoba was found to be toxic to guinea pigs and small animals. Cattle feed from jojoba therefore requires removal of the toxic component from the fodder. Analysis of jojoba oil revealed that it is actually a non-glyceride liquid wax which maintains its viscosity at high pressure and temperatures up to about 300 °C. It is therefore suitable for lubricating fast moving machinery and used for other industrial purposes. Its antioxidant qualities ensure a long shelf life and powerful ally in the fight against oxidative stress and lipid peroxidation. Jojoba oil analysis also shows that it resembles sperm whale oil which makes it a suitable substitute to save this endangered and protected species. Further studies showed that jojoba plant produces wax esters that are found in human skin sebum (Naqvi and Ting 1990). Jojoba can prevent the human skin from becoming too oily. Because the structure of golden jojoba so closely resembles wax esters that comprise up to 30% of the natural sebum in skin, jojoba floods sebaceous glands signaling the glands to reduce sebum production (Abobatta 2017; Mills et al. 2004; Reddy and Chikara 2009; Undersander et al. 1990).

Besides the seed-extracted oil uses, jojoba leaf extract was found to have a nematocidal effect on the root-knot nematode (*Meloidogyne incognita*) in experiments on onion and tomato seedlings (Khatab and Seehy 2013). Jojoba leaf extract may be a safe substitute for the highly toxic and widely banned chemical Furadan.

In the 1970s, jojoba became the focus of numerous commercial research and cultivation projects. Since then, the industry has struggled with development and marketing problems. Work is underway to select plants for desired traits and suitable for mechanical harvest. The plant is currently being grown commercially in Argentina, the USA, Israel, Australia and a few other countries.

This review presents a historical overview of the origin and habitat of jojoba and its industrial and economic importance. It also highlights the agronomic aspects and nutrient requirements of the plant and the role of recent biotechnology and molecular biology findings in jojoba breeding and research.

8.1.1 Distribution and Ecology

Jojoba is native to the islands in the Gulf of California, the Sonoran Desert of North America extending east of Riverside and San Diego counties through Imperial County in California, and in Arizona to southern Yavapai and Greenlee counties, and southward to Cochise, Pima and Yuma counties. Jojoba is also distributed throughout the Baja California Peninsular and the state of Sonora, Mexico. In Sonora the annual rainfall range is 75–450 mm. Jojoba tolerates high ambient temperatures, but temperatures below −6.7 °C may destroy seedling plants and damage adult jojoba shrubs. Fires may kill jojoba shrubs, but often they are known to grow back (Undersander et al. 1990; Yermanos 1979). Jojoba is native to the ambient conditions of the Sonoran Desert (Fig. 8.1) and its biotic and abiotic factors; it is not known to occur naturally anywhere else. With efforts of domestication, jojoba has shown that it can survive in various regions in the world (Fig. 8.2) that have climates similar to the Sonoran Desert.

8.1.2 Morphology

Jojoba is a deep-rooted, dioecious, succulent evergreen woody perennial shrub. In its natural habitat it tolerates high temperatures and drought, and may be classified as a true xerophyte (Yermanos 1974). In humid areas, jojoba may reach a height of 3–6 m, whereas in arid areas near the ocean, it exhibits a semi-prostrate form of 20–50 cm in height (Gentry 1958). Jojoba may have several taproots and have been observed at depths of 10 m. This extensive root structure enables the plant to survive under arid

Fig. 8.1 Jojoba in Sonora Desert. (Http://www.cabezaprieta.org)

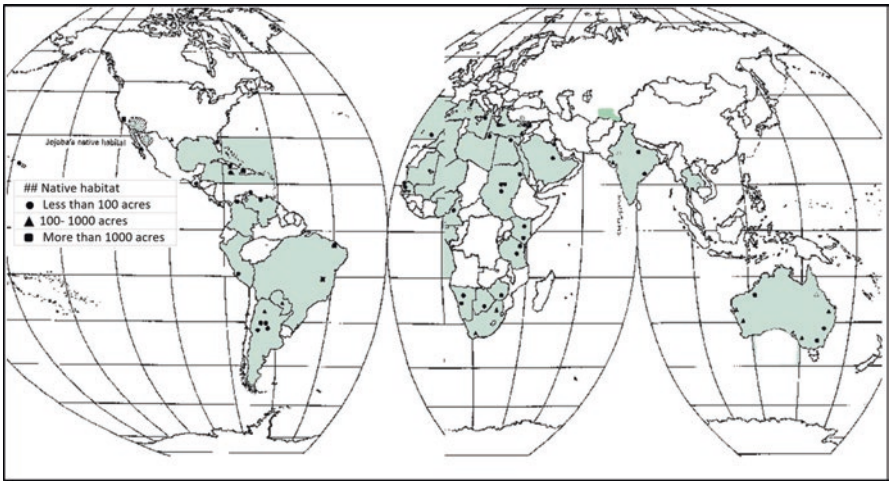

Fig. 8.2 Jojoba cultivation around the world. (Source: National Research Council 1985)

conditions. Several stems arise from the root crown (Fig. 8.3a). In full light, lateral branching is prolific near the base (National Research Council 1985). As the plant ages, the lower foliage is pruned by the shade and a high canopy develops. Young jojoba stems are green, hairy and flexible, measuring 15–75 cm length and 2–7 mm in diameter. Old branches are monopodially branched, hard, erect and cylindrical in shape with a brown color and slightly rough surface, and small longitudinal fissures measuring 2–4 m in length and 1–5 cm in diameter. Jojoba bark is smooth (Sherbrooke and Haase 1974). An anomalous growth of successive rings of xylem and phloem have been noted in jojoba roots and stems. Judging from counting the annual rings of roots and stems wild plants, it is believed that jojoba is long lived, living probably for 100–200 years (Brooks 1978; Gentry 1958, 1973; Matthews 1994).

Leaves are opposite, oval, 2–3 cm long and 1–1.5 cm wide, leathery, oblong, gray-green or bluish-green, vertically orientated as an adaptation to the extreme desert heat. During midday in summer, when high leaf temperatures make photosynthesis less efficient, the sun is shining on the edges of the leaves. In morning and late afternoon, light reaches the flat leaf surfaces more directly, at a time when temperatures are more favorable for energy capture (Phillips et al. 2015) (Fig. 8.3b). The epidermis of the leaves is covered with hairs, stomata are equally numerous on both leaf surfaces, the mesophyll consists wholly of palisade cells and lime oxalate crystals occur abundantly in the peripheral portions of the leaf. Jojoba is dimorphic in secondary sexual characters in some populations in which females have larger leaves and more open canopies than males (Kohorn et al. 1994).

Flowers are small and greenish-yellow, apetalous, with 5–6 sepals. Male flowers are yellow, larger and occur in clusters. Female flowers may be single or in clusters. Jojoba is dioecious, male and female flowers on separate plants, occasionally monoecious with male and female flowers on the same plant; rare hermaphroditic plants also occur. Female flowers are borne singly, 10–20 mm in size with 3 styles; male

Fig. 8.3 (**a**) Multiple stemmed jojoba plant (University of Oregon, College of Agric., Horticulture, https://landscapeplants.oregonstate.edu), (**b**) Upright jojoba leaves and green fruits. (Amber's Organics; http://www.gardenofcures.com)

flowers measure only 3–4 mm, borne in small clusters, greenish with 8–12 pale yellow stamens. Jojoba fruit is about 2.5 mm in size, green but finally brown, nut-like, ovoid, tough, leathery, obtusely 3-angled, edible, tasting somewhat like hazelnut, but is slightly bitter until cured. In the Northern Hemisphere, pollination occurs during February and March; in the Southern Hemisphere, during August and September (Benzioni 1997) (Fig. 8.4a, b, c). Jojoba is a tetraploid; the number of chromosomes is $2n = 4x = 52$.

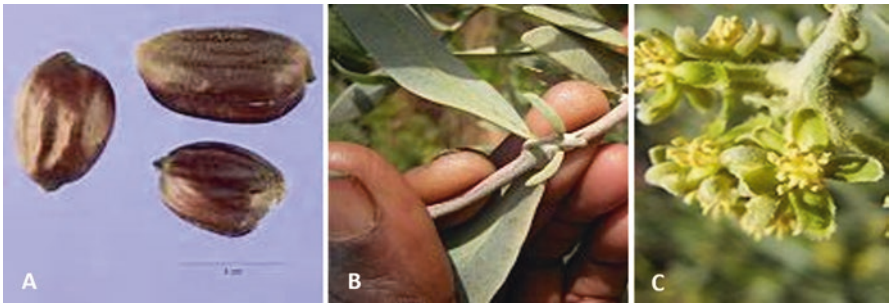

Fig. 8.4 Jojoba fruits and flowers. (**a**) Jojoba fruits, (**b**) Female jojoba flowers, (**c**) Male jojoba flowers. (Sources: https://en.wikipedia.org/wiki/Jojoba, Shebs 2005, Palm Canyon, California)

8.1.3 Taxonomy

Jojoba is known by several other common names including buck nut, coffee berry, coffee bush, coffee nut, deer nut, goat nut, gray box bush, jojowi, lemon leaf, pig nut, quinine nut, quinine plant and wild hazel. The accepted scientific name is (*Simmondsia chinensis* (Link) Schneider). Despite the specific epithet, jojoba is not native to China. The genus *Simmondsia* is monotypic.

Kingdom:	Plantae
Division:	Angiosperms (Magnoliophyta)
Class:	Eudicots (Magnoliopsida)
Order:	Caryophyllales
Family:	Simmondsiaceae
Genus:	*Simmondsia*
Species:	*chinensis* (Link) Schneider
Synonyms:	*Buxus chinensis* Link, *Simmondsia californica* Nuttal

8.1.4 Domestication

Natural stands of jojoba have provided inconsistent commercial quantities of seed to meet the growing market demand for jojoba wax. Yields fluctuate greatly and very little seed can be harvested due to inaccessibility of stands, harvesting costs and land ownership issues. The need to establish domesticated plantations is therefore evident. Although jojoba is susceptible to cold injury under low temperature conditions, it is its ability to withstand high salinity (EC up to 12 dS m^{-1} at pH 9), the capability to combat desertification and the high value of its products that make

it attractive for domestication. The first experimental jojoba planting was established in the USA at The Boyce Thompson Southwestern Arboretum, in Superior, Arizona, in 1925 and jojoba cultivation expanded from there. Further interest developed in 1935 when University of Arizona chemists declared that jojoba oil is actually a wax similar to sperm whale oil which was commonly used as an important high-temperature and high-pressure lubricant. Jojoba from the wild was collected and plantations were established from seedlings. As jojoba is dioecious, yields between individual trees showed great variation (Hogan and Bemis 1984).

Beyond the countries of origin, interest in jojoba as an industrial desert crop spread worldwide. In many countries where climates are similar to that of the jojoba native area, there were initiated experimental plantings and semi-commercial and commercial plantations. Currently, jojoba is grown commercially in Argentina, Australia, Chile, Egypt, Israel, Mexico, Peru and the USA. Plantations in South Africa and India are also reported. The most recent data found is from 2003, when the area of commercial jojoba plantations exceeded 7000 ha, with the majority grown in Argentina and the USA (Table 8.1).

In Argentina, the main jojoba plantations are located in the provinces of La Rioja and Catamarca (Ayerza and Coates 1996). In 2003, the cultivated area in Argentina was around 3000 ha, which represented 43% of the world total.

In Australia, jojoba was developed as an alternative economic enterprise in low-rainfall areas. The Centre for Sustainable and Responsible Organizations (CSIRO) introduced the plant from North America in 1978 and selectively bred cultivars especially suited to Australian conditions. A joint research program, undertaken by CSIRO and the New South Wales Department of Agriculture in the late 1970s and 1980s, developed jojoba cultivars to suit commercial farming in the dry inland regions of Australia. Five cultivars of the male and female plants were selected from this program; three female (Barindji, Wadi-Wadi, Waradgery) and two male (Dadi-Dadi, Guyambul). The cultivars developed for Australian conditions are reported to be more productive and better suited to the temperate and winter dominant rainfall

Table 8.1 Annual production of jojoba 2003

Country	Area (ha)
Argentina	3000
USA	2290
Israel	550
Australia	480
Peru	350
Egypt	240
Mexico	100
Chile	45
Total	7055

Source: Ash et al. (2005) based on data from the International Jojoba Export Council 2003

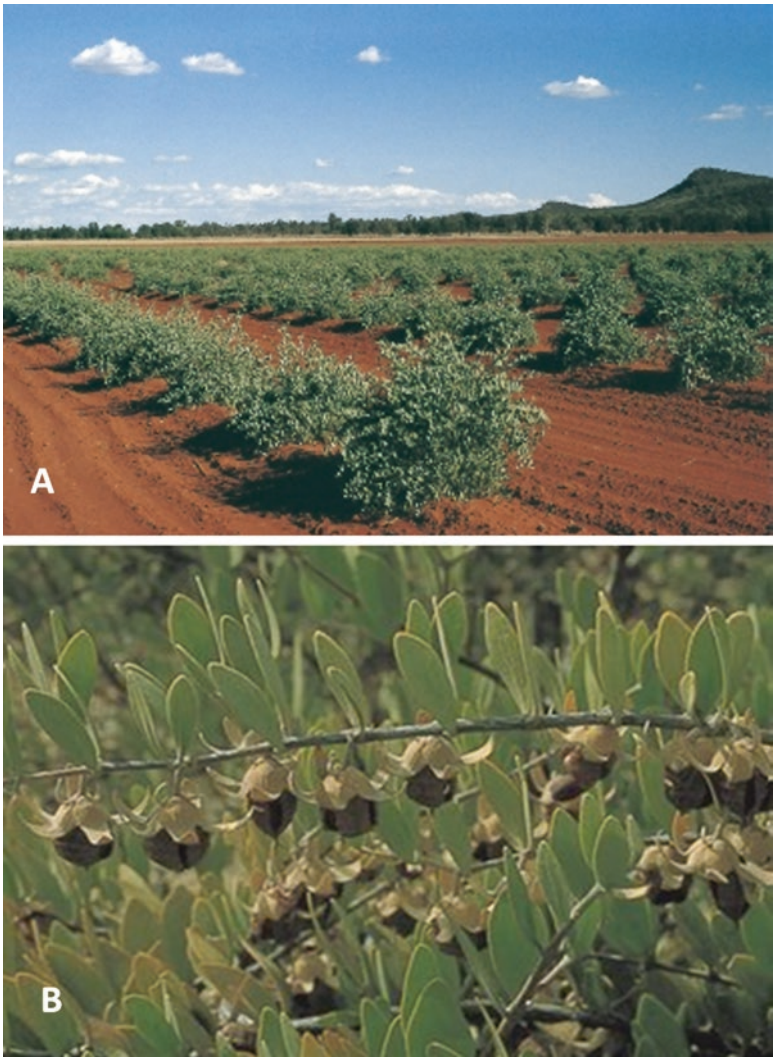

Fig. 8.5 (**a**) Jojoba cultivation field in Australia, (**b**) Fruiting jojoba in Australia. (Source: Agrifutures, Australia, agrifutures.com.au)

areas of Australia than introduced cultivars. Commercial plantations are now established across the country, including in Kalannie, Wagin, Geraldton and York in Western Australia (Agrifuture) (Fig. 8.5a, b).

In Brazil, some prolific semi-commercial jojoba plantations were established from rooted cuttings of selected clones in arid northeast of Brazil. Since 1976, jojoba performed well in test plots near Fortaleza, Ceara (latitude 4°S) where the first experimental plot was established on the Campus do Pici, of the Federal University of Ceara. Between 1977 and 1980, additional small plots were installed

in surrounding areas with corporations and private individuals getting involved in the business. Some 6- and 7-year-old plants, grown without irrigation, were reported to have reached heights of almost 3 m and produced seed in the third year, but the area's high humidity caused outbreaks of fungal disease in the rainy season. In 1982 about 1200 ha of commercial plantations were established; some unirrigated, and many inter-planted with cowpea, cassava, and cotton in the rainy season. In the northern part of the state of Minas Gerais and the southern part of Bahia, a private company initiated plantations covering more than 1000 ha (National Res CO 1985). Today, Brazil has well established jojoba plantations and commercial companies involved in jojoba oil processing and manufacturing products. In pilot trials, stable Nano-emulsions with great potential for commercial use have been successfully produced (Costa et al. 2014).

In Chile, jojoba is a potentially profitable crop for the arid regions. Liquid wax ester extracted from the seeds of the jojoba plant is used commercially in the cosmetics industry (Cappillino et al. 2003).

Egypt initiated jojoba trials in the Sinai where the neglected un-irrigated desert plantations all failed to produce any significant yield of jojoba seeds (Elmogy 1999). In an evaluation trial of ten jojoba clones that were selected from a private jojoba farm at Behira Governorate in Egypt, data were taken to determine the genetic relationships among these genotypes and select the best, using the difference of total protein band and Random Amplified Polymorphic DNA (RAPD). Growth parameters, tree volume, branch length (cm), branch diameter (mm), number of nodes forming branches, mean length for secondary branches (per branch, leaf area, chlorophyll A and B), flowering date, flowering percentage, fruit set percentage and seed yield per plant (g) were measured. Further, seed samples were analyzed for content of oil, protein, minerals and carbohydrates. Traits EI 1 and EI 4 were selected as the best traits for further evaluation and planting (Genaidy et al. 2016).

Jojoba was introduced to India from Israel in 1978 and cultivated in fertile soils of Thar Desert of Rajasthan State and other areas to serve as a renewable source of its high-quality oil (Agrawal et al. 2007). The Biodiesel Business Academy (BBA), Rajasthan, India, has developed the technology for the establishment of a plant nursery and of semi-commercial jojoba plantations. Commercial jojoba plantations were then established from rooted cuttings of high quality pedigree mother trees. BBA provides proprietary services and planting stocks for creating Jojoba Failsafe Farms. High quality oil is produced through a drip irrigation system and improved cultivars, and sold in domestic and foreign markets.

The jojoba plant has assumed a position as the Israeli Negev's main crop in the global cosmetics market. Increased cultivation of jojoba is leading to an increase in jojoba oil and a reduction in the need for whaling (Fig. 8.6).

Sudan was the first African country to introduce jojoba to the continent. A UNDP sponsored project was implemented during 1977–1981 as a joint venture between University of California, Riverside and the Sudanese Government. The objectives were to test the opportunities for success of jojoba as a crop for development of marginal lands unsuitable for production of food crops, a cash crop for generating income to the inhabitants of these marginal lands, an industrial and commercial

Fig. 8.6 Jojoba on an Israeli kubbutz. (Source: Jojoba Hazerim Ltd., Israel, https://www.jojoba-hatzerim.com/en/home)

crop, a browse plant and a plant for abatement of desert creep. Two semi-commercial plantations showed extremely impressive success. On the Red Sea Hill Summit, at Arkaweet, Sudan, where the native flora is similar to jojoba's natural habitat, and irrigation water and precipitation do not support the production of food crops, jojoba has shown better growth than in its natural habitat, without irrigation, for 40 years (Fig. 8.7a, b, c). The Bara site in western Sudan showed success of jojoba without irrigation as a desert abatement crop in an area where sand dunes migrate south at the rate of 1.6 km per year (Khairi 1982; National Research Council 1985).

8.1.5 Jojoba Oil

8.1.5.1 Jojoba Oil Properties

Jojoba oil is a natural plant oil, composed of a mixture of long chain liquid esters, which is extracted by pressing seeds (nuts) from the jojoba tree *Simmondsia chinensis*. This oil is unique in its molecular simplicity as it has a high viscosity index, high flash and fire points, high dielectric constant, high stability and freezing point (Reddy and Chikara 2009). The physical properties of jojoba give it low volatility and the ability to maintain its composition up to about 300 °C. Unlike most crude

Fig. 8.7 (**a**) 40-year-old jojoba without irrigation, (**b**) Habitat of Arkaweet, Sudan, (**c**) Sonora Desert habitat, North America. (Source: Khairi 1982)

triglyceride seed and nut, natural jojoba oil is very low in or virtually devoid of tars, gums, free carboxylic acids, hydro peroxides, phosphatides, chlorophylls, color bodies and malodorous low molecular weight carbonyl compounds. Expelled, crude jojoba is naturally of high quality and purity as it flows from the mechanical presses. Structurally, jojoba oil is a polyunsaturated liquid composed almost entirely of mono-esters of long-chain fatty acids and alcohols, with no side branching, accompanied by only a tiny fraction of triglyceride esters (Fig. 8.8a). The alcohol molecules in jojoba are long chains which range from 40–42 carbon atoms that qualifies it as a wax. The dominant fatty alcohols and fatty acids of jojoba are of C-20 and C-22 in length. The unbranched, long-chain nature of the alcohol and acid moieties give jojoba esters their non-polar behavior (Miwa 1971). This unique chemical configuration accords jojoba special characteristics of extreme shelf-life stability and extraordinary resistance to high temperatures unparalleled in the plant kingdom.

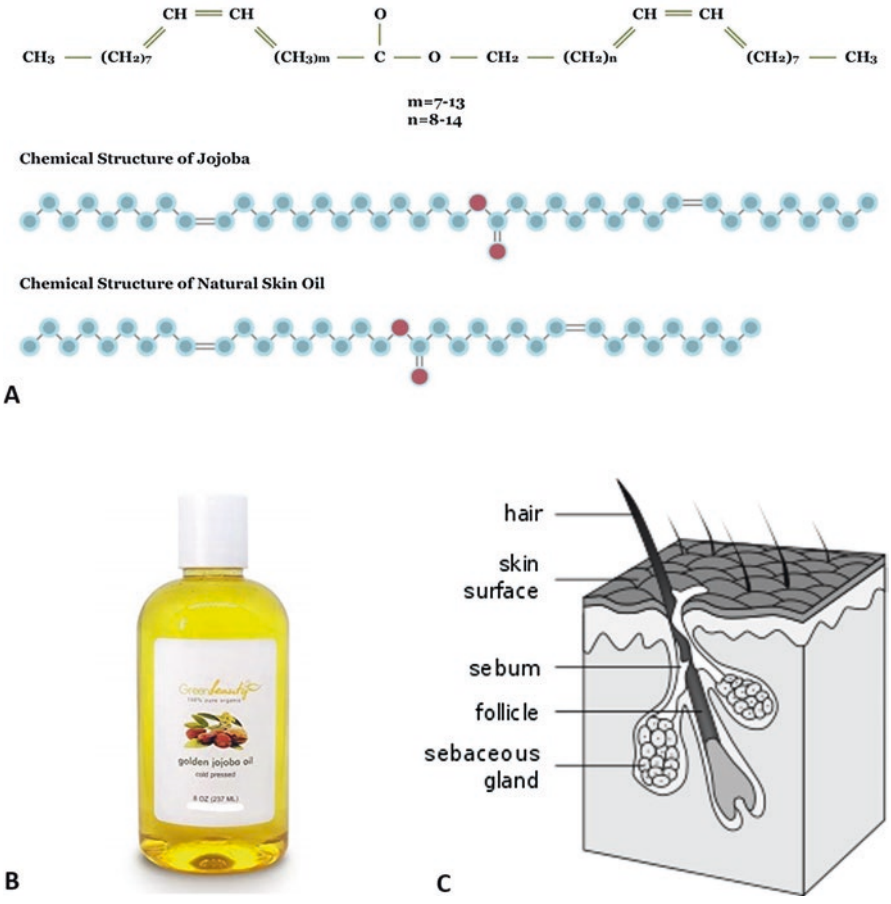

Fig. 8.8 (**a**) Molecular structure of jojoba oil. (Source: www.jojobanatturals.com), (**b**) Unrefined jojoba wax, (**c**) Sebum of human skin. (Source: www.hairscienceak.com)

8.1.5.2 Uses of Jojoba Oil

Jojoba oil is the only known botanical wax (as defined by its chemical classification) which is liquid at room temperature (Fig. 8.8b) and is used commercially in the cosmetic, pharmaceutical and lubricant industries (Mills et al. 2004; Reddy and Chikara 2009; Sherbrooke and Haase 1974). The global jojoba oil market was USD 160.3 million in 2016 and is projected to reach around USD 250 million by 2024. Jojoba oil is likely to experience greater popularity due to increased demand from the cosmetics industry and for personal uses, coupled with the growing trend of producing herbal medicines. This projection of growing demand for jojoba oil arises from the fact that jojoba has several special properties such as: structurally and chemically similar to human sebum; having a bright and golden color when pure and becoming clear and transparent once it undergoes processing; it has quite a long

shelf life; stable and does not oxidize easily; it will not become rancid like other oils because it is not a triglyceride; it contains a high amount of minerals and vitamins B5 and E which promote healthy skin; it contains antioxidants, which add protection to the skin and hair; it is highly antibacterial and quite safe to use.

The principal market for jojoba is the cosmetic industry either mixed with other substances in the form of either golden or refined (lite) jojoba, hydrogenated jojoba, jojoba esters, hydrolyzed jojoba, ethoxylated jojoba and other value-added jojoba derivatives or unrefined as it is. Human sebaceous glands are microscopic glands in the skin that secrete an oily or waxy matter called human sebum (Undersander et al. 1990) (Fig. 8.8c). Jojoba oil very closely resembles human sebum which makes it an ideal moisturizing treatment to keep the skin healthy with less risk of irritation. As humans age, sebaceous glands produce less sebum, which cause the skin and hair to become dry. Jojoba oil can therefore be used to treat acne and is well suited for other dry-skin conditions. Jojoba is applied directly to the skin for acne, psoriasis, sunburn and chapped skin treatments.

In manufacturing, jojoba is used as an ingredient in shampoo, lipstick, makeup, and cleansing products for the face, hand and body. When applied to the skin, jojoba is an emollient, which soothes skin and unclogs hair follicles (WebMed). It can work as a higher quality skin product and does not clog pores or cause allergic reactions, which is why it has become an ingredient in cosmetics and toiletries.

Jojoba oil has potential application in a wide range of industries as lubricants, anti-foaming agents, and in electrical insulators and the plastics industries (Reddy and Chikara 2009). In 1972, Key Oils & Lubricants Inc. began manufacturing motor oils, transmission fluids and gear oils using jojoba in USA. Although impractical, jojoba biodiesel has been explored as a sustainable fuel to serve as a substitute for petroleum diesel. The pour point of jojoba oil, acid value and oxidative stability were found to be the limiting factors for its use as a base stock for biofuel lubricants. Research investigations, however, indicated that these properties can be modified by physical and chemical methods and also by additive viscosity of jojoba oil by partial sulfurization to widen the scope of its utilization in developing lubricant formulations (Sivasankaran et al. 1988; Bhatia et al. 1990). Wildlife browse on both the leaves and the nuts, although the oil that constitutes nearly 50% of the nut is generally indigestible. It passes unchanged through the digestive tract of mammals. Jojoba oil is anti-fungal, high in vitamins and minerals that support healthy skin (B-complex, zinc, copper, selenium, chromium, high levels of iodine) helps in healing and is anti-inflammatory (https://www.stylecraze.com/articles/amazing-benefits-of-jojoba-oil-for-skin-and-hair).

8.1.6 Adaptation of Jojoba to Environment

In its natural habitat, the Sonoran Desert of North America, native stands of jojoba occur in non-saline to slightly saline and near neutral to alkaline soils. Jojoba is mostly limited to well drained, coarse desert soils such as sandy alluviums and

coarse mixtures of gravels and clays (Brooks 1978; Gentry 1958, 1973). Jojoba is a drought tolerant plant. The leaf water potential of well – watered plants average − 20 bars and are generally below −10 bars at night. These are low compared with most other mesophytes (Ash et al. 2005). Reasonable production of jojoba seed cannot be expected below 250 mm annual rainfall unless the plants are located in water drainage areas or areas where water accumulates (Fischer and Turner 1978). But jojoba grows exceptionally well without irrigation in the mild climate of Sudan's Red Sea Summit Resort at Arkaweet with average annual rainfall of about 100 mm and poor ground water resources (Khairi 1982). Jojoba grows in soil of marginal fertility with a pH 5–8. It has greatly reduced reliance on fertilizers when compared with other horticultural crops in this pH range (Yermanos 1979). The optimum temperature range for vegetative growth of jojoba is 27–30 °C (Dunstone and Begg 1979), for seed dry matter accumulation it is 28–30 °C and seed maturity is greatest at day/night temperatures of 18/13 °C. This is also close to optimum temperature for flower bud development (Dunstone and Dawson 1980). Frost susceptibility can differ noticeably between different clonal lines of jojoba and there are instances where severe frost has caused complete loss of crops (National Research Council 1985). Young plants have been damaged or completely killed if subjected to −7.2 °C for 3 h (Hogan and Bemis 1984).

8.2 Cultivation and Traditional Breeding of Jojoba

8.2.1 Current Cultivation Practices of Jojoba

Jojoba may be propagated by seed, stem cuttings, air layering, grafting or micropropagation (Benzioni 1997; Undersander et al. 1990). Seed propagation is less expensive, faster and requires less manual labor. Seed is sown when soil temperatures warm up and become favorable for germination. The most successful seed germination requires darkness, warm temperatures and high moisture availability over a considerable period of time, approximately 20 days, before seedlings break above ground (Burden 1970). But jojoba is dioecious and seed propagation produces a heterogeneous progeny of about 50% males. This makes seed propagation uneconomical and growers have switched to propagation by rooted cuttings. Rooted cuttings facilitate better control of male: female ratio, improve cultivars by selection but is a slow process; a much faster and reliable method is in vitro jojoba propagation.

The basic requirements for jojoba production have been established. Jojoba grows best in sandy, decomposed granite or rocky soils. Growth is slowest in heavy clay soils. Fertilizer may be required depending on soil fertility. Ideally, soils should have a pH of 6 or more and available potassium (K) levels of at least 100 ppm. Dolomitic lime should be applied according to soil test recommendations to raise

Fig. 8.9 Jojoba in Argentine Jorge Arizu's farm, Andes, Argentine. (Source: https://drhausch-kaaus.wordpress.com/2016/10/12/jojoba/)

soil pH to 6. Approximately 4.5–6.8 kg of potash fertilizer should be applied if available K levels are less than 100 ppm.

Irrigation is required depending on annual rainfall. Low temperature areas that harm jojoba should be avoided. Based on plant selection and the development of agronomic practices, advanced cultivation practices appear to have been developed and implemented in Argentina, currently the leading jojoba producing country (Fig. 8.9).

Advanced criteria include suitable site selection, seed and oil yield improvement, selection of cultivars with long chilling requirements, cloning, rooting capacity, frost tolerance, pruning, land preparation for seed harvest, fertilization, artificial pollination, as well as oil extraction and refining (Ayerza 2016). Selecting clones is based on seed yields, late flowering, plant architecture, shattering, rooting capacity, seed size and oil content and quality. Withholding irrigation water from late summer through fall to early winter was found to decrease plant metabolic activity and reduce frost damage. Rather than increasing the ratio of male to female plants in jojoba populations, the technique of assisted supplemental pollination was found to be more economical (Coates and Ayerza 2008; Coates et al. 2006). Equipment has been developed in Argentine for transplanting jojoba cuttings, pruning plants and preparing the soil for harvest.

Over 70% of jojoba seed is reported to be mechanically harvested with a vacuum suction system after seed drop to the ground. Jojoba oil is extracted locally, called *gold*, or deodorized, bleached and called *light*, and marketed in Europe and the USA (Ayerza 2016).

8.2.2 Current Agricultural Challenges of Jojoba Production

8.2.2.1 Production Requirements

Jojoba prefers light, sandy soils that have a good drainage in order to allow adequate moisture. It survives in a wide range of pH 5–8. The optimum growth temperature for jojoba is 19–25 °C and it is sensitive to frosts below 5 °C, which can damage flowers and the ends of young branches (Butler 2013). Late frosts may cause additional damage, as they prevent any second flowering that may occur after early frosts. Hence site selection is a crucial factor that contributes to success of commercial plantations. Required optimum rainfall for jojoba is 500–600 mm, and subsidiary irrigation may be required if annual rainfall is less than 400 mm. Among other factors, success of jojoba plantations depends on installation of efficient irrigation systems. The highest-yielding cultivars need to be propagated by in vitro techniques and grown to boost yield. There are few animals that adversely affect jojoba, depending on location, including birds, rabbits and rodents. Grazing cattle can also severely damage or kill jojoba plants. Fencing and individual plant guards are the most effective ways to combat these challenges.

Minor damage is caused by bugs, aphis and *Heliothis* moths and caterpillars. These pests may be controlled by chemicals or left for predator control. On poorly-drained soil, jojoba is susceptible to fungal wilts, including *Verticillium*, *Fusarium*, *Pithium* and *Phytopthora*. Collar rot is a major disease of jojoba, caused by the fungus *Fusarium oxysporum,* which results in stem and root decay. Control of this disease is through prevention by planting clean stock, and avoiding physical damage. Research suggests that some cultivars are more susceptible than others. The fungal disease, black scab (*Elsinoë* sp.) can also be a problem for jojoba in wet or high humidity years, and particularly in areas with high summer rainfall. The disease causes lesions on the leaf, calyx and young stems, and may have an impact of the opening of the flowers. It can be controlled by common horticultural fungicide sprays. Ash et al. (2005) reported black scab of jojoba in Australia caused by the fungus *Elsinoë australis*. Omar et al. (2017) recently reported a new bacterial infection (*Candidatus phytoplasma*) of jojoba in Saudi Arabia. In spite of these disease reports, there is no study describing the economic impact of these pathogens on jojoba plantations and how these pathogens affect the oil productivity (Al-Obaidi et al. 2017a). Weeds must be cleared from jojoba plantations in early years.

8.2.2.2 Infrastructure Requirements

Producing economic yields of high quality jojoba requires gearing management of farm operations to be lucrative and cost effective. The infrastructure of a jojoba plantation can be divided into four types: (1) Irrigation pipes and nozzles, (2) Irrigation wells and pumps, (3) Electricity lines to the motors of pumps, and (4) Other necessary infrastructure including infrastructure of the processing

station, efficient irrigation system, fencing to exclude stock and wild animals, and tree guards to protect seedlings from animals.

The main management operations are establishment and floor, soils and nutrition, pruning and training systems, pollination, protection, crop load management, harvest, and automation and mechanization. Orchard floor management involves both tree rows and alleyways. This includes weed suppression, soil stabilization, maintaining beneficial insect populations, and minimizing maintenance inputs, mainly irrigation and mowing. Initial site preparation is done only once and may not require purchasing machinery and equipment. It may be cost effective to rent the necessary machinery and equipment for implementation of the operation, or to have the operation implemented by a contractor.

Basic machinery and equipment for jojoba plantations include material for planting and trellis building, mulch spreader, fertilizer spreader and hand and power pruning equipment. Hand operated pruners include hand-held clippers, loppers, saws, pole pruners, and pole saws. Handsaw is used to prune larger braches. Loppers are used to prune shoots. Pole pruner is used to reach shoots at the tops of trees. There are three types of power pruners: engine driven, hydraulic driven, and electric. The growers can decide on the best type that suits their needs in the orchard.

There are two types of mower**s:** rotary and flail mowers: (1) Rotary mowers are useful to mow the ground cover close to the tree trunks and are often wider than flail mowers to enable coverage of more ground with a single pass; (2) Flail mowers use a horizontally-mounted spinning drum on which knives are mounted that shred materials during operations. There are three types of farm tractors: spray, general purpose and tractors with buckets.

In 2002, a program for development and implementation of the mechanical application of supplemental pollen was launched and run by the University of Arizona, funded by Fincas de Ambato and the State of Arizona. This program developed the machinery required to collect pollen and apply management techniques and preservation. Evaluation of testing the performance of the machine under conditions of Arizona, USA and Catamarca in Argentina proved the success of operation of the machine in both countries. The machine achieved a 6-month reduction in the period between harvest and application of pollen. Although the success of this machine has been confirmed experimentally, its use is not yet commercialized (Coates et al. 2006; Ayerza 2016).

8.2.2.3 Harvesting & Processing

A well-managed plantation, with appropriately-selected cultivars, planted at 1250 plants/ha will yield about 0.5 mt of seed/ha after 10–11 years under rainfed conditions, and up to 1 mt/ha if irrigated. Production should commence 3–5 years after planting and reach full levels about 8–10 years after planting. Certain cultivars are reportedly yielding up to 3 mt/ha or more (Naqvi and Ting 1990). After flowering in early spring, pods form on the female plants, in which the seed develops and matures. When the seed is fully mature and dry, the pod splits open in late summer

and autumn and the seeds fall to the ground. The season of seed fall is predetermined by both environmental and genetic factors involved in seed maturation (Sherbrooke and Haase 1974). In very few instances, the jojoba pod may not split open readily and tree shakers are required to dislodge the seed. The seed on the ground is collected using sweeper harvesters, which are specially-developed harvesters equipped with rotating brooms. Alternatively, modified almond harvesters may be used. Seed cannot be harvested from the shrub as it would not be fully ripe and unsuitable for cold-pressing due to moisture levels. Stones, soil and organic matter are separated from the seed by winnowing and passing over a gravity table. Some producers wash the seed to remove any residual soil after winnowing, using an industrial washing machine. Seeds comprise up to 55% oil, of which 40–45% is extracted by cold-pressing. The first pressing produces 80–90% of the oil, used for pharmaceuticals and cosmetics, while the second pressing (or solvent extracted) oil can be used for lubrication or chemical applications. Once pressed, the oil is filtered (*polished*) and may be bleached before storage in sealed drums until required, or sold. Growers can purchase their own presses, or engage contract processors.

8.2.2.4 Markets and Marketing

The major jojoba producing countries are Argentina, USA, Israel, Australia and Peru, in descending order. The global jojoba oil market has witnessed strong growth over the years owing to the increasing applications of jojoba oil in the cosmetics and pharmaceutical industries. This is a result of its diverse functional properties that make it an essential ingredient in skin- and hair-care products. The major applications of jojoba oil are found in cosmetic, pharmaceuticals and industrial sectors. The cosmetics sector is the largest application area, accounting for the majority of the total share. The market is also segmented on a regional basis, covering various regions including North America, Europe, Asia Pacific and Latin America. In 2016, the global jojoba oil market reached USD 160.3 million and is anticipated to increase to around USD 250 million in 2024 (https://www.Researchester.com). INCA OIL, Germany (www.incaoil.org) indicated that jojoba seed production of exporting nations in 2018 was about 10,000 mt and the forecasted production for 2019 to be around 12,000 mt. The estimated average oil yield is 45% www.pera-ingredients.de.

8.2.2.5 Risks and challenges

Jojoba production presents a commercial risk involved in the capital investment and time to establish the gardens. Jojoba requires investment from the development stage, which is followed by a lengthy maturation period of 3–5 years before first harvest and 8–10 years until full production.

8.2.3 Improvement Strategies for Jojoba Development

Jojoba is a valuable industrial and cash crop. Its industry requires great attention and research backup to meet the challenges of finding ways to improve productivity and quality of the products. It is dioecious in nature, and exhibits tremendous variability at the clonal level, and in morphological and yield contributing characters. Research work is required to understand jojoba's unique properties that can be manipulated to coax the crop to reach its fullest potential.

To enhance yield and improve the oil quality, it is crucial to identify the factors that contribute to the extreme variability observed in different genotypes by selecting and propagating desirable male and female plants, and their most compatible combinations. The criteria for selection includes plant habit and consistency in production and seed yield. Seed waxes are analyzed for acidity, peroxide and saponification values, and for fatty acids and fatty alcohols compositions. A multi-disciplinary approach based on molecular genetics, functional genomics, plant reproductive biology, biochemistry and agronomy may provide accurate information to identify genotypes with stabilized yields in various production systems. At present, flowering is variable and multiple harvests must be made to collect the seeds, because they ripen at different times. Improvement practice is selecting superior males that produce pollen at the time when ovules are receptive, synchronizing the fertilization process. Researchers indicate that mild water stress before the onset of frost may help reduce frost damage suggesting that jojoba has the capacity to *supercool* to many degrees below the freezing point (Benzioni et al. 1992; Nelson and Bartels 1998; Nelson et al. 1993).

8.2.4 Traditional Propagation Methodologies and Limitations

Traditionally jojoba has been propagated by seed or rooted cuttings; each has its limitations. Jojoba is dioecious and extremely heterogynous and has a long rotation period, long flowering and seed set time and gives an excess of male plants. This makes propagation by seeds commercially uneconomic. The alternative of propagation by cuttings is a slow process and time consuming. These drawbacks call for development of biotechnology techniques to improve jojoba propagation. In vitro tissue culture has been reported to be more feasible as compared to seed and rooted cutting techniques.

8.2.4.1 Propagation by Seed

Jojoba propagation by seed results in very diverse populations and low average yields. In seedling plantations in Israel up to 72% of inferior yielding pistillate plants were considered not economically viable and were removed (Benzioni 1995).

It is impossible to ensure quality in terms of oil content. Genetic uniformity in planting materials is difficult. One-half the seedlings are male which should be rouged, as 8–10% males are sufficient. Jojoba has a long flowering and seed set 3–4 years after transplantation. Nevertheless, jojoba propagated from seed allows the possibility of genetic selection, either through seeds or vegetatively (Yermanos 1977). It is now considered essential that future plantations be established from high-yielding selected cultivars propagated by vegetative means if reliable, economical yields are to be obtained (Benzioni 1997; Hogan 1979).

8.2.4.2 Vegetative Propagation

Vegetative propagation enables the production of true to type jojoba clones by rooting of cuttings, air layering or in vitro cultures. Plantations with the desired proportion of male to female plants of preselected superior clones has enabled improvement of the crop performance (Dunstone 1988). Selected clones have produced up to ten times the average yield of unselected, seeded crops in the USA (Hogan and Palzkill 1983; Hogan et al. 1978; Yermanos 1982), Mexico (Bernal 1982), Israel (Forti and Heiman 1978) and Australia (Milthorpe and Dunstone 1989a, b). A systematic screening of plants suggests that jojoba germplasm at the University of California Riverside was capable of producing 4 mt of seeds per plant (Naqvi and Ting 1990). Nelson and Watson (2001) achieved a maximum of 2.7 mt of seed per plant from irrigated clonal lines (Arya and Khan 2016; Hogan and Bemis 1984).

Rooting of Cuttings

Commercial plantations of jojoba are mainly established using cuttings, or in a few cases grafting, resulting in a narrow genetic diversity that may cause the low seed yield. Eed and Burgoyne (2015) reported the suitability of jojoba propagation by semi-hard wood cuttings. The key is to get the seedlings well established before the heat of summer or the cold of winter. The propagating medium best includes an equal mix of peat moss, perlite and vermiculite, which favors good aeration and moisture retention, and should be free of diseases, weed seeds and insects. Ash et al. (2005) reported that jojoba cuttings produce roots well in peat moss or peat moss and sand mixtures, and that peat-based *jiffy* containers are a well-suited medium (Fig. 8.10a).

It is desirable that the substrate temperature for cuttings have a temperature range of 27–30 °C. Cuttings are most successfully rooted with misting techniques, using bottom heat to accelerate the process. In mist-propagation chambers, kept at about 22 °C, stem- and apical-wood cuttings (treated with fungicide and rooting hormone) produce roots within 3–8 weeks, depending on the season. Cuttings grow true to the sex of the parent material and mature sooner than seed-derived material (Low and Hackett 1981). Genetic variability among jojoba plants is likely to have an effect on rooting success, as does the season in which cuttings are taken. High rooting per-

Fig. 8.10 Jojoba cuttings rooting. (**a**) Jojoba cuttings in soil mixture. (Source: https://goo.gl/images/WoYqyo), (**b**) Significant effect of IBA auxin on rooting of jojoba cuttings. Cuttings treated with 4000 ppm IBA (left group) and control without IBA (right group). (Source: Low and Hackett 1981)

centages strongly correlate with cuttings taken from plants during the active growing period of spring and summer with winter cuttings yielding the poorest results (Khattab et al. 2014; Low and Hackett 1981). The root systems for five node cuttings were found to be larger than those of single node cuttings. A comparison of differing single node cuttings showed double-eye cuttings had significantly greater roots than single-eye cuttings (Lee and Paskill 1984). Generally a rooting percentage range of 15–95% is possible, depending on the clone and the season during which the cutting is taken (Feldman et al. 1989; Mills et al. 1997). Successful propagation from cuttings has been achieved. Cuttings tend to respond well to indole butyric acid (IBA)-based rooting powders or gels (Low and Hackett 1981) (Fig. 8.10b). However, IBA has been reported to be effective only when cuttings are collected during the active growing period of the source plant. In Egypt, cuttings taken for rooting in winter were unsuccessful even with addition of IBA in the culture media compared to cuttings taken in April (spring) and July (summer time) (Khattab et al. 2014).

Clonal material should be exposed to a constant humidity regime; rooting takes places over a period of 3–5 weeks. The rooted cuttings are transferred to final pots and gradually hardened on unheated tables. Cuttings are then hardened in a greenhouse with approximately 30% shade. Plants are ready for transplantation to the field after 3–6 months. Field soil substrate temperature variation (10–12 °C and 20–25 °C) appears to make very little difference to the rooting percentages in clonal material (Pratt et al. 1998). It has been found that high phosphorus in water has an inhibitory effect on root development in clonal plantlets and a fertilizer low in P is recommended during the hardening and maintenance of jojoba cuttings. The lack of phosphorus results in decreases in magnesium and calcium content in the leaves of the cuttings, but there was no effect on shoot growth or chlorophyll concentration (Benzioni and Ventura 1998). Treatments such as shading of cuttings aid survival and the resulting reduction in photosynthesis does not appear to adversely affect successful rooting percentages (Feldman et al. 1989).

Although jojoba is difficult to root plant, propagation through cuttings is the most commonly used asexual method with limited success (Bashir et al. 2009; Palzkill and Feldman 1993).

Air Layering

Cuttings are the most common form of vegetative cloning used to commercially propagate new plants, but success in asexual reproduction has also been achieved by using air layering. Jojoba plants respond well to air layering using a peat moss or sphagnum moss ball to retain moisture with the addition of 0.02 M IBA and 0.02 M naphthalene acetic acid (NAA) in a base of lanolin applied to the girdle. Pistillate plants achieve better success in rooting using this method, but there is no significant difference in the number of roots on new plants between staminate and pistillate clones once they are successfully rooted (Alcaraz and Ayla-Rocha 1982).

Veneer Grafting

A veneer grafting experiment was performed in Bangladesh on jojoba to compare the better timing for veneer grafting between the 2 months of August and February, 2002–2004. The results showed that grafting practiced in August performed better than that of February. The August grafting resulted in longer sprouts (16.21 cm) with more grafting success (76.39%) than that practiced in February. The study indicated that the optimum time for grafting jojoba in Bangladesh was the month of August (Bashir et al. 2006). A top wedge and veneer grafting comparison trial was conducted in Egypt to find out whether April or August was the better time for conducting the operations. Data showed that grafting practiced in April showed a significantly higher percentage of success and sprout length compared to that of August; but grafting in August showed significantly lower number of days to sprouting. Top wedge grafting scored a significantly higher percentage of success

compared to veneer grafting and sprout length, but on the other hand, veneer grafting scored significantly less number of days to sprouting. April was therefore recommended to be the better time for jojoba grafting and top wedge grafting as well (Khattab et al. 2013). Success of operations seems to depend on weather conditions of the propagation trials.

8.3 Micropropagation

Micropropagation is the in vitro vegetative propagation of plants under controlled environmental conditions of temperature, humidity, light intensity and photoperiod, laminar airflow system and a defined nutrient medium. It is an advanced technique to produce a large number of genetically superior and pathogen-free transplants in a limited time and space. The number of jojoba shoots to be produced in vitro was reported to depend on the stock plant material, culture media and conditions of the culture media.

Investigators used explants from nodal stem segments, apical meristems or various tissue. The number of jojoba shoots produced in vitro was reported to depend upon the type of explants (Hassan 2003), source of explant, the plant genotype (Elhag et al. 1998) and the type of vessels and cultural conditions (Mills et al. 2004). Agrawal et al. (2002) reported that the per cent of nodal explants inducing multiple shoots increased significantly when in vitro-raised shoots were used as the source of explants. Virus-free material was obtained from apical meristems and nodal segments. Hegazi et al. (2014) concluded in a study that shoot tips were the best jojoba explants for micropropagation, followed by the terminal, subterminal and basal stem node segments, respectively. Organogenesis has been induced in vitro both from juvenile explants (Roussos et al. 1999; Singh et al. 2008) and mature explants (Chaturvedi and Sharma 1989; Kumar et al. 2009; Mills et al. 1997; Singh et al. 2008). A protocol developed for the induction, maturation and germination of somatic embryos from leaf tissue of jojoba has been described by Hamama et al. (2001). Shehata et al. (2018) reported that nodal segments were the best explant material for callus formation followed by the leaf disks (leaves) and seeds, respectively. In vitro culture of immature jojoba zygotic embryos has been demonstrated by a number of studies since the 1980s (Benzioni 1995; Chaturvedi and Sharma 1989; Hamama et al. 2001; Kacker et al. 1993; Lee and Paskill 1984; Roussos et al. 1999). Tissue culture has been successful up to and including root elongation in many trials. Kacker et al. (1993) developed a technique allowing the successful regeneration of female jojoba plants using coppice shoots. Chaturvedi and Sharma (1989) developed a standardized method for production of cloned plants of known sexuality by in vitro proliferation of auxiliary buds.

In plant tissue culture, MS medium (Murashige and Skoog 1962) is the most frequently used medium with varying concentrations of different plant growth regulators. Several investigators have attempted to propagate jojoba in vitro on various

culture media containing different concentrations of plant growth regulators with varying levels of success (Agrawal et al. 2002; Bashir et al. 2007a, b, c, 2008; Benzioni 1995; Elhag et al. 1998; Kumar et al. 2009; Lee and Paskill 1984; Singh et al. 2008). A highly successful protocol for the rapid multiplication of jojoba seedlings by in vitro culture has been developed using a modified Driver and Kuniyuki (DKW) growth medium incorporating varying concentrations of 6-benzyladenine alone and in combination with silver nitrate (Roussos et al. 1999). Chaturvedi and Sharma 1989 developed a protocol for clonal production of jojoba by using modified Schenk & Hildebrand (SH) medium fortified with different plant growth regulators (PGRs).

Öncel and Erişen (2017) managed to obtain in vitro propagated jojoba shoots and transfer them to MS medium containing different concentrations of NAA, indole 3-acetic acid (IAA) and IBA for rooting. Eed and Burgoyne (2016) discussed the progress and difficulties of micropropagation, in vitro oil production, gene transformation and DNA fingerprinting of jojoba. An increase in rooting following treatment with cyclodextrins, in the absence of IBA, has also been observed in vitro (Apostolo et al. 2001) (Fig. 8.11a, b).

A protocol was developed for clonal production of jojoba by using modified SH medium fortified with different PGRs by Chaturvedi and Sharma (1989). Llorente and Apostolo (2013) developed an in vitro protocol for obtaining elite jojoba clones. For culture initiation, single-node explants were cultivated on MS medium supplemented with Gamborg's vitamins (B5, 11).1 μM BA (N(6)-benzyladenine), 0.5 μM

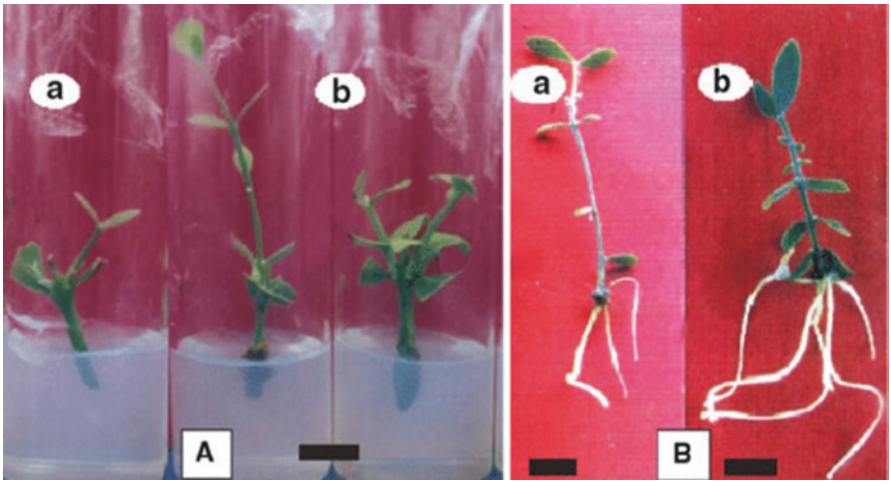

Fig. 8.11 Effect of oligosaccharides on jojoba micropropagation. (**a**) Trehalose effect on initiation cultures: (a) Shoots in basal BM medium supplemented with 0.49 μ M IBA, 1.44 μ M GA 3 and 11.10 μ M BA, (b) Shoots in BM supplemented with trehalose 1 mM. Bar = 1 cm, (**b**) β-Cyclodextrins (β-Cd) effect on in vitro rooting: (a) Shoot rooting in rooting medium (RM) supplemented with 15 μM IBA (control treatment), (b) Shoot rooting in RM medium supplemented with 15 μ M IBA and 30 μ M β-Cd. Bar = 1 cm. (Source: Apostolo et al. (2001); https://www.floraldesigninstitute.com)

Fig. 8.12 In Vitro Jojoba micropropagation. (**a–d**) Initiation culture, (**e–g**) Shoot proliferation, (**h**) In vitro rooting, (**i, j**) Acclimatization, (**k**) Micropropagated plants in glasshouse. Bars: a – e, g, h, j = 1 cm; f, i = 2 cm; k = 5 cm. (Source: Llorente and Apostolo 2013)

IBA and 1.4 μM gibberellic acid (GA3). A large number of uniform plants from selected genotypes of jojoba were produced (Fig. 8.12).

Mills et al. (2009) studied the effect of substrate, medium composition, irradiance and ventilation on jojoba plantlets at the rooting stage of micropropagation. Rooting percentage, and root morphology and functioning (mineral uptake) were investigated. Plantlets grown on Sorbarod plugs embedded in perlite accumulated more biomass and showed better rooting than plantlets grown on Sorbarod plugs

embedded in agar. However, the addition of sucrose to water or salts media promoted rooting and accumulation of shoot and root biomass in plants grown on plantlets grown on Sorbarod plugs embedded in agar substrate. Nevertheless, attempts to harden plantlets and transfer them to outdoor soil failed for many years. This continued to occur until Chaturvedi and Sharma (1989) developed a standardized method for the production of cloned plants of known sexuality by in vitro proliferation of auxiliary buds.

As an alternative to the laborious conventional method of jojoba propagation by rooted cuttings, which produces limited amounts of shoot material and subjects the product to pest and environmental field storage hazards, Bekheet et al. (2016) studied methods for short to medium-term storage that may eliminate the obstacles of field storage. Polyethylene Glycol, mannitol and low temperature effects were studied to observe the treatments' induction of in vitro minimal growth of jojoba for short-term storage of shootlet cultures. Observations proved that both mannitol and Polyethylene Glycol could be used for short-term storage of jojoba shootlet cultures. Limitation of growth was stronger using Polyethylene Glycol with risk of more lethal shootlets. Mannitol slightly increased the induction of new branches. Low temperature reduced both number of shootlets and shootlet height and encouraged new branches regeneration. No necrosis with low temperature storage for 9 months was observed with the conclusion that cold storage at 5 °C is better for healthy storage of in vitro grown shootlets of jojoba. Despite the existence of a number of protocols for in vitro cultivation of jojoba (Prakash et al. 2002), it must be acknowledged that there are difficulties which have limited the reliability and efficiency of micropropagation. As yet the aim of an economically-viable process of in vitro wax production has not been achieved.

8.4 Jojoba Germplasm Biodiversity and Conservation

Biodiversity is the variability among living organisms from all sources including terrestrial, marine, and other aquatic ecosystems and the ecological complexes of which they are a part; this includes diversity within species, between species and of ecosystems. It can be observed at three levels: (1) Genetic diversity: each member of any animal or plants species differs widely from another individual in its genetic makeup; (2) Species diversity: number of species of plants and animals that are present in a region constitutes its species diversity; (3) Ecosystem diversity: conservation of genetic diversity in the face of rapidly depleting natural resources has considerable significance and worldwide importance.

Various types of biological materials preserved in germplasm and tissue banks can ensure the genetic diversity of a species almost indefinitely. Stored gametes, embryos and somatic tissues represent a means of overcoming barriers of space and time, allowing the transfer of genetic material between populations and its use many years after extinction of the donor organism.

Jojoba has many special characteristics which make it an inherently sustainable, renewable source of raw material for industry, land conservation and browsing. These characteristics include long life span of over 100 years; its nature of producing seed oil that is non-perishable, highly stable and versatile; disease, pest and drought resistance; growth in arid climates, drought tolerance, often farmed on marginal land, in poor soils unsuited for many other crops.

Jojoba is dioecious in nature, and exhibits tremendous variability at the clonal level in morphology as well as yield contributing characters. It is desirable to select prolific male and female plants, and their most compatible combinations to enhance yield. A multidisciplinary approach based on molecular genetics, functional genomics, plant reproductive biology, biochemistry and agronomy can provide accurate information to identify genotypes with stabilized yields in various production systems.

Bekheet et al. (2016) conducted trials to develop an in vitro method for short-term conservation of planting materials of jojoba. MS medium supplemented with 1 mg/l of benzyladenine was used for in vitro proliferation of jojoba shootlets from node explants. Slowing down shootlet growth was induced by maintenance under low temperature (5 °C) and storage in three concentrations of mannitol and Polyethylene Glycol. Results showed that shootlet storage at 5 °C pronouncedly caused a decline in number of shootlets and shootlet height with an enhanced number of the proliferated branches. Pronounced decline in both number of proliferated shootlets and shootlets height were observed on low levels of mannitol. All Polyethylene Glycol treatments and the high levels (40 and 60 g/l) of mannitol caused a progressive reduction of the two growth parameters. More growth retardation was observed in the presence of Polyethylene Glycol than in the presence of mannitol. Slowing down jojoba growth was also induced by modifying culture media with supplements of osmotic agents, growth inhibitors, or by removing growth promoters.

Bhardwaj et al. (2010) analyzed genotypes of a collection of jojoba males and females with 50 random amplified polymorphic DNA (RAPD) and 55 inter simple sequence repeat (ISSR) markers to compare the efficiency and utility of these techniques for detecting genetic polymorphism. Yields of 442 and 566 scorable amplified products were obtained from analysis by RAPD and ISSR, respectively, of which 60.7 and 69.3% were polymorphic. Heikrujam et al. (2015) employed start codon targeted (SCoT) polymorphism and CAAT box-derived polymorphism (CBDP) to test their efficiency in analyzing genetic relationships among genotypes of male and female jojoba. CBDP data analysis also revealed a similarity range of 0.751–0.958 within female genotypes and 0.754–0.976 within male genotype populations, indicating a genetically diverse jojoba population. Genetic diversity is of great significance for breeding, management and conservation of elite (high yielding) jojoba germplasm.

The production of synthetic seeds in jojoba have been reported by Hassan (2003) and Kumar et al. (2011b). Hassan (2003) indicated that it is possible to use the apical and axillary buds of jojoba for encapsulation and utilization of the technology for clonal propagation and regeneration of complete plants. Regenerated plants

showed no morphological variations when compared with the original mother plant. Environmental variation has a significant effect on yields of clonal plantation. This was first detailed at the Coit plantation in Vista, California, where environmental variability in the non-irrigated clonal material was reflected in the high variability of yields achieved from year to year. Environmental variation did not affect the uniformity of the clonal material in terms of anthesis and maturation (Yermanos and Holmes 1973).

Positive relationship has been observed between branching frequency and an increase in jojoba plant nodes (Ravetta and Palzkill 1993). In general, highly branched plants have more flowers and are more productive, which could be a useful trait to select for. Based on this evidence, selection for branching frequency, which is related to flower bud production and presumably yield, should be effective. Benzioni and Vaknin (2002) found that both male and female jojoba genotypes additively influenced the composition of the wax esters in the presence of high and low saline irrigation regimes, concluding that jojoba wax composition is influenced by both female and male genotypes as well as by environmental factors.

8.5 Molecular Breeding

8.5.1 Molecular Marker-Assisted Breeding

Traditionally plant breeders have selected desirable plants based on their phenotypical characters, a process which can be time consuming, is influenced by environment, difficult, slow and costly. Marker-assisted selection (MAS), currently used by breeders as a short-cut, however, makes the process of selecting plant traits easier and faster.

Amarger and Mercier (1995, 1996) developed a technique using RAPD for discrimination between jojoba clones at the genomic level. Among a set of 30 primers tested, a simple reproducible pattern with three distinct fragments was identified for one jojoba clone and two distinct fragments for another clone. The main goal of this work was to assess the amount of genetic variability among and within individual plants in cultivated populations. High genetic variation was found in seeds of unknown origin. Very little variation was found within clones, as would be expected, but there were some differences in a few individuals within clones with the presence of different rDNA unit types. There was also a minor difference between acclimatized plants and tissue-cultured explants with a discrete 2.5 kbp DNA fragment missing in the tissue-cultured plants. This may be due to the unavailability of restriction sites due to the plant's biological process of methylation or DNA methyl group tagging (Amarger and Mercier 1996).

Heikrujam et al. (2015) investigated the genetic diversity among different jojoba genotypes using the molecular markers start codon targeted (SCoT) polymorphism and CAAT box-derived polymorphism (CBDP) markers. CBDP proved to be the more effective.

Sex determination in plants is not always supported by biochemical and cytological analyses so molecular markers became a focus of interest for gender differentiation (Grewal and Goyat 2015; Hosseini et al. 2011). This is an area where biotechnology has made great advancements in sex determination in early stages of plant life, especially in dioecious species like jojoba, where a number of molecular markers have been employed (Agrawal et al. 2007, 2011; Eed and Burgoyne 2016; Heikrujam et al. 2014a, b; Hosseini et al. 2011; Ince and Karaca 2011; Jangra et al. 2014; Mohasseb et al. 2009; Sharma et al. 2008).

In a study to develop protein molecular markers for early gender differentiation, a comparative proteomic study was conducted on male and female jojoba leaves by Al-Obaidi et al. (2012, 2017a). The study concluded that results could help in better understanding the molecular mechanism of gender differentiation in jojoba. The results demonstrated the feasibility of using RAPD-PCR in distinguishing between jojoba sexes.

Grewal and Goyat (2015) reported that sex-linked markers pave the way for further discoveries, but do not alone explain the molecular mechanism of sex determination in several plant species. Table 8.2 summarizes the molecular biology studies involved in jojoba compiled by Al-Obaidi et al. (2017b).

Table 8.2 Summary of molecular biology-based research on jojoba (*Simmondsia chinensis*)

Molecular techniques	Targets	References
RAPD	Genetic variability	Amarger and Mercier (1995)
RAPD	Gender differentiation	Agrawal et al. (2007)
ISSR	Gender marker-assisted selections	Sharma et al. (2008)
RAPD, ISSR	Genetic relationship among and between gender	Sharma et al. (2009)
RAPD, ISSR	Assessment of ISSR and RAPD marker assays for genetic diversity analysis	Bhardwaj et al. (2010)
Touch-down PCR assay	Gender diagnostic	Ince et al. (2010)
RAPD, ISSR	Clone maintenance	Kumar et al. (2011a, b)
AFLP	Gender-linked AFLP markers	Agarwal et al. (2011)
CAPS	Early gender differentiation	Ince and Karaca (2011)
RAPD	In vitro and in vivo gender differentiation	Al-Obaidi et al. (2012)
ISSR	Genetic variation and chemical traits of selected plants	Al-Soqeer et al. (2012) and Al-Soqeer (2014)
ISSR, SCAR	Early gender differentiation	Jangra et al. 2014
ISSR	Male-specific sequence tagged sites marker	Heikrujam et al. (2014a)
ISSR	Male-specific sequence tagged sites marker	Heikrujam et al. (2014b)
SCoT, CBDP	Gender genetic diversity	Heikrujam et al. (2015)
RAPD, GC-MS technique	Validation of different jojoba accessions for commercial applications	Arya and Khan (2016)

Source: Al-Obaidi et al. (2017b)

8.6 Enhanced Traits of Jojoba

Several researchers have studied the relationship between botanical traits and oil yield and quality in jojoba, as an early means to detect and eliminate undesirable genotypes in jojoba breeding (Al-Soqeer et al. 2012; Al-Taweel et al. 2018; Tobares et al. 2004). The traits studied were vegetative, reproductive and oil content and quality. The jojoba industry is always seeking cultivars that produce the highest yield and best quality of wax. Tobares et al. (2004) examined some agronomical and chemical traits as descriptors for discrimination and selection of jojoba clones. Their criteria for selection included: plant habit, consistence in production and seed yield. Seed waxes were analyzed for acidity, peroxide and saponification values, and for fatty acids and fatty alcohols compositions. Intraspecific variations in the concentrations of fatty acids and alcohols were smaller than differences in agronomical characters. Significant differences were found in most of the parameters evaluated. Al-Taweel et al. (2018) in Egypt studied the response of jojoba to foliar sprays with three levels of GA3 (50,100,150 ppm) on vegetative, reproductive and seed oil content. Results revealed that all treatments significantly enhanced vegetative growth characteristics (branch length, secondary branch length, number of branched nodes) and reproductive characteristics (flowering date, initial fruit set, final fruit set, seed yield, chemical composition of jojoba seed) compared to the control. Al-Soqeer et al. (2012) analyzed the contents of seeds from six selected jojoba genotypes for oil protein, total carbohydrate, and simmondsin. The genetic relationships among jojoba genotypes were also determined. Highly significant differences among the genotypes were found for oil, protein, total carbohydrate and simmondsin content. These genotypes also had almost similar values for most chemical traits.

8.7 Genetic Engineering

Molecular techniques were developed as advances over traditional procedures, precisely targeting individual genes in plants rather than relying on scattershot techniques like mutagenesis. Genetic engineering is the extraction of DNA from the genome of one organism and combining it with the genome of another, overcoming the species barriers set up by nature. In agriculture, it is used by scientists to enhance the characteristics of plants by induction of higher nutritional values, disease resistance or eliminating genes of undesirable nutritional values. Modern forms of genetic engineering are precise ways to add desirable or subtract undesirable traits. The most common form of genetic engineering is transgenesis, usually using an *Agrobacterium*-mediated transformation, and has the ability to transfer a portion of its DNA, carrying a new desirable trait, into the DNA of the recipient plant.

Limited availability of jojoba oil keeps its price quite high relative to petroleum-based products. Three key enzymes are involved in the process of oil production:

wax synthase, fatty acyl-CoA reductase and b-ketoacyl-CoA synthase. DNA sequences for genes specifying these enzymes were identified from jojoba (and silver dollar, *Lunaria annua*, another plant that accumulates long-chain fatty acids in its seeds), cloned into plasmid vectors of *Agrobacterium* and inserted into the *Arabidopsis* genome. The enzymes were expressed in these transgenic plants and produced waxy substances that constituted up to 70% by weight of the mustard seeds. Hussein et al. (2014) and Canoira et al. (2006) reported processes to convert jojoba oil-wax to biodiesel by transesterification with methanol and catalyzed with sodium methoxide (1 wt % of the oil) carried out in an autoclave at 60 °C. Accordingly, geneticists are seeking ways to engineer more traditional crop species so that they, too, can produce jojoba oil. Toward that end, the experimental mustard family plant (*Arabidopsis*) has once again been employed.

In Japan, researchers at Lusaka University (2017) established the first genetic transformation procedure in jojoba by using *Agrobacterium* for breeding improvement, and developed DNA markers as indices for screening. Genetically modified jojoba plants are being used for abatement of desertification in Japan and Egypt.

A number of key genes encoding the enzymes involved in wax synthesis have been isolated from jojoba (Lassner et al. 1999). The aim is to introduce these genes into cultivars of field mustard (*Brassica rapa*) via transgenic *Arabidopsis thaliana*. *Brassica rapa* is a member of the mustard (*Brassicaceae)* family, which includes cultivated species such as cabbage and radish. At present the levels of jojoba wax achieved in transgenic *B. rapa* are quite high with up to 50% of the seed oil present as *wax* in the transgenic pCGN8559 plant with the best phenotype.

8.8 Mutation Breeding

The traditional farm methods of altering crop gene structures, known as simple selection**,** was to choose the best looking seeds or plants to grow the next season. As the science of genetics became better understood, plant breeders developed techniques of transferring specific traits for cultivar improvement. Biotechnology techniques dramatically speeded up the process of transfer of traits and cultivar improvement. Variation is the source from which plant breeders are able to produce new cultivars. Mutations are caused by mutagen treatment within the genome of an organism. Mutagenesis, an acceptable alternative for genetic engineering, involves radiating or chemically dousing seeds in laboratories to create thousands of unknown random mutations in the hopes of finding beneficial traits. There are several physical mutagens such as gamma radiation, x-rays, protons, neutrons, alpha particles and beta particles; chemical mutagens include ethylmethane sulfonate and sodium azide for mutation induction in plants. Genfa et al. (2006) reported ion implantation using N^+ on *Arabidopsis thaliana*, as a new biophysical mutagenic technique that showed great potential for crop breeding. A few of these traits occasionally arise spontaneously through a process called mutation, but the rate of natural mutation is very slow and unreliable to produce all the plant traits that breeders would like to

see. Chikara et al. (2001) reported that in a mother jojoba plantation at Zanjmer, Gujarat, India, they identified a few abnormal plants. While the height of normal plants was 1.5–3 m, the height of dwarf jojoba mutants had a range of 0.75–1.2 m, in addition to significant differences in internode and leaf sizes between mutant and normal plants. This may have been a spontaneous mutation.

Mutation may be induced in jojoba by in vitro regeneration via organogenesis and somatic embryogenesis. Factors affecting organogenesis as well as production of synthetic seeds using shoot tips and axillary buds need to be investigated to develop an efficient genetic transformation system in jojoba.

8.9 Conclusions and Prospects

As a source of a unique liquid wax that resembles sperm whale oil and human sebum in its chemical structure and biological characters, jojoba has been moved from being a neglected Sonora Desert shrub known to Native Americans for personal care, to a renowned domesticated worldwide industrial, cosmetic and commercial crop. Commercial jojoba plantations exist today in several countries mainly Argentina, USA, Israel, Australia, Peru, Egypt, Mexico, Chile and Sudan. Golden Jojoba Oil is the unrefined version of the Clear Jojoba Oil, obtained from the fruit of the *Simmondsia chinensis* plant. The golden color of this liquid plant wax potentially makes it a very attractive addition to personal care products. Jojoba products such as cosmetics, industrial oil, pharmaceuticals, animal feed and other products are found in several commercial centers. Yet the jojoba industry faces serious challenges and it must be acknowledged that there are difficulties, which have limited the impetus of reliability and efficiency of expansion in jojoba plantations. Jojoba is propagated by rooted cuttings, despite the existence of a number of protocols for in vitro propagation (Prakash et al. 2002); high cost hampers commercial micropropagation of jojoba. There may be few productive jojoba cultivars on a limited scale, but there are as yet no available cultivars for growers. Researchers have identified wild jojoba, plants with desired merits, which were selected as parent candidates for commercial cultivars, and research work to resolve various problems is progressing.

The processes of material selection, propagation, storage and multiplication is very tedious, time consuming and costly. Jojoba is known to require less water for irrigation, yet experience has proved that consistent stable production requires the availability of irrigation water as required. Climatic factors, specially frost and occasionally extreme heat, have caused serious losses that forced some commercial jojoba growers to abandon their plantations, early growers in the USA being an example. Finally, while jojoba oil is a very attractive industrial and cosmetic commodity, and investment in the business looks lucrative, caution is advised for careful consideration in site selection, availability of finance and climatic conditions. Research work is underway to trace desirable biological traits to find prolific commercial cultivars that can be harvested mechanically. Haque et al. (2018) reported that the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9

(CRISPR-associated protein9) genome editing technology has shown great promise for quickly addressing emerging challenges in agriculture to achieve desired traits. Compared to other genome editing tools such as zinc finger nucleases (ZFNs) and transcriptional activator-like effector nucleases (TALENs), CRISPR/Cas9 is faster, cheaper, and precise and highly efficient in editing genomes even at the multiplex level. Much more research needs to be pursued to understand jojoba's unique properties that can be manipulated to coax production towards maximum seed yield and high quality oil. Some of the main areas for support are: (a) Reduce plant size and synchronize flowering by selections of compatible male and female plants; (b) Help oil-based industries to promote jojoba oil marketing; (c) Encourage research to promote mechanized jojoba harvesting; and (d) Explore areas of utilization of jojoba to combat desert creep.

Appendices

Appendix I: Research Institutes Relevant to Jojoba

Institute name	Specialization research activities	Contact and location
American Jojoba Association	Provides jojoba services	1688 W Adams, Phoenix, AZ 85007 Tel: (520) 624-6663 Fax: (520) 624-6842
Arid Lands Studies	Maintains a comprehensive collection of jojoba literature, and may provide photocopies of any jojoba-related article.	The University of Arizona, Tucson, Arizona 85706, USA https://alrs.arizona.edu/
International Jojoba Association	Nonprofit organization of growers, processors, researchers, and suppliers promoting the advancement of the jojoba industry. It holds conferences and symposia and distributes jojoba information, including a membership directory), reprints of a feature article on jojoba from the Journal of the American Oil Chemists' Society), and this report	Wm Howard O'Brien, 4350 East Camelback Road, Phoenix, Arizona 85018, USA
International Jojoba Export Council	Identify new jojoba market opportunities, derivatives and co-products; promote the use of jojoba as a natural, renewable and biodegradable resource; promulgate and distribute uniform standards for jojoba products; keep an open forum for technical collaboration; improve farming and harvesting technique	4250 North Civic Center, Scottsdale, Arizona, 85251–3900 Email: office@ijec.net http://ijec.net/
Jojoba Association of Australia	Devoted to jojoba	325 Riley Street, Surry Hills, New South Wales 2010, Australia

(continued)

Institute name	Specialization research activities	Contact and location
Jojoba Happenings	An official publication of Jojoba Grower's Association. Provides information on the latest jojoba developments and editorials, literature reviews, and advertisements for organizations selling such things as seed, oil, equipment, and land	805 North Fourth Avenue, Suite 404, Phoenix, Arizona 85003, USA. Phone: (602) 253-5470
Latin America Association for Jojoba	Devoted to jojoba	Ricardo Ayerza, La Magdalena, Cerrito 822 – 7° piso, (1010) Buenos Aires, Argentina
Texas Jojoba Growers Association	Devotes to jojoba	Fred Jordan, 3616 Howell, Dallas, Texas 75204, USA
The Jojoba Society of America	Devoted to jojoba	T. K. Miwa, 2086 East La Jolla Drive, Tempe, Arizona 85282, USA

Appendix II: Jojoba Genetic Resources

Cultivation location	Important traits[a]
El-Behara, Egypt	EAI 1 and EAI 4 recommended for commercial production
Australia dry inland areas: New South Wales	Three female varieties (Barindji, Wadi-Wadi, Waradgery) and two male varieties (Dadi-Dadi, Guyambul) selected by CSIRO
Bañado de los Pantanos, La Rioja, Tucuman and Mendoza, Catamarca Province, Argentina	The maximum values corresponded to lipids (58.47% in clone 206), proteins (25.58% in clone 403) and tannins (3.95% in clone 503). Long chilling requirement traits to withstand cold weather
Negev Desert, Israel	Some selected clones

[a]No commercial jojoba cultivars have been released officially. Research work in several countries is still in progress to establish reliable commercial cultivars

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Chapter 9
Flax (*Linum usitatissimum* L.) Genomics and Breeding

Frank M. You, Sylvie Cloutier, Khalid Y. Rashid, and Scott D. Duguid

Abstract Flax (*Linum usitatissimum* L.) is a self-pollinating crop grown for its stem fiber and seed oil. Owing to long-term domestication, cultivated flax has diversified into two main morphotypes, namely fiber and oil or linseed types. In the last decade, significant progress has been achieved in flax genomics, such as the development of genetic and physical maps, chromosome-scale reference genome sequences, a large number of molecular markers (SSRs, SNPs) and quantitative trait loci (QTL) associated with traits of importance. Genome-wide markers and QTL can be effectively used for the evaluation of flax germplasm. Rust, *Fusarium* wilt, powdery mildew, and pasmo are four major diseases of concern in flax breeding. Biparental hybridization breeding with pedigree selection and off-season operations remains the predominant method of improvement in Canadian linseed breeding programs, requiring about ten years to reach registration of a cultivar. However, use of the doubled haploid (DH) method may shorten this breeding cycle by approximately two years or three generations and simultaneously be advantageous for application of genomic selection (GS). The mapping of QTL for target traits may accelerate application of molecular breeding in flax improvement programs. Genome-wide QTL have shown potential in GS for the prediction of complex quantitative traits. With more QTL identified, it is possible to pyramid all superior alleles of target traits by multiparental crossing combining DH and GS to develop superior cultivars.

Keywords Flax · Linseed · Fiber · Genetic map · Molecular breeding · Marker-assisted selection · Genomic selection · Doubled haploid · Quantitative trait locus/loci

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9.1 Introduction

Flax (*Linum usitatissimum* L.) is a self-pollinating crop from the Linaceae family that is cultivated for the production of stem fiber and seed oil (Singh et al. 2011). Due to its long-term domestication, cultivated flax has diversified into two main morphotypes, namely fiber and oil types, as well as an intermediate type (Liu et al. 2011). The oil type is also referred to as linseed or flaxseed. The two morphotypes differ primarily in terms of morphology and agronomic traits. Fiber-type accessions are usually taller, have fewer branches, and have greater straw weight, seed protein content and resistance to pasmo and *Fusarium* wilt diseases while linseed types are often shorter, have more branches, greater 1000-seed weight, seed oil content, resistance to powdery mildew and produce more seeds (Diederichsen and Ulrich 2009; He et al. 2019b; You et al. 2017).

Most cultivars are either linseed or fiber types as opposed to dual purpose (Deyholos 2006), but the intermediate type opens the door for the development of a true dual-purpose flax (Irvine et al. 2010). The currently grown linseed cultivars have up to 50% oil (Cloutier et al. 2011) that contains five main fatty acids (FAs): palmitic (PAL, C16:0, ~6%), stearic (STE, C18:0, ~2.5%), oleic (OLE, C18:$1^{\Delta 9}$, ~19%), linoleic (LIO, C18:$2^{\Delta 9, 12}$, ~13%) and linolenic (LIN, C18:$3^{\Delta 9, 12, 15}$, ~55%) (Diederichsen et al. 2013; Westcott and Muir 2003). LIN is also referred to as α-linolenic acid (ALA), an omega (ω)-3 FA. LIO and LIN, collectively called polyunsaturated FAs (PUFAs), are the precursors of essential fatty acids of the ω-6 and ω-3 families, respectively (Simopoulos et al. 1999). Although the majority of oilseed crops contain LIO, LIN is only present in oils from certain fish, microalgae, and a few crops such as canola and flaxseed (Simopoulos et al. 1999). High-LIN linseed cultivars contain as much as 60–73% LIN, making flax the richest source of plant-based omega-3 FAs (Fofana et al. 2010). Fiber flax produces fibers for woven (linen) or nonwoven textiles, twine, and rag-based paper (Deyholos 2006). Both flax morphotypes can serve as feedstocks for the production of biomass energy in the biofuel industry (Naik et al. 2010).

Flax domestication is hypothesized to have occurred during the Neolithic period between 8000–10,000 years ago in the Near-Middle East, from where it spread to Europe, the Nile Valley, and the rest of the world (Fu 2011). Flax grows best in regions with temperate climates under favorable growing conditions, such as moderate warmth, high moisture, and well-drained, medium to heavy soils (Worku et al. 2015). Currently, flax is primarily cultivated in Western Canada (linseed), the northern regions of China (fiber and linseed), north-central USA (linseed), and Western Europe and Russia (fiber and linseed) (Foulk et al. 2004; Liu et al. 2011; You et al. 2016b). As of 2011, flax was the third largest textile fiber crop and fifth largest oil crop in the world (Worku et al. 2015). Canada remains the most important linseed producer of the world, but, it has now been joined by Russia and Kazakhstan whose production has steadily increased in the last few years (Fig. 9.1).

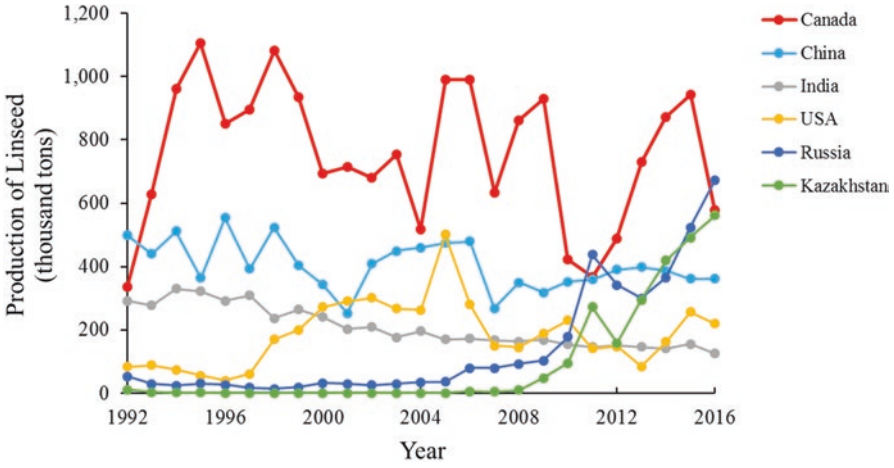

Fig. 9.1 Production of linseed by major world producing countries. (Source: FAOSTAT 2016)

Flax was initially introduced to Canada in 1617 by Louis Hébert (FCC 2015) and widely grown for fiber use in Québec. However, flax was not broadly marketed until the early 1900s when the demand for industrial oil increased, particularly during World War II (You et al. 2016b). By the 1950s, flax products were distributed and utilized globally, marking the beginning of active flax breeding in Canada. Today, linseed production in Canada is primarily located in the Prairie provinces, namely Saskatchewan, Manitoba and Alberta (FCC 2015). The bulk of the production is exported, with Canada being the world's largest exporter of flax seeds. During the last decade, significant investments were made in Canada to create and utilize flax genomics resources to accelerate flax breeding in the Total Utilization Flax Genomics (TUFGEN) project. Large amounts of genomic data and products thereof have been created, such as a consensus genetic map (Cloutier et al. 2012b), a bacterial artificial chromosome (BAC)-based physical map (Ragupathy et al. 2011), BioNano optical maps (You et al. 2018c), the first reference genome reference (Wang et al. 2012), the first version of chromosome-scale pseudomolecules (You et al. 2018c), the first generation of a flax haplotype map with 407 diverse flax accessions and ~1.7 million single nucleotide polymorphisms (SNPs) (He et al. 2019a, b; You et al. unpublished data), and a large number of molecular markers and quantitative trait loci (QTL) associated with traits of importance. These outcomes lay a solid foundation and provide the necessary resources for accelerating flax improvement using genomics-based technologies and strategies. This chapter emphasizes recent progress in genomics studies and flax breeding with an emphases on the most recent advancements in Canada.

9.2 Flax Genome

Cultivated flax is a diploid (2n = 2x = 30) with 15 chromosomes in its haploid genome whose size is estimated to be ~368 Mb based on the BAC-based physical map (Ragupathy et al. 2011) or ~373 Mb based on flow cytometry (Wang et al. 2012).

9.2.1 Genetic Maps

A genetic map is a valuable tool for QTL mapping, map-based cloning and for the finishing of genome assemblies. Genetic maps are recombination-dependent and are constructed based on molecular markers organized in linkage groups. Several genetic maps of flax have been generated (Table 9.1). Early genetic maps use traditional molecular markers such as amplified fragment length polymorphism (AFLP), random amplification of polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) markers (Cullis et al. 1995; Oh et al. 2000; Spielmeyer et al. 1998a). These genetic maps had low marker density because RFLPs are labor intensive and, AFLP and RAPD are dominant and plagued with lower reproducibility (Kumari and Thakur 2014). Simple sequence repeat (SSR) markers are widely used to construct genetic maps in flax (Asgarinia et al. 2013; Cloutier et al. 2011;

Table 9.1 Genetic maps constructed using flax biparental populations

Population	Markers	Linkage group	Total size (cM)	References
50 F_2 individuals	19 RFLPs, 69 RAPDs	15		Cullis et al. (1995)
59 DH lines	213 AFLPs, 8 RFLPs	18	1400	Spielmeyer et al. (1998a)
50 F_2 individuals	1 STS, 13 RFLPs, 80 RAPDs	15	1000	Oh et al. (2000)
78 DH lines	5 SNPs, 114 SSRs	24	834	Cloutier et al. (2011)
300 F_2 individuals	143 SSRs	15	1241	Asgarinia et al. (2013)
Combined from three populations of 243 RILs, 90 RILs, and 78 DH lines	665 SSRs, 5 SNPs, 1 seed coat color, 6 gene loci	15	1551	Cloutier et al. (2012b)
243 RILs	329 SNPs, 362 SSRs	15		Kumar et al. (2015)
100 F_2 individuals	4145 SLAF SNPs	15	2633	Yi et al. (2017)
112 F_2 individuals	2339 SLAF SNPs	15	1483	Wu et al. (2018)
Combined from two populations of 110 and 123 RILs	4497 SNPs	15	1658	Zhang et al. (2018)

DH doubled haploid, *RIL* recombinant inbred line, *SLAF* specific length amplified fragment, *AFLP* amplified fragment length polymorphism, *RAPD* random amplification of polymorphic DNA, *RFLP* restriction fragment length polymorphism, *SSR* simple sequence repeat

Kumar et al. 2015). The first SSR-based consensus genetic map contained 665 SSRs, 5 SNPs, 1 yellow seed coat color trait, and 6 gene loci (*dgatA*, *dgatB*, *fad2A*, *fad2B*, *fad3A, fad3B*), spanning 1551 cM with a mean marker density of 2.0 cM, and covered ~274 Mb or 74% of the estimated flax genome size of 370 Mb (Cloutier et al. 2012b). This high resolution consensus map was successfully used for association mapping (Soto-Cerda et al. 2013b, 2014), population linkage disequilibrium analysis (Soto-Cerda et al. 2012) and genome finishing (You et al. 2018c). With recent developments and applications of genotyping by sequencing (GBS) techniques, higher density genetic maps with up to 4497 SNP markers have been developed (Table 9.1) (Wu et al. 2018; Yi et al. 2017; Zhang et al. 2018). These high-density genetic maps are useful resources for QTL mapping, map-based gene cloning, comparative genomics, genome organization, evolution studies and reference genome finishing.

9.2.2 Physical Maps

Two types of physical maps have been constructed for flax: a BAC-based physical map and optical maps. In the first instance, BAC clones are digested with restriction enzymes to produce DNA fingerprints that are assembled into BAC contigs (Luo et al. 2013). A BAC-based physical map can provide the backbone for anchoring sequences and generating high-quality reference genome sequences. BACs from minimal tiling paths have been used as templates for BAC-by-BAC sequencing of complex genomes such as maize (Schnable et al. 2009) and wheat (Choulet et al. 2014; Luo et al. 2017). Ragupathy et al. (2011) constructed the first genome-wide physical map of flax (cv. CDC Bethune), consisting of 416 contigs assembled from 32,025 fingerprints with an N50 size of 1494 kb and a longest contig of 5562 kb (Table 9.2). This map spanned ~368 Mb, representing 99% of the estimated haploid genome (373 Mb). A total of 80,337 BAC-end sequences (BES) from 43,776 clones were also generated, used for validating and anchoring the whole genome shotgun assembly (You et al. 2018c) and, for developing putative genomic SSR markers (Cloutier et al. 2012a).

Optical genome mapping is a high-resolution recombination-independent approach to assist genome assembly (Ganapathy et al. 2014; Hastie et al. 2013; Lam et al. 2012; Shearer et al. 2014; Zhang et al. 2015). Large single DNA molecules are digested to generate ordered restriction maps. This approach is routinely employed to finish microbial and complex plant genome sequence assembly (Latreille et al. 2007). The BioNano genome (BNG) mapping technique is one of such optical mapping platforms (Hastie et al. 2013; Stankova et al. 2016). The use of optical maps as backbones substantially increases the scaffold N50 of assemblies and facilitates their sorting into pseudomolecules representing chromosomes (Luo et al. 2017; Shearer et al. 2014; Zhang et al. 2015). In flax, three BioNano optical maps for two cultivars (CDC Bethune, MacBeth) and one wild flax accession (LN1917) have been generated, consisting of 251, 357 and 354 contigs with total sizes of 317, 327 and 357 Mb, respectively (Table 9.2). The optical maps have much higher alignment

Table 9.2 Flax physical maps

Map type	Genotype (species)	No. of contigs	N50 size (Mb)	Longest contig (Mb)	Total size (Mb)	References
BAC-based map	CDC Bethune (*Linum usitatissimum*)	416	1.49	5.56	368	Ragupathy et al. (2011)
BioNano optical maps	CDC Bethune (*L. usitatissimum*)	251	2.15	7.02	317	You et al. (2018c)
	MacBeth (*L. usitatissimum*)	357	1.23	5.55	327	You et al. (2018a)
	LIN1917 (*L. bienne*)	354	1.30	5.10	357	You et al. (2018a)

Table 9.3 Flax reference genome

	Scaffold length (bp)	Number of scaffolds
N50	693,492	132
Longest	3,087,368	1
≥1 kb	300,552,207	3852
With genes	293,519,352	1028
With genes (≥1 kb)	293,484,382	980
Total (≥100 bp)	318,247,816	88,384

Source: Adapted from Wang et al. (2012)

accuracy than the BAC-based map and are very useful to identify discrepancies in sequence assembly and scaffolding, to order and orient scaffolds, to estimate gaps, and, eventually, to assist with super-scaffolding and the construction of flax pseudomolecules.

9.2.3 Flax Reference Genome

The flax genome has been sequenced using a whole genome shotgun (WGS) sequencing strategy (Wang et al. 2012). Seven paired-end libraries ranging in size from 300 bp to 10 kb from cv. CDC Bethune were sequenced using an Illumina genome analyzer and approximately 69× filtered short-reads (44–100 bp) were generated. A *de novo* assembly generated 116,602 contigs (302 Mb) or 88,384 super contigs (greater than 100 bp) with an N50 of 694 kb, totaling 318 Mb and representing ~81% of the flax genome (Table 9.3). A total of 43,384 protein-coding genes were predicted in the whole-genome shotgun assembly. All data for this assembly can be downloaded from https://phytozome.jgi.doe.gov/pz/portal.html.

9.2.4 Chromosome-Scale Pseudomolecules

The BNG optical, BAC-based physical and consensus genetic maps were used to generate chromosome-scale pseudomolecules (Table 9.4). A total of 622 scaffolds (286.6 Mb, 94.9% of the WGS assembly) were aligned to the 15 flax chromosomes (You et al. 2018c). The draft pseudomolecules contained ~316 Mb of sequences and covered 97.5% (42,277) of all protein-coding genes (43,384) predicted in the WGS assembly (Wang et al. 2012). Chromosome size ranged from 15.6 Mb for chromosome (Chr) 15 to 29.4 Mb for Chr 1. Of the 316.2 Mb pseudomolecules, gaps of 47.6 Mb were introduced from the original scaffold sequences and from the merging of scaffolds. The 15 chromosome sequences are available from the NCBI database (CP027619–CP027633).

In an attempt to fill some of the large gaps remaining in the reference genome sequence, PacBio long read-based reference sequences are under construction for both cultivated (cv. CDC Bethune) and wild flax (*Linum bienne*, accession LIN1917) (You et al. 2018a). Approximately 60× PacBio Sequel sub-reads and 54× RS II sub-reads were generated with an N50 of 9537 bp and 18,108 bp for CDC Bethune and LIN1917, respectively. For CDC Bethune, the polished assembly generated 2315 gap-free contigs totaling 359 Mb with an N50 of 2.13 Mb. For LIN1917, the polished assembly resulted in 886 contigs for a final genome size nearing 309 Mb. The BNG maps (Table 9.3) and the first version of the chromosome-scale pseudomolecules (Table 9.4) will be used to create the next iterations of the flax reference genome sequences for both cultivated and wild flax.

Table 9.4 Flax reference genome sequence

Chromosome	NCBI accession	No. of refined scaffolds	Scaffold length (Mb)	Chromosome length (bp)	No. of genes
Lu1	CP027619	66	25.2	29,425,369	4071
Lu2	CP027626	61	21.7	25,730,386	2552
Lu3	CP027627	67	24.1	26,636,119	3476
Lu4	CP027628	43	18.3	19,927,942	2936
Lu5	CP027629	42	16.5	17,699,757	2436
Lu6	CP027630	42	17.0	18,078,158	2732
Lu7	CP027631	53	16.6	18,299,719	2315
Lu8	CP027632	73	21.1	23,785,339	3181
Lu9	CP027633	70	19.4	22,091,576	2527
Lu10	CP027620	32	17.4	18,203,127	2192
Lu11	CP027621	38	18.3	19,887,771	3035
Lu12	CP027622	41	18.2	20,889,232	2885
Lu13	CP027623	48	19.3	20,483,506	2714
Lu14	CP027624	55	17.5	19,392,306	2938
Lu15	CP027625	41	13.9	15,636,771	2287
Total		772	284.5	316,167,078	42,277

Source: Adapted from You et al. (2018c)

9.2.5 *Flax Genome Evolution*

The genus *Linum* belongs to the Linaceae family and comprises approximately 200 diploid species ($2n = 2x$) that have a broad range of chromosome numbers ($x = 7$, 8, 9, 10, 12, 13, 14, 15, 16, 18, 19, 21, 30, 32, 36, 42, 43) (Goldblatt 2007; Rice et al. 2014). Cultivated (*L. usitatissimum*) flax possesses the same haploid chromosome number of 15 as its wild progenitor called pale flax (*L. bienne* Mill.). *L. usitatissimum* ($x = 15$), *L. bienne* ($x = 15$), and *L. grandiflorum* ($x = 8$) belong to the same clade in the phylogenetic tree and share a common ancestor with $n = 8$ (Fu et al. 2016; McDill et al. 2009; Sveinsson et al. 2014; You et al. 2018c). The variable chromosome number implies chromosomal duplication events in the speciation events that took place within the *Linum* genus (Ray 1944; Rogers 1982). A recent study also indicates that all angiosperms may have undergone at least two rounds of ancient whole-genome duplication (WGD) (Jiao et al. 2011). Evidence in the lineage of cultivated flax corroborates the occurrence of two WGD events. The first polyploidization event may have occurred 20–40 (Sveinsson et al. 2014) or 23–44 million years ago (MYA) (You et al. 2018c), while the most recent WGS was estimated at 3.7–9 million years ago (MYA) (Wang et al. 2012; You et al. 2018c).

9.3 Molecular Markers

Molecular markers are a genomic tool for characterizing genotypes or target organisms. They have been intensively used for genetic variation evaluation and diversity studies of populations, genetic map construction, QTL mapping and association studies, marker-assisted selection (MAS) and genomic selection (GS). The success of a useful marker system depends on several factors, such as abundance, ease of design, high throughput (automation), identification ability of phenotype (dominance/codominance), and cost. Various types of molecular markers have been developed and used in flax, such as RFLP, RAPD, AFLP, sequence-specific amplified polymorphism (SSAP), inter simple sequence repeat (ISSR), SSR or microsatellite and SNP. SSRs and SNPs are the two predominant marker systems used in flax and other plants.

Some early generation markers including RFLP, RAPD and AFLP were developed in flax mainly to construct genetic maps (Cullis et al. 1995; Oh et al. 2000; Spielmeyer et al. 1998a). However, RAPD markers (Bolsheva et al. 2015; Diederichsen and Fu 2006; El-Nasr and Mahfouze 2013; Fu 2005, 2006; Fu et al. 2002, 2003a, b; Muravenko et al. 2003, 2009, 2010; Ottai et al. 2012; Stegnii et al. 2000; Wiesner and Wiesnerovà 2004; Yurkevich et al. 2013), ISSR markers (Pali et al. 2015; Rajwade et al. 2010; Uysal et al. 2012; Wiesner and Wiesnerovà 2004) and retrotransposon-based SSAP markers (Melnikova et al. 2014) were also used in genetic diversity studies in *Linum*.

9.3.1 Simple Sequence Repeats

An SSR marker represents a locus that harbors a variable number of short tandem repeats that produce amplicons of different lengths. Because of their frequent codominance, genome-wide distribution, multi-allelism, heritability, polymorphism and robustness (Powell et al. 1996), SSRs are a valuable tool for genetic mapping and studies of genetic diversity (Hwang et al. 2009). Owing to the availability of large-scale genome-wide sequences such as BESs (Ragupathy et al. 2011), expressed sequence tags (ESTs) (Venglat et al. 2011), and the subsequent flax genome sequence (Wang et al. 2012), a large number of SSR markers have been developed from these genomic resources (Cloutier et al. 2009, 2012a; Deng et al. 2010). For example, 416 SSR assays derived from ESTs detected 447 loci (Cloutier et al. 2009, 2012a; Soto-Cerda et al. 2011b) and 733 SSR assays mined from genomic sequences detected 786 loci (Cloutier et al. 2012a; Soto-Cerda et al. 2011a). Additional SSR markers were recently developed from new genome sequencing (Choudhary et al. 2017; Wu et al. 2016). These markers have been used for consensus genetic map construction (Cloutier et al. 2012b), QTL mapping (Cloutier et al. 2011; Kumar et al. 2015; Soto-Cerda et al. 2013b, 2014), and genetic diversity evaluation of germplasm collections (Choudhary et al. 2017; Soto-Cerda et al. 2012, 2013a).

9.3.2 Single Nucleotide Polymorphisms

SNPs are the most popular DNA markers in genomics studies and breeding applications because of their abundance in most species through the massively parallel next-generation sequencing (NGS) and high-throughput genotyping technologies. One of the popular high-throughput SNP genotyping platforms is the Illumina's Infinium SNP oligonucleotide assay, which can simultaneously interrogate between 3000 and 1 million SNPs. Kompetitive allele specific PCR (KASP, LGC Genomics) is another scalable and flexible genotyping system that is useful for genetic mapping and marker-assisted breeding. NGS based GBS is also a popular genotyping method, owing its popularity to the ever-decreasing cost of sequencing.

The first sets of SNPs in flax were discovered using 454 pyrosequencing combined with genomic reduction (Fu and Peterson 2012) and Illumina sequencing of reduced representation libraries (Kumar et al. 2012). In the first case, 1067 SNPs were identified from 16 cultivated and wild accessions (Fu and Peterson 2012) and, in the second, 55,465 SNPs culled from eight genotypes of cultivated flax (Kumar et al. 2012). Additional SNPs were recently identified from biparental populations, including 17,288 SNPs from 260 individuals of one DH and two RIL populations (You et al. 2018b), 4497 SNPs from two RIL populations (Zhang et al. 2018), and 7399 and 5505 SNP from two RIL populations (Zhang et al. 2018). F_2 populations were also used to identify SNPs. A total of 260,380 SNPs from 100 F_2 individuals

(Yi et al. 2017) and 23,115 SNPs from 112 F_2 plants using GBS and specific length amplified fragment sequencing (SLAF-seq) library construction (Wu et al. 2018) were detected. The purpose of SNP discovery in these studies was to construct genetic maps and find QTL associated with traits of importance. Using the same GBS approach with SLAF library construction, Xie et al. (2018b) reported 584,987 SNPs from a diverse panel of 224 fiber and linseed cultivars. These SNPs were used to identify QTL of agronomic traits using genome-wide association study (GWAS). A much larger number of SNPs (1,775,461) were discovered from the resequencing effort of a flax core collection of 407 diverse genotypes (You et al. unpublished). A haplotype map based generated on this diverse genetic panel was used to identify QTL related to pasmo resistance (He et al. 2019b) as well as seed mucilage and hull content (Soto-Cerda et al. 2018).

9.3.3 Quantitative Trait Loci

A quantitative trait locus represents a chromosomal location associated with a percentage of the phenotypic variation of a quantitative trait in a population. The definition of a QTL region depends on the genetic variation, the size of the population, and the marker density. QTL can be identified using traditional QTL mapping approaches based on recombination-based genetic linkage maps constructed from biparental populations such as F_2, RIL, DH and backcross (BC) populations (Asgarinia et al. 2013; Cloutier et al. 2011; Fu 2011; Kumar et al. 2015; Soto-Cerda et al. 2012; Wiesner and Wiesnerovà 2004). Low representation of allelic diversity and finite genetic recombination limit the QTL dissection power of biparental population (Bandillo et al. 2013); hence, attempts have been made to expand the genetic diversity through more diverse populations, such as natural populations that possess tremendous phenotypic and genetic diversity (Huang et al. 2010), nested association mapping (NAM) populations (Yu et al. 2008) and multiparent advanced generation intercross (MAGIC) populations (Cavanagh et al. 2008; Mackay and Powell 2007). These populations incorporate diversity while retaining the advantages of biparental populations and, they hold several advantages in GWAS. Association mapping (AM) overcomes the phenotypic diversity limitation of biparental populations and thereby increases the QTL mapping power (Meng et al. 2016) by using a diverse germplasm panel and taking advantage of ancient genetic recombination events in the population (Huang and Han 2014).

To date, a total of 267 major QTL for 29 traits including 11 seed yield and agronomic traits, 11 seed quality traits, four fiber traits and three disease resistance traits have been identified in flax in 13 studies (Table 9.5). These QTL were mapped mainly by AFLP, SSR or SNP markers. A total of 67 QTL (SNP markers) for pasmo resistance (He et al. 2019b) were directly mapped on the recently released chromosome-scale pseudomolecules (You et al. 2018c). Another 200 QTL were mapped based on different references, including genetic maps (Asgarinia et al. 2013; Cloutier et al. 2011; Kumar et al. 2015; Soto-Cerda et al. 2013b; Spielmeyer et al. 1998a; Wu et al.

2018; Zhang et al. 2018), scaffold sequences (Wang et al. 2012; Xie et al. 2018a, b) or the pre-released version of the chromosome-scale pseudomolecules (Soto-Cerda et al. 2018; You et al. 2018b). All of these QTL/markers have now been mapped onto the recently released chromosome-scale pseudomolecules, rendering them comparable and thereby enhancing their usefulness for genome-wide QTL analyses, candidate gene predictions, and breeding applications (You and Cloutier 2019).

QTL are identified primarily by two types of statistical models: single locus models that perform one-dimensional genome scans by testing one marker at a time, and multi-locus models that simultaneously test multiple markers. The general linear model (GLM) and mixed linear model (MLM), the two main single-locus methods, tend to identify large-effect quantitative trait nucleotides (QTNs) or QTL while multi-loci models, such as the multi-locus random-SNP-effect mixed linear model (mrMLM) (Li et al. 2017; Wang et al. 2016), are able to detect QTNs/QTL with smaller effects (He et al. 2019b). A total of 500 QTL including 67 major QTL were identified using four single-locus and six multi-locus models for QTL identification of pasmo resistance. The combined utilization of multiple statistical methods was specifically powerful to identify QTL with small effects for traits with a complex genetic base and low heritability.

9.4 Germplasm

9.4.1 Germplasm Collections and Conservation

Approximately 48,000 accessions of *Linum* germplasm are preserved *ex situ* in 33 gene banks in 23 countries (Diederichsen 2007). In this germplasm, approximately 46,500 accessions are cultivated flax (*L. usitatissimum*) and close to 900 accessions belong to 53 *Linum* species other than *L. usitatissimum*. Of these, 279 accessions are *L. bienne* Mill, the wild progenitor of cultivated flax. Most accessions are stored in Russia (11,773), China (2680), Canada (3325), Ethiopia (3110), the USA (3017), Romania (2880) and the Czech Republic (2011) (Appendix 1). Many accessions are duplicated and, only approximately 10,000 accessions are deemed unique (Diederichsen 2007).

9.4.2 Germplasm Evaluation

Genetic resources serve as the basis for introduction of new genes to improve flax cultivars. There is a need to evaluate the diversity from various global sources to capitalize on yield potential, disease resistance and other important gene sources. Flax genetic resources have been evaluated at different levels. Phenotypic assessment in fields, greenhouses, or laboratories for different traits is an initial but critical step for parental selection and for crossing in breeding (Diederichsen 2001;

Table 9.5 QTL identification in flax

Population type	Pop. size	Markers	ENVs	Method	Statistical model	No. of QTL identified/trait	References
DH	59	8 RFLPs, 213 AFLPs	1	QTL mapping	Interval mapping	2/FW	Spielmeyer et al. (1998a)
DH	78	113 SSRs, 5 SNPs, 4 genes	4	QTL mapping	CIM	2/LIO, LIN, IOD; 1/PAL; 2/SC	Cloutier et al. (2011)
F_3-F_4	300	143 SSRs	2	QTL mapping	CIM	3/PM	Asgarinia et al. (2013)
Core collection	390	464 SSRs	8	GWAS	MLM	5/TSW; 1/DTF; 2/PLH; 1/BSC; 2/LDG	Soto-Cerda et al. (2013b)
Core collection	390	460 SSRs	8	GWAS	MLM	1/OIL; 1/STE; 3/LIO; 3/LIN; 1/IOD	Soto-Cerda et al. (2014)
RIL	243	329 SNPs, 362 SSRs	8	QTL mapping	MIM	1/PAL; 3/STE; 3/OLE; 2/LIO; 1/LIN; 2/IOD; 1/OIL; 1/PRO; 1/CEW; 1/STW; 1/TSW; 1/SEB; 1/YLD; 1/DTM	Kumar et al. (2015)
RIL(2) populations	233	4497 SNPs	2	QTL mapping	CIM	14/PLH; 9/TL	Zhang et al. (2018)
F_2	112	2339 SNPs	1	QTL mapping	CIM	1/PLH; 1/TL; 3/YLD; 3/STW; 2/FY; 2/FC	Wu et al. (2018)
Flax collection	224	146,959 SNPs	3	GWAS	GLM, MLM	9/PLH; 3/TL; 13/NB; 8/FN; 10/TSW	Xie et al. (2018a)
Flax collection	224	584,987 SNPs	1	GWAS	GLM, EMMAX	2/PLH; 1/FN; 8/TSW; 1/PAL; 2/STE; 1/LIO; 3/LIN; 2/FC	Xie et al. (2018b)
Flax core collection	200	771,914 SNPs	2	GWAS	GLM, MLM	7/MC; 4/HC	Soto-Cerda et al. (2018)
RIL(2) and DH(1)	260	17,288 SNPs	6–8	GWAS	GLM, MLM	1/YLD; 8/OIL; 5/PLH; 4/PAL; 3/IOD, LIN, LIO, 2/DTM, 2/STE; 1/PRO, OLE	You et al. (2018b)
Flax core collection	370	258,873 SNPs	5	GWAS	3 single-locus and 7 multi-locus models	67/PAS	He et al. (2019b)

(continued)

Table 9.5 (continued)

DH doubled haploid, *RIL* recombinant inbred line, *Pop. size* population size, *AFLP* amplified fragment length polymorphism, *RFLP* restriction fragment length polymorphism, *SNP* single nucleotide polymorphism, *SSR* simple sequence repeat, *ENVs* the number of years and/or locations for phenotyping, *GWAS* genome-wide association study, *QTL* quantitative trait loci, *CIM* composite interval mapping, *EMMAX* efficient mixed-model association expedited, *GLM* general linear model, *MIM* multiple interval mapping, *BSC* branching score, *CEW* cell walls (%), *DTF* days to flowering, *DTM* days to maturity, *FC* fiber content (%), *FIB* fiber (%), *FN* fruit number, *FW Fusarium* wilt, *FY* fiber yield, *HC* seed hull content, *IOD* iodine value, *LDG* lodging, *LIG* lignin (%), *LIN* linolenic acid content (%), *LIO* linoleic acid content (%), *MC* seed mucilage content, *MIL* powdery mildew rating, *NB* number of branches, *OIL* oil content (%), *OLE* oleic (%), *PAL* palmitic (%), *PAS* pasmo rating, *PLH* plant height (cm), *PM* powdery mildew rating, *PRO* protein content (%), *SC* seed color, *SEB* seeds per boll, *STE* stearic acid content (%), *STR* straw weight (g), *TL* technical length (cm), *TSW* 1000-seed weight (g), *YLD* seed yield

Diederichsen and Raney 2006; Diederichsen et al. 2006; Kulpa and Danert 1962; Nozkova et al. 2006; You et al. 2017). Phenotyping of important traits for diverse genetic panels is also a prerequisite for identifying QTL and constructing GS models used in molecular breeding.

The Canadian national plant germplasm repository, Plant Gene Resources of Canada (PGRC), preserves more than 3000 accessions of cultivated flax (*Linum usitatissimum* L.) and 76 accessions of other *Linum* species, originating from 72 countries and representing all historical and present cultivation regions for linseed or fiber production (Diederichsen and Fu 2008). In the last two decades, more than 90% of these accessions have been evaluated for their morphological and agronomic characters, such as plant height, seed color, seed weight, seed oil content, fatty acid composition, seed coat, mucilage content, fiber content, adaptation to dry growing conditions, disease resistance, seed vigor and seed lignin content. (Diederichsen 2001; Diederichsen and Fu 2006, 2008; Diederichsen and Raney 2006; Diederichsen and Ulrich 2009; Diederichsen et al. 2006). The wide variation in agrobotanical characteristics of this germplasm is useful for flax breeding (Diederichsen 2001). The weak correlation between fiber content and other agronomic traits demonstrates that the variation in fiber content of flax germplasm is valuable for identifying germplasm relevant for breeding dual-purpose flax (Diederichsen and Ulrich 2009).

To meet breeding needs, a core collection comprising 381 worldwide accessions was assembled from the Canadian collection (Diederichsen et al. 2013). Twenty-six additional breeding lines and cultivars from Canadian flax breeding programs have since been added to this subset, resulting in a current core collection of 407 flax accessions (Soto-Cerda et al. 2013a; You et al. 2017). This core collection preserves the major variation present in the Canadian flax collection of different geographical origins (38 countries), both morphotypes (fiber, linseed), and varying improvement statuses including landraces, breeding lines and cultivars (Diederichsen et al. 2013), and also includes many core ancestors of currently registered cultivars (You et al. 2016b). Twenty-seven important traits such as seed yield, seed oil and protein contents, fatty acid composition, fiber traits and disease resistance have been evaluated at multiple locations over several years (Fig. 9.2 and Table 9.6) (You et al. 2017).

Fig. 9.2 Flax germplasm field evaluation in Morden, Manitoba, Canada

The diverse phenotypic and genetic variabilities of this core collection render it a useful resource for expanding the genetic base of future flax cultivars and dissecting the genetic architecture of traits for future molecular breeding applications.

Genetic diversity evaluation of flax genetic resources has been widely performed at the molecular level. RAPD markers have been used to evaluate the genetic variations and geographic patterns of more than 2700 resampled cultivated flax accessions and cultivars (Fu 2005, 2006; Fu et al. 2002, 2003a, b). The core collection and 125 pale flax accessions were characterized using 448 SSR markers (Soto-Cerda et al. 2013a). These large-scale assessments for cultivated and wild flax germplasm demonstrate the value of genetic variation for breeding. These variations exist largely within geographical regions.

Genome-wide molecular markers are useful for the overall evaluation of the genetic diversity of a population, but identification of all potential QTL with both major and minor effects provides a way to screen individual germplasm for specific traits. A total of 500 QTL, including 67 stable and large-effect QTL and, many additional small effect and environment-specific QTL for pasmo severity, were identified from the core collection (He et al. 2019b). The number of QTL with positive-effect or favorable alleles (NPQTL) in each accession was highly correlated with the observed pasmo severity ($R^2 = 0.73$) (Fig. 9.3), indicating that the NPQTL of an accession is a good indicator for evaluation and screening of accessions for pasmo severity. The top ten most pasmo-resistant accessions listed in Table 9.7 reveals that highly resistant or immune germplasm in the core collection is rare and that some levels of disease incidence was always observed even in the most resistant germplasm.

Table 9.6 Phenotypic evaluation of 27 agronomic, seed quality, fiber, and disease resistance traits of the flax core collection

Trait group	Trait	$\bar{x} \pm s$	Range	$\widehat{GCV}$	$\hat{H}^2$
Agronomic	Seed yield (t·ha^{-1})	0.78 ± 0.27	0.07–1.46	17.90	0.41
	Seeds (boll^{-1})	6.10 ± 0.88	2.80–8.12	12.23	0.31
	Seeds (m^{-2})	10,804 ± 3039	2638–27,714	23.45	0.35
	1000-seed weight (g)	5.36 ± 0.86	2.90–8.42	14.01	0.77
	Bolls (m^{-2})	1763.33 ± 386.55	736.32–3821.00	17.22	0.32
	Lodging	1.36 ± 0.40	0.87–3.32	25.74	0.14
	Days to flowering	51.13 ± 3.07	45.53–70.74	5.11	0.66
	Days to maturity	97.40 ± 3.85	88.06–110.7	3.77	0.21
	Plant height (cm)	51.15 ± 12.81	23.00–95.49	24.63	0.59
	Branching score	3.50 ± 1.41	1.00–6.00	38.85	–
Seed quality	Protein content (%)	26.88 ± 1.80	16.73–31.3	5.30	0.72
	Oil content (%)	42.13 ± 1.88	37.22–50.59	13.17	0.64
	Iodine value	186.10 ± 7.02	145.41–202.85	3.70	0.80
	Palmitic (%)	5.48 ± 0.60	3.30–8.45	10.92	0.82
	Stearic (%)	4.33 ± 1.01	2.33–9.4	21.67	0.85
	Oleic (%)	21.21 ± 3.03	13.82–37.97	13.13	0.77
	Linoleic (%)	14.22 ± 4.15	6.84–68.43	29.01	0.93
	Linolenic (%)	54.75 ± 4.70	5.02–66.07	8.32	0.66
Fiber	Straw weight (g)	23.80 ± 12.56	4.58–75.01	39.58	0.65
	Fiber (%)	38.50 ± 1.99	34.47–46.52	5.14	0.33
	Lignin (%)	9.48 ± 0.29	8.32–10.08	3.02	0.32
	Shive (%)	62.08 ± 2.01	53.98–66.11	3.11	0.33
	Cell walls (%)	79.17 ± 0.96	75.86–82.01	1.07	0.17
	Cellulose (%)	60.14 ± 1.99	54.21–65.21	3.22	0.31
Disease resistance	Pasmo rating	3.40 ± 0.96	1.35–7.15	24.24	0.25
	Powdery mildew rating	4.13 ± 1.46	1.36–8.45	29.49	0.52
	Fusarium wilt rating	7.77 ± 1.37	4.00–10.1	13.71	0.60

Source: You et al. (2017)
$\bar{x}$ population mean, s standard deviation, $\widehat{GCV}$ genetic coefficient of variation, $\hat{H}^2$ broad-sense heritability

9.5 Disease Resistance

Rust, *Fusarium* wilt, pasmo and powdery mildew are the four major diseases afflicting flax in Canada. Though rust and *Fusarium* wilt were historically major factors limiting flax production, these two diseases have been controlled by genetic improvement of flax cultivars. Powdery mildew is a common and widespread foliar disease of flax in most flax-growing areas of the world and most Canadian commercial cultivars are moderately resistant. Pasmo is a widespread disease in the Western Canadian prairies but no genetic resistance exists in commercial flax cultivars. Maintaining and improving genetic resistance to rust, wilt, powdery mildew and pasmo are objectives in developing new cultivars.

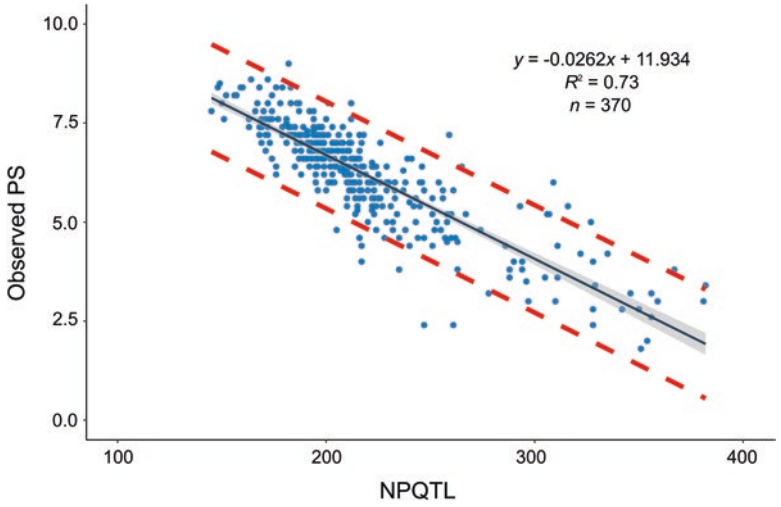

Fig. 9.3 Relationship of observed pasmo severity (PS) (y) with the number of QTL with positive-effect alleles (NPQTL) (x) in 370 flax accessions. The grey band represents the 95% confidence interval. The red dashed lines represent the upper and lower boundaries of the 95% prediction interval. (Source: He et al. 2019a)

Table 9.7 Ten accessions most resistant to pasmo identified from the core flax collection according to the number of QTL with positive-effect alleles (NPQTL) per accession

Accession	Origin	Morphotype	NPQTL	Pasmo severity
CN40081	Netherlands	Fiber	382	3.4
CN33390	Netherlands	Fiber	381	3.0
CN101053	China	Fiber	359	3.0
CN100929	Netherlands	Fiber	356	2.6
CN18982	France	Fiber	356	3.2
CN19001	Netherlands	Fiber	354	2.0
CN101367	Georgia	Linseed	351	1.8
CN18983	Netherlands	Fiber	350	2.8
CN101298	Russia	Linseed	342	2.8
CN100939	Russia	Linseed	328	2.4

9.5.1 Rust

Rust, a fungal disease, caused by *Melampsora lini* that overwinters through teliospores on flax debris. Early infections produce aeciospores on flax seedlings that subsequently advance to urediospores that can cycle through several generations during the growing season. The sexual life cycle of flax rust can be finished on the flax plant, and does not require an alternate host.

Flax rust is characterized by bright orange and powdery pustules, called uredia (Fig. 9.4). Uredia develop mostly on leaves (Fig. 9.4a), but can also be seen on

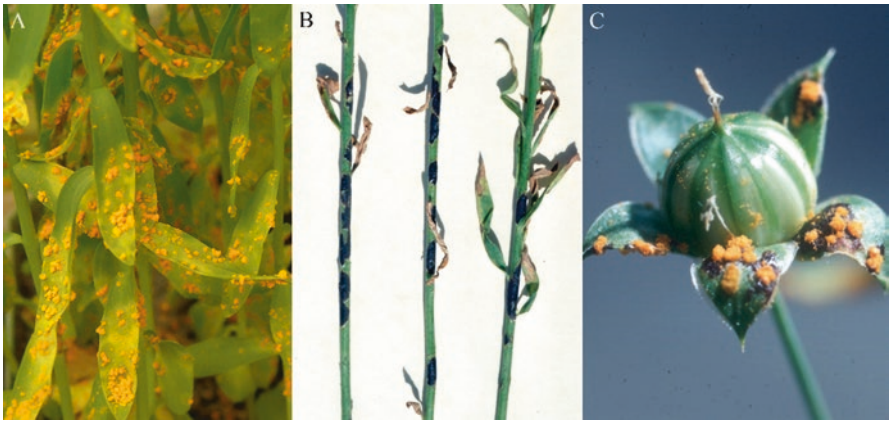

Fig. 9.4 Flax rust: (**a**) Uredia on leaves, (**b**) Telia on stems, and (**c**) Uredia on a boll

stems (Fig. 9.4b) and bolls (Fig. 9.4c). The orange uredia turn black and produce overwintering telia and teliospores during the season. The black telia are most common on stems (Fig. 9.4b). The uredia produce numerous airborne urediospores that cause new cycles of infection during the season. High humidity during cool nights, warm day temperatures and vigorously-growing plants facilitate infection and spread of the disease. Rust can result in completely defoliated plants and reduction of seed yield and fiber quality.

Rust could potentially be the most destructive flax disease. The frequent development of rust races has been a major challenge of breeding new resistant cultivars. The last major rust epidemic in Canada occurred in the 1970s when a new group of races, including races 300, 370 and 371, was identified (Hoes and Tyson 1963; Hoes and Zimmer 1976; Zimmer and Hoes 1974). More than 500 flax rust races have been identified over the last 70 years, mostly by Dr. Harold Henry Flor, a research scientist at the United States Department of Agriculture (USDA) (Flor 1956), and by oilseed crop pathologists at Agriculture and Agri-Food Canada (AAFC). All registered flax cultivars developed in North America (USA and Canada) are currently immune to local races of rust (Fig. 9.5). In Canada, genetic improvement has succeeded in delivering immunity to all Canadian modern cultivars, including to rust race 371 which is the most recently identified race in Western Canada. Rust remains a constant threat to flax production worldwide because the fungus evolves to produce new races that can attack resistant cultivars (Rashid unpublished).

The types of pustules developed on a host cultivar following inoculation with a race of rust is indicative of the reaction of that cultivar to the race and of the pathogenicity of that race to the cultivar. Rust races and virulence genes are identified by their reactions on a set of cultivars termed *rust differentials* carrying resistance genes to different rust races (Flor 1956). Table 9.8 lists some reactions of selected flax rust races to a host differential set of 30 single-gene inbred lines. Inheritance of rust resistance in flax is primarily dominant although dominance with some genes is not complete, whereas inheritance of virulence in *Melampsora lini* is recessive (Flor

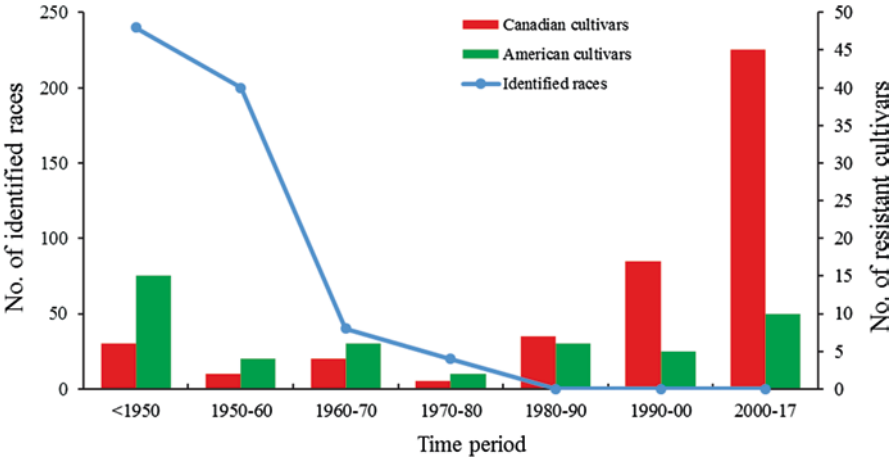

Fig. 9.5 Number of new flax rust races identified and the number of resistant flax cultivars developed in the USA and Canada from <1950–2017

1956). Flax rust is genetically controlled by a few major genes (Rashid and Kenaschuk 1994). Several gene families (K, L, M, N and P) have been identified, of which *L*, *M*, *N* and *P* genes have been cloned (Table 9.9).

9.5.2 Fusarium Wilt

Flax wilt or *Fusarium* wilt is a soil-borne fungus disease caused by *Fusarium oxysporum* f. sp. *lini*. The fungus persists in the soil because mycelia and spores can survive for many years in flax debris and other organic matter. Wind-blown soil and water runoff may spread the fungus from one field to another. The fungus invades plants through the roots at any growth stages and spreads inside their water-conducting tissues, thereby interfering with water uptake, a phenomenon exacerbated by warm weather and, consequently being exacerbated by warm weather. Early infections may kill flax seedlings (Fig. 9.6), while delayed infections cause yellowing and wilting of leaves followed by browning and death of the plants. Plants are infected more commonly in patches.

Resistance to flax wilt is genetically controlled by some major genes and polygenes (Spielmeyer et al. 1998b). Two independent genes with additive effects were identified to confer wilt resistance in 143 DH lines derived from a cross between the wilt-resistant cv. Linola and the wilt-susceptible Australian flax cv. Glenelg under greenhouse and field conditions (Spielmeyer et al. 1998b). Two independent and recessive genes were also identified from a RIL population developed by crossing

Table 9.8 Reactions of selected flax rust races to a host differential set of 30 single-gene inbred lines

		Race									
Inbred line or cultivar	Locus	22	X36	41	79	97	191	218	218S61	258	371
B^{12} × CI 1188	*K*	+	−	−	+	−	+	−	−	−	−
Raja	*K1*	+	−	−	+	−	−	−	−	−	−
B^{6} × Kugler C	*Ku*	+	+								−
B^{12} × CI 355	*L*	+	−	−	−	−	−	−	−	−	+
B^{7} × CI 1180	*L1*	+	+	+	+	+	−	−	−	−	−/+
B^{12} × CI 708	*L2*	−	−	−	−	−	−	−	−	−/+	−
B^{8} × CI 647	*L3*	+	−	−	+	−	−	−	−	−	−
B^{12} × CI 709	*L4*	+	−	−	+	−	−	−	−	−	−
B^{6} × CI 1193	*L5*	+	+	−	+	+	+	+	+	−	−
B^{12} × CI 1085	*L6*	+	+	−	+	−	+	+	+	−	−
B^{6} × CI 1190	*L7*	+	+	+	+	+	+	+	+	−	−
B^{12} × CI 1336	*L8*	+	+	−	−	−	−	−	+	−	−
Bison	*L9*	+	+	+	+	+	+	+	+	+	+
B^{6} × CI 1183	*L10*	+	+	−	+	−	−	+	−	−	−
Linore	*L11*	+	+	−	−	−	−	+	+	−	−
B^{12} × CI 1071	*M*	+	+	−	−	−	−	−	+	+	+
B^{12} × CI 803	*M1*	+	+	+	+	+	+	+	+	+	+
B^{14} × CI 1181	*M2*	+	+	−	+	+	−/+	+	+	+	−/+
B^{12} × CI 1182	*M3*	+	−	−	−	+	+	−	+	−	−
B^{12} × CI 1170	*M4*	+	+	−	+	+	+	+	+	+	+
B^{8} × CI 1512	*M5*	+	−	−	−	+	−	−	−	−	−
CI 2008	*M6*	+	+	−	−	−	−	−	−	−	−/+
B^{12} × CI 42	*N*	−	−	−	−	−	−	−	−	−	+
B^{12} × CI 1191	*N1*	+	+	−	+	+	+	−	+	−	+
B^{8} × CI 1335	*N2*	+	+	−	−	+	−	−	+	−	−
B^{12} × CI 842	*P*	+	+	−	+	+	−	−	−	+	+
B^{10} × CI 515	*P1*	+	+	−	−	+	+	+	+	−	−
B^{4} × CI 701	*P2*	+	−	−	−	+	−	−	−	−	−
B^{13} × CI 836	*P3*	+	+	−	−	+	−	−	−	−	−
CI 1911	*P4*	−	−	−	−	−	−	−	−	−	−

(+) compatible susceptible reaction. (−) non-compatible resistant reaction. (−/+) mixed reaction due either to segregating flax line or a mixture of the rust race. B^{x}: a cultivar with a rust resistant allele has been backcrossed into Bison X times. CI 1188: Clay; CI 355: Ottawa 770b; CI 1180: Burke; CI 708: Stewart; CI 647: Pale Blue Crimped; CI 709: Kenya; CI 1193: Wilden; CI 1085: Birio; CI 1190: Barnes; CI 1336: Bisbee; CI 1183: Bolly Golden Sel; CI 1071: Dakota; CI 803: Williston Brown; CI 1181: Ward; CI 1182: Cass; CI 1170: Victory; CI 1512: Cortland; CI 42: Bombay; CI 1191: Polk; CI 1335: Marshall; CI 842: Koto; CI 515: Akmolinsk ACC; CI 701: Abyssinian Brown; CI 836: Lenoa; CI 1911: Punjab

Table 9.9 Cloned flax rust genes

Gene and alleles		Accession for nucleotides	Accession for proteins	Lines	References
L	*L*	AF093638	AAD25965	Ottawa 770B	Ellis et al. (1999)
	L1	AF093639	AAD25966	Burke	Ellis et al. (1999)
	L2	AF093642	AAD25969	Stewart	Ellis et al. (1999)
	L3	AF093643	AAD25970	Pale blue crimped	Ellis et al. (1999)
	L4	AF093644	AAD25971	Kenya	Ellis et al. (1999)
	L5	AF093645	AAD25972	Wilden	Ellis et al. (1999)
	L6	U27081	AAA91021, AAA91022	Birio	Lawrence et al. (1995)
	L7	AF093646	AAD25973	Barnes	Ellis et al. (1999)
	L8	AF093647	AAD25974	B^{14} × Towner	Ellis et al. (1999)
	L9	AF093648	AAD25975	Bison	Ellis et al. (1999)
	L10	AF093640	AAD25967	Bolley Golden Seln.	Ellis et al. (1999)
	L11	AF093641	AAD25968	Linore	Ellis et al. (1999)
	LH	AF093649	AAD25976	Hoshangabad	Ellis et al. (1999)
M	*M*	U73916	AAB47618	Dakota	Anderson et al. (1997)
	M1	GQ141888	ACS91452	Williston brown	Lawrence et al. (2010)
	M3	GQ141889	ACS91453	Cass	Lawrence et al. (2010)
	M-X39	U76370	AAB47619	F1 mutant of forge × Hoshangabad	Anderson et al. (1997)

(continued)

Table 9.9 (continued)

Gene and alleles		Accession for nucleotides	Accession for proteins	Lines	References
N	*N1-A*	AJ310152	CAC35327	Polk	Dodds et al. (2001a)
	N1-B	AJ310153	CAC35328	Polk	Dodds et al. (2001a)
	N1-C	AJ310154	CAC35329	Polk	Dodds et al. (2001a)
	N1-D	AJ310155	CAC35330	Polk	Dodds et al. (2001a)
	N2-A	AJ310156	CAC35331	Marshall	Dodds et al. (2001a)
	N2-B	AJ310157	CAC35332	Marshall	Dodds et al. (2001a)
	N2-C	AJ310158	CAC35333	Marshall	Dodds et al. (2001a)
	N2-D	AJ310159	CAC35334	Marshall	Dodds et al. (2001a)
	Ngc-A	AJ310150	CAC35323	Bombay	Dodds et al. (2001a)
	Ngc-B	AJ310150	CAC35325	Bombay	Dodds et al. (2001a)
	Ngc-D	AJ310150	CAC35321	Bombay	Dodds et al. (2001a)
	Ngc-C	AJ310151	CAC35326	Bombay	Dodds et al. (2001a)
P	*P1-A*	AF310958	AAK28803	Akmolinsk	Dodds et al. (2001b)
	P1-B	AF310959	AAK28804	Akmolinsk	Dodds et al. (2001b)
	P2-A	AF310960	AAK28805	Abyssinian	Dodds et al. (2001b)
		AF310960	AAK28806	Abyssinian	Dodds et al. (2001b)
	P3-A	AF310961	AAK28808	Leona	Dodds et al. (2001b)
	P3-B	AF310962	AAK28809	Leona	Dodds et al. (2001b)
	P4-B	AF310964	AAK28810	CI 1911 (Punjab)	Dodds et al. (2001b)
	P	AF310966	AAK28811	Koto	Dodds et al. (2001b)

Source: Partially from Islam and Mayo (1990)

Fig. 9.6 Flax fusarium wilt disease: (**a**) Early, and (**b**) Partial wilt symptoms

cv. Aurore (wilt resistant) and cv. Oliver (wilt susceptible) (Edirisinghe 2016). Germplasm evaluation for susceptibility to flax wilt showed that germplasm from North America and East Asia had above-average resistance compared to accessions from the Indian subcontinent and Europe (Diederichsen and Fu 2008). None of the existing Canadian flax cultivars show resistance to all isolates of *Fusarium oxysporum* f. sp. *lini* collected in Western Canada, and resistance has broken down on occasion (Mpofu and Rashid 2001). Despite the large variability observed in the flax core collection of 407 accessions, only a few accessions are highly resistant to Fusarium wilt (Table 9.6) (You et al. 2017). All commercial flax cultivars in Canada are resistant to moderately resistant (You et al. 2016b). Genetic improvement remains the best strategy for resistance to flax wilt because alternative control methods are challenging with soil-borne diseases.

9.5.3 Pasmo

Pasmo is caused by *Septoria linicola*, a fungus that attacks above-ground parts of the flax plant and overwinters in the soil on infected flax stubble. Infected flax is characterized by circular brown lesions on leaves (Fig. 9.7a) and brown to black infected bands alternating with green healthy bands on stems (Fig. 9.7b). Infected tissues contain tiny black pycnidia. Debris carry numerous pycnidia that overwinter and produce masses of spores that cause the initial infections on leaves and stems. Spores are subsequently dispersed by rain and wind. Flax is infected by this pathogen from the seedling to maturity. High moisture and temperature promote the disease. Lodging facilitates the development of pasmo owing to increased humidity within the flax canopy, which may result in patches of dead plants completely covered with the fungus (Vera et al. 2014). Pasmo can weaken the pedicels, cause defoliation, and premature ripening, resulting in heavy boll-drop under rain and wind

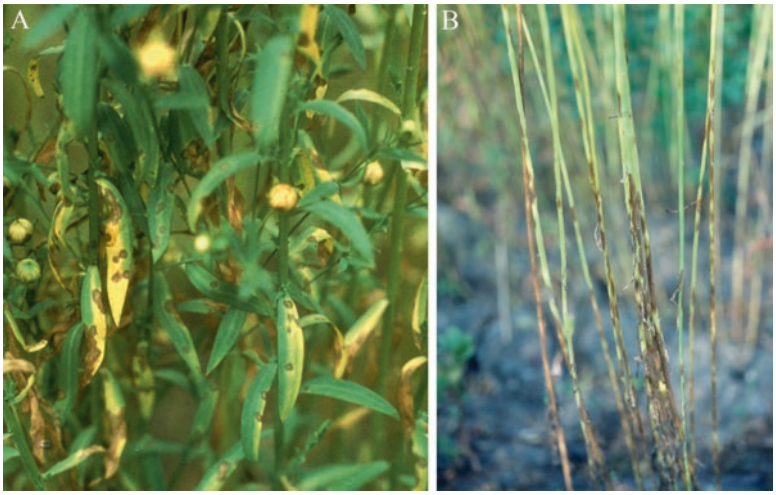

Fig. 9.7 Flax pasmo disease on (**a**) Leaves, and, (**b**) Stems

conditions. Depending on the earliness and severity of the infection, pasmo reduces seed yield as well as seed and fiber quality.

Resistance to pasmo is characterized by low broad-sense heritability (0.25) and a strong genotype × environment interaction (Table 9.6) (You et al. 2017). Large variations in pasmo disease severity have been observed in the flax core collection but few highly resistant accessions were identified. A total of 500 QTL including 67 large-effect QTL with major additive effects were identified from the flax core collection (He et al. 2019b). Fiber flax is more resistant to pasmo than linseed accessions and, most major QTL exist in the fiber flax germplasm (He et al. 2019b; You et al. 2017). At present, no commercial flax cultivar with resistance to pasmo is available in Canada. However, researchers at the AAFC and at the Crop Development Centre (CDC) of the University of Saskatchewan have identified several genotypes with moderate resistance, under field conditions and, several genotypes with specific host genotype-fungal isolate resistance that can be capitalized on to develop resistant cultivars by pyramiding major genes to achieve good field resistance against natural pathogen populations with multiple pathotypes.

9.5.4 Powdery Mildew

Powdery mildew is caused by the fungus *Oidium lini* and little is known about the overwintering and host range of this fungus in Western Canada, where it was first reported in 1997 (Rashid et al. 1998). Since then, it has spread and, its incidence and severity have increased sharply in the Canadian provinces of Manitoba and Saskatchewan. Early infections may cause severe defoliation of the flax plant and eventually influence seed yield and quality. A white powdery mass of mycelia starts

Fig. 9.8 Effect of powdery mildew disease on susceptible cv. AC Linora (left) and resistant cv. Linda (right)

as small spots and rapidly spreads to cover the entire leaf surface (Fig. 9.8). Heavily infected leaves wither, dry up and die. Early infections may cause complete defoliation of flax plants.

Most Canadian flax cultivars are moderately resistant to powdery mildew under field conditions with natural inoculum (You et al. 2016b). Several major genes or QTL for resistance to powdery mildew have been identified. One dominant gene, designated *Pm1*, was identified from three Canadian cvs. (AC Watson, AC McDuff and, AC Emerson) and two introduced cvs. (Atalante and Linda). Two additional dominant genes were found in the cv. Linda (Rashid and Duguid 2005). Three powdery mildew resistance QTL were identified on LG1, 7, and 9 using phenotyping data from both field and growth chamber studies in F_3 and F_4 families derived from a cross between the susceptible cv. NorMan and the resistant cv. Linda. These QTL explained 97% of the phenotypic variation highlighting the contribution of mainly dominant genes (Asgarinia et al. 2013). Powdery mildew has a moderate heritability (0.52) (Table 9.6). On average, linseed accessions are more resistant to powdery mildew than fiber flax accessions. Similar to pasmo and *Fusarium* wilt, only a few accessions of the flax core collection are highly resistant to powdery mildew. Additional resistant germplasm is required for breeding and genetic studies of resistance to powdery mildew, as is also the case for pasmo and *Fusarium* wilt (You et al. 2017).

9.6 Linseed Breeding

Canadian flax cultivars are developed for the quantity and quality of their oil. Three major linseed breeding programs have developed linseed cultivars in Canada: (1) the AAFC program located at Morden Research and Development Centre in Morden, Manitoba; (2) the CDC program located at the University of Saskatchewan in Saskatoon, Saskatchewan and, (3) the Saskatoon R&D facility of Crop Production Services Canada Inc. (CPS), previously known as the Viterra program and, currently renamed as Nutrien Ag Solutions, located at Vegreville, Alberta (Duguid 2009; FCC 2015). Since 2000, CÉROM (Centre de recherche sur les grains), located in Saint-Mathieu-de-Beloeil, has maintained a breeding program targeting flax for crop diversification in the Province of Québec (FCC 2015). At present, the CDC program is the only remaining program conducting linseed breeding for Western Canada.

9.6.1 Breeding Objectives

The overall goals of linseed breeding are to maximize grain yield while maintaining high seed oil (>45%) and ALA (>50%) contents, increasing resistance to diseases and lodging, and shortening the time to maturity (Duguid 2009; FCC 2015; Hall et al. 2014; You et al. 2016b). As described in the previous section, the major diseases of concern include rust, *Fusarium* wilt, powdery mildew and pasmo. The area of flax production in the Canadian Prairies is limited by the risk of frost and is often subject to periods of water deficit. Expanding flax production northward to higher latitude regions can increase water availability, but the increased risk of frost remains. To adapt to shorter growing seasons and climate uncertainties, cultivars with early flowering and adapted to short growing seasons are needed.

9.6.2 Breeding Strategies and Methods

Flax breeding is a process involving the creation of genetic variability by parent selection and crossing, followed by selection of the best recombinants by phenotypic and genomic selection, and eventually the fixation of desirable traits by inbreeding to develop a new cultivar (Hall et al. 2014). Hybridization or crossbreeding is the predominant approach in Canadian linseed breeding programs. Most cultivars released in the last 35 years were developed by crossing two parents, even though three and four-way crosses were also used. Parental selection and crossing schemes are the first factors contributing to the success of conventional crossbreeding methods. To develop segregating populations, genetically diverse parents with complementary characteristics may be chosen. Two major offspring selection methods from segregating populations have been used in flax breeding: pedigree selec-

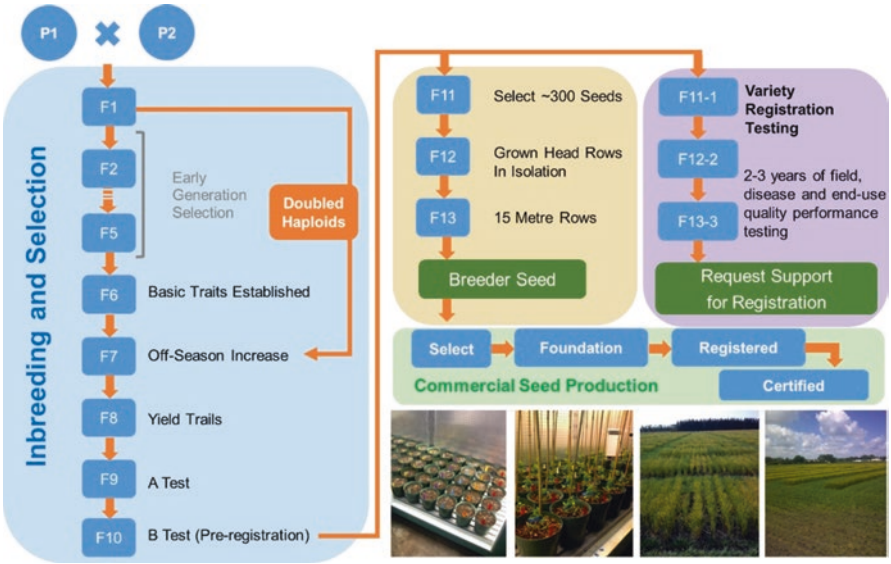

Fig. 9.9 Schematic of the AAFC flax breeding program, cultivar registration and commerical seed production in Canada

tion and doubled haploid (DH). The AAFC breeding program uses both methods, while the CDC program strictly adheres to the pedigree selection method. Figure 9.9 depicts the AAFC flax breeding program, Canadian cultivar registration system and the production of certified seed.

Generally speaking, development of a new flax cultivar from crossing of two parents to registration requires ten years, that is when the conventional pedigree method is combined with contra season nursery operations. Development of flax cv. AAC Bravo is described as a typical example in Table 9.10. Crossing and F_1 advancement in the winter nursery or greenhouse took one year. Advancement and early generation selection from F_2 to F_6 with two contra season nursery operations in Brawley, California, spanned the next three years. Two options for off-season increase are available (Fig. 9.10). Brawley, California, located at a latitude of 32.98°N and a longitude of 115.53°W, features irrigated conditions. Seeding usually takes place around the end of October or early November with harvesting the following April. Temuco, Chile is located at a latitude of 38.44°S and longitude of 72.40°W. In both locations seeding takes place at the end of October with harvesting in March of the following year. Starting from the F_7 generation, multi-location yield trials with seed quality and disease resistance tests are conducted for a period of 3 years: 1 year for yield trials (F_7), 1 year for preliminary yield trials (F_8) and one year for evaluation trials or A tests (F_9). An additional three years are needed for cooperative tests (F_{10}–F_{12}) (Table 9.10).

A doubled haploid (DH) is a genotype formed when haploid cells undergo chromosome doubling. Artificial production of doubled haploids from haploids produces fully homozygous lines in one generation, which can be tested directly in the field because they only possess additive variance. Therefore, application of the DH

Table 9.10 Breeding procedure of flax linseed cultivar AAC Bravo

Generation	Year/ season	Location	Operation
Flanders × Pacific	1995	Germany	Crossing
F_1	1995	Morden, Manitoba	Grown in greenhouse
F_2	2002	Morden, Manitoba	Grown in field; F_2 plants were selected.
F_3	2003	Morden, Manitoba	Row plots grown in the field; three single plants selected and harvested from each of the selected F_3 generation rows based on height, maturity, lodging resistance, and general appearance.
F_4	2003–2004	Brawley, California	Three hills grown in the contra season nursery; individual hills selected based on oil content, oil quality, and lodging resistance, and concurrently tested for resistance to flax rust using local race 371 in a growth room at Morden.
F_5	2004	Morden, Manitoba	Same as F_3.
F_6	2004–2005	Brawley, California	Same as F_4.
F_7	2005	Two sites in Manitoba and one in Saskatchewan	Bulk rows of selected hills grown; selection based on seed yield, height, maturity, lodging resistance, general appearance, preliminary seed quality analysis (i.e., oil and protein content, IOD, ALA), and resistance to flax rust.
M7970 (F_8)	2006	Two sites in Manitoba and three in Saskatchewan	Preliminary yield trial. One selected line, M7970, grown in replicated yield trials; same selection criteria as for F_7 plus resistance to wilt in the wilt nurseries at Morden, Manitoba and Indian Head, Saskatchewan.
M7970 (F_9)	2007	Three sites in Manitoba, three in Saskatchewan, and one in British Columbia	Canadian flax evaluation trial.
FP2270 (F_{10}-F_{12})	2008–2010	Multiple sites in different Western Canadian provinces	Flax cooperative test.
AAC Bravo	2010		Registered.

Source: Adapted from Duguid et al. (2014)
IOD iodine value, *ALA* α-linolenic acid

method can save two years or three generations through bypassing early generation selection (Fig. 9.11), thereby reducing the number of years for the development of a new cultivar from ten down to eight. DH lines have an added advantage in genomic selection whose prediction accuracy is greater in homozygous germplasm which would further improve selection efficiency (Fig. 9.11).

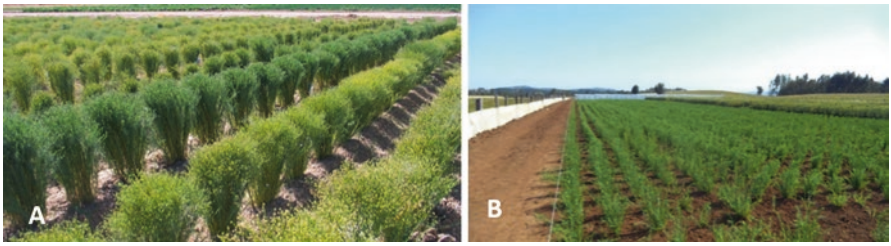

Fig. 9.10 Winter nursery operations of the AAFC flax breeding program in (**a**) Brawley, California, and, (**b**) Temuco, Chile

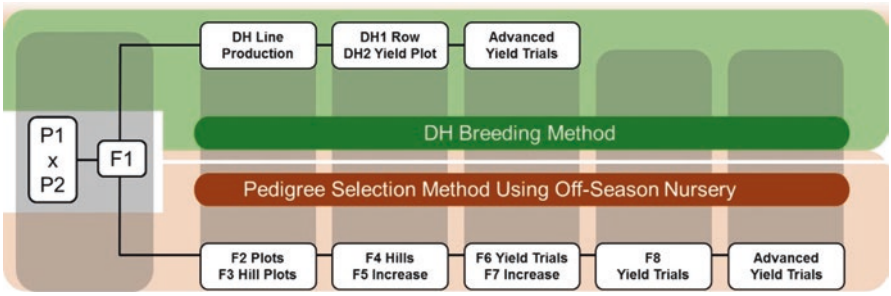

Fig. 9.11 Comparison of two breeding methods. The doubled haploid (DH) breeding method can save ~two years (or three generations) compared to the traditional pedigree selection method

Although the DH method has not yet produced a cultivar for release from the AAFC flax breeding program, this method has been successfully employed to produce mapping populations for genetic map construction and QTL mapping (Cloutier et al. 2011, 2012b; Spielmeyer et al. 1998a, b; You et al. 2018b).

In addition to the traditional breeding method based on crossing, mutation breeding, transgenic techniques and tissue culture have been applied to flax cultivar development in Canadian breeding programs. X-ray irradiation was used to improve cv. Redwood (Larter et al. 1965) to produce the higher yielding cv. Redwood 65. Mutation breeding was also used to develop low LIO cultivars in Australia and in Canada. Linola 947 cv. was the first one with low LIO (2.4%) (Dribnenki and Green 1995). It originates from Australian cv. Glenelg (Green and Marshall 1984; Rowland 1991). Transgenic methodology was exploited in the development of CDC Triffid to provide tolerance to sulfonylurea herbicides (McHughen et al. 1997). This herbicide was often used on cereals and its residues limited the use of flax in crop rotations because the herbicide does not degrade easily in the soil, affecting the establishment and growth of flax in the subsequent year (McHughen et al. 1997). Introduction of a sulfonylurea resistance gene into NorLin yielded the transgenic cv. CDC Triffid, which was developed to grow in such soil environments (McHughen et al. 1997). CDC Triffid was the first and only genetically modified flax cultivar registered in Canada, but it never went into commercial production (Booker et al. 2014). Tissue culture was used to produce two cultivars: Andro and CDC Normandy.

Andro was created by in vitro selection on high salt concentration media and resulted in a cultivar capable of withstanding high-saline environments (Rowland et al. 1989). Using the same method, CDC Normandy cv. had improved yield, oil content and oil quality.

9.6.3 Cultivars Released in Canada

A total of 82 flax cultivars were registered in Canada between 1910 and 2015, of which 58 were developed by Canadian breeders and the remaining 24 were introductions from foreign countries that were registered for production in Canada (You et al. 2016b). In the early stages of Canadian flax breeding, most registered cultivars were directly introduced from foreign countries. Some of them, including Redwing (1932), Bison (1930), Ottawa 770B (1910), J.W.S. (1930), and Buda (1930) were frequently used as core parents and contributed to the genetic base of modern flax cultivars. Since 1951, a total of 61 cultivars have been registered, 46 of which date to the last 35 years (You et al. 2016b).

Solin (low ALA) and NuLin™ (high ALA) are two special linseed types with ALA contents lower than 5% or higher than 65%, respectively. The CPS/Viterra cultivars were trademarked as Linola™ and NuLin™, respectively. Seven Solin (Linola™ 947, 989, 1084, 2047, 2090, 2126, 2149) (1.4–2.6% ALA) and one NuLin (NuLin™ VT 50) (68% ALA) cultivars have been released. CDC Gold is also a low ALA cultivar (13.3%), although its ALA content is not as low as the Linola™ series. Solin oil is suitable for the margarine industry because of its higher solidification temperature. However, the markets for these specialty linseed cultivars remain immature; thus all Solin cvs. have been deregistered in Canada. The yellow seed kernel visual distinguishability trait, originally exclusive to the Solin type, is no more, and Canadian cultivars can now be produced as a yellow- or brownseeded type regardless of the ALA content (FCC 2015).

The major characteristics of most registered cultivars are high yield, oil and high ALA contents. Almost all modern flax cultivars are immune to all North American races of rust caused by *Melampsora lini* (Ehrenb.) Lev. Currently registered flax cultivars must be immune to flax rust race 371 conditioned by the *K1*, *L6* or *M3* genes (Rashid and Kenaschuk 1994). Most registered flax cultivars are moderately resistant to *Fusarium* wilt and powdery mildew. Some cultivars, such as Royal and Rocket, have unique resistance to anthracnose, and the sole deregistered transgenic cultivar CDC Triffid is tolerant to sulfonylurea herbicides.

9.7 Molecular Breeding

Plant breeding pyramids elite complementary alleles from distinct parents using different approaches such as conventional crossing, mutation, in vitro selection or transgenic methods for crop improvement. Molecular breeding incorporates molec-

ular markers correlated with specific traits to improve selection efficiency. Molecular breeding is superior to conventional phenotype selection in several aspects, such as (1) the shortening of breeding cycles, (2) the selection for traits that are difficult to evaluate or for which evaluations in fields, greenhouses or laboratories are costly, e.g. disease resistance traits, (3) the selection in complex crosses to combine traits, (4) the introduction of new genes in backcrossing and, (5) the pyramiding of genes or favorable alleles for complex quantitative traits.

Two basic methods are widely adopted in crop molecular breeding. MAS uses DNA markers, especially diagnostic markers for specific traits to predict phenotypes, thus increasing the precision and efficiency of offspring selection. MAS is efficient in early generation screening for phenotype prediction of traits that have high heritability or that are controlled by a few major genes or QTL. However, the most important traits in crops are quantitative and genetically controlled by polygenes with small effects. MAS is not suited for accurate prediction of those traits because the associated QTL only explain a very small portion of the total phenotypic variation. GS provides a better approach to predict genomic estimated breeding values of quantitative traits of individuals based on genome-wide genetic markers, rather than a limited number of markers.

Based on the molecular breeding methods, Peleman and van der Voort (2003) proposed the *breeding by design* concept, which aims to bring together superior alleles for all genes of breeding target traits from potential germplasm resources to generate better cultivars. The superior alleles for traits can be identified through genome-wide high-resolution allele detection via precise QTL mapping and dissection of the genetic structure for potential parental resources. Thus, MAS and GS provide tools to implement this concept.

Flax has a small to medium size genome of ~370 MB (Table 9.2). Its chromosome-scale pseudomolecules for the genome have been generated based on CDC Bethune (Wang et al. 2012; You et al. 2018c). Genomic studies in the last decade have led to the production of a significant amount of genomic resources, including numerous molecular markers (SSRs, SNPs) and QTL associated with major breeding target traits (Table 9.5). With the declining costs of high-throughput sequencing and genotyping technologies, molecular breeding strategies have become feasible in flax cultivar development. Although there are no reports on the applications of molecular strategies in flax breeding programs, these genomic resources lay the foundation and offer a concrete potential for flax molecular breeding implementation. For instance, *Fusarium* wilt resistance is controlled by two major independent genes as well as some minor resistance genes in a DH population (Spielmeyer et al. 1998b), and two major QTL have been identified and respectively contributed 38% and 26% to the phenotypic variation (Spielmeyer et al. 1998a). For powdery mildew, three major QTL have been identified, explaining 97% of the phenotypic variation in an F_2 population (Asgarinia et al. 2013). These two disease traits may be candidates for application of MAS. In addition, seed fatty acid composition traits such as IOD, LIN and LIO are also suitable for MAS because they have high heritability, and major QTL have been identified (Tables 9.5 and 9.6).

GS has been evaluated in flax. Prediction accuracy of GS (r) and the efficiency of GS relative to phenotypic selection (RE) were initially assessed using three SSR

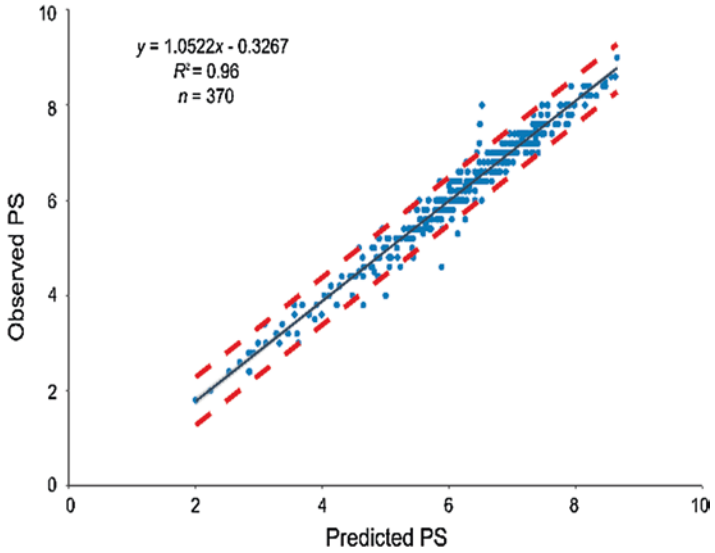

Fig. 9.12 Relationship of observed pasmo severity (PS) (y) to PS predicted (x) by the genomic prediction model. The red dashed lines represent the upper and lower boundaries of the 95% prediction intervals. (Source: He et al. 2019b)

marker sets and three GS models—ridge regression best linear unbiased prediction (RR-BLUP), Bayesian LASSO (BL) and Bayesian ridge regression (BRR)—in three biparental populations segregating for seed yield, oil content, IOD, LIN and LIO (You et al. 2016a). The three GS models generated similar *r* and *RE* values. A significantly higher selection efficiency of GS over direct phenotypic selection (a mean *RE* of 1.52) across populations and marker sets was obtained. Further evaluation of GS was performed for pasmo resistance using the diverse core collection of 370 accessions, 500 pasmo resistance associated QTL, and the field observations of pasmo severity scores (PS) across five consecutive years with the RR-BLUP model (He et al. 2019a). With five-fold random cross-validation, the prediction accuracy of the GS models was as high as 0.92. The PS values predicted by the GS models are highly correlated with the observed PS values ($R^2 = 0.96$) (Fig. 9.12), demonstrating that GS model based on information from all markers identified QTL very effectively for pasmo resistance prediction, a low heritability and complex trait.

9.8 Conclusions and Prospects

In the last decade, significant advances have been made in flax genomics, including the development of genetic and physical maps, scaffold-based and chromosome-scale reference genome sequences, a large number of molecular markers (SSRs, SNPs) from genomic sequences, different types of populations, and QTL associated with important breeding target traits. Biparental hybridization breeding with pedi-

gree selection and contra season operations remains the predominant methodology for Canadian flax breeding programs which requires approximately ten years from crossing to cultivar registration. Application of the DH method may shorten the breeding cycle by two years or three generations while simultaneously be advantageous for application of GS. Recent advances in flax genomics lay the foundation for molecular breeding. MAS is expected to be most fruitful for selection of resistance to diseases such as powdery mildew and *Fusarium* wilt for which several major QTL have been identified, while GS using genome-wide QTL with both large and minor effects has potential for the selection of complex quantitative traits. With more QTL identified, pyramiding superior alleles associated with breeding target traits by multiparental crossing and combining DH and GS will enhance the ability of breeders to make leaps in cultivar development whether quantitative, qualitative or simply through accelerated deliveries.

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Appendices

Appendix 1: Flax Research Institutes and Germplasm Resources

Country	Genbank and location	Number of accessions	Website
Russia	VNIIL, All-Russian Flax Research Institute, Torzhok	6302	http://www.carbo-extreme.eu/index.php/Consortium/VNIIL
Russia	VIR, N.I. Vavilov All-Russian Scientific Research Institute of Plant Industry, St. Petersburg	5471	http://www.vir.nw.ru/
Canada	PGRC, Plant Gene Resources of Canada, Saskatoon, Saskatchewan	3328	http://pgrc3.agr.gc.ca/about-propos_e.html
Ethiopia	PGRC/E, Plant Genetic Resources Centre, Addis Ababa	3110	http://www.ebi.gov.et/
USA	NC7, North Central Regional Plant Introduction Station, USDA-ARS, NCRPIS, Ames, IA	3017	https://www.cals.iastate.edu/ncrpis
Romania	ICCPT, Research Institute for Cereals and Industrial Crops Fundulea, Fundulea – Calarasi	2880	http://www.incda-fundulea.ro/informatii_en.htm

(continued)

Country	Genbank and location	Number of accessions	Website
China	Institute of Crop Germplasm Resources, Chinese Academy of Agricultural Sciences	2680	http://www.cgris.net/cgris_english.html
Czech Republic	SUMPERK, AGRITEC, Research, Breeding and Services Ltd., Sumperk	2011	http://www.agritec.cz/en
France	INRA, National Institute for Agricultural Research, Versailles	1700	http://www.inra.fr/en/
Germany	IPK, Institute of Plant Genetics and Crop Plant Research, Gatersleben	1638	https://www.ipk-gatersleben.de/en/institute/about-us/
Germany	Rheinische Friedrich-Wilhelms-Universtät, Bonn	1250	https://www.uni-bonn.de/startpage?set_language=en
Argentina	IRB-INTA, Banco Base Nacional de Germoplasma, Castelar, Prov. de Buenos Aires	1125	https://inta.gob.ar/unidades/211000/banco-base-de-germoplasma
Lithuania	LIA, Lithuanian Institute of Agriculture, Upytės Experimental Station, Upyte – Panevezio distr.	1060	http://lsdi.lt/en/
Ukraine	ILK, Institute of Bast Crops, Glukhiv, Sums'ka obl.	1042	http://ibc-uaas.at.ua/
Bulgaria	IPGR, Institute for Plant Genetic Resources "K.Malkov", Sadovo, Plovdiv district	957	http://ipgrbg.com/en/
Netherlands	CGN, Centre for Genetic Resources, the Netherlands Plant Research International, Wageningen	929	http://ipgrbg.com/en
Poland	INF, Institute of Natural Fibers, Poznan	927	http://www.istc.int/en/institute/13948
Australia	ATFCC, Australian Temperate Field Crops Collection, Horsham Victoria	639	https://grdc.com.au/research/reports/report?id=325
Poland	IHAR, Plant Breeding and Acclimatization Institute, Blonie, Radzikow near Warsaw	547	http://www.ihar.edu.pl/about_institute.php
Romania	BRGV Suceava, Suceava Genebank, Sučeava	634	http://www.svgenebank.ro/activities_collecting_ro.asp
India	NBPGR, Regional Station Akola, NBPGR, Akola, Maharashtra	442	http://www.nbpgr.ernet.in/Regional_Stations/Akola_Maharashtra.aspx
Romania	Livada Agricultural Research Station, Livada	420	http://www.scdalivada.ro/
Hungary	ABI, Institute for Agrobotany, Tápiószele	409	

(continued)

Country	Genbank and location	Number of accessions	Website
Sweden	NGB, Nordic Gene Bank, Alnarp	359	https://www.nordgen.org/skand/
United Kingdom	Department of Plant Science, Scottish Agricultural College, Auchincruive – Ayr	350	https://www.sruc.ac.uk/
Japan	NIAR, National Institute of Agrobiological Resources, Tsukuba-shi, Ibaraki-ken	287	https://www.naro.affrc.go.jp/archive/nias/eng/about/index.html
Bulgaria	ABI, AgroBioInstitute, Kostinbrod	283	ftp://nas.gov.ua/ciptt/www/eu/old_site_copy/bulgarian/eng/new_page_17.htm
Denmark	KVL, The Royal Veterinary and Agricultural University, Copenhagen	250	https://www.science.ku.dk/english/
United Kingdom	Northern Ireland Horticultural and Plant Breeding Station, Loughgall, Armagh	200	http://varieties.ahdb.org.uk/organisations/view/Northern%20Ireland%20Horticulture%20and%20Plant%20Breeding%20Station
Slovakia	SVKPIEST, Research Institute of Plant Production Piestany, Piestany	170	http://www.vurv.sk/en/introduction/
Ukraine	IZT, Institute of Agriculture & Cattle-breeding of the Western Region, S. Obroshyno, Pustomytivs'kyi r-n	104	https://www.cwrdiversity.org/data-sources/

Source: Adapted from Diederichsen (2007)

Appendix 2: Reaction Type of Canadian Commercial Flax Cultivars to Major Flax Diseases

Name	Release year	Rust	Fusarium wilt	Powdery mildew
AAC Bravo	2010	I	MR	MR
AAC Marvelous	2017	I	MR	MR
AAC Prairie Sunshine	2016	I	MR	MR
AC Emerson	1994	I	R	R
CDC Bethune	1998	I	MR	MR
CDC Buryu	2016	I	MR	MR
CDC Glas	2012	I	MR	MR
CDC Neela	2012	I	MR	MR
CDC Plava	2015	I	MR	MR
CDC Sanctuary	2009	I	MR	MR
CDC Sorrel	2005	I	MR	MR
Hanley	2001	I	R	MR
Lightning	2001	I	R	MR
Prairie Blue	2003	I	MR	MR
Prairie Grande	2007	I	R	MR
Prairie Sapphire	2009	I	MR	MR
Prairie Thunder	2006	I	R	MR
Taurus	2000	I	MR	MR
Topaz/WestLin 74	2017	I	MR	MR
Vimy	1986	I	MR	MS
VT50	2009	I	MR	MR
WestLin 60/WestLin 73	2016	I	MR	MR
WestLin 70	2013	I	MR	R
WestLin 71	2015	I	MR	MS
WestLin 72	2014	I	MR	MR

I immune, *R* resistant, *MR* moderately resistant, *MS* moderately susceptible
All listed cultivars are immune to the local races of flax rust and susceptible to pasmo

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Chapter 10
Breeding Strategies to Improve Production of Agave (*Agave* spp.)

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Abstract Agaves are perennial species that originated in the arid lands of North America, Mexico and Central America. They are well adapted to high temperatures and drought which makes them important species for the future of agriculture under water scarcity. Agaves are economically-important source of industrial products, food industry, steroids for animal feedstuffs, ornamental plants and as a raw material for biofuels. Because of their long-life spans and inefficient sexual reproduction, it is very difficult to genetically improve agaves and relatively little has been done in this respect. There is only one example of a breeding program, implemented by the British in what was then Tanganyka, now Tanzania, during the first half of the twentieth century, which produced the only hybrid (H11648) that has ever been commercially exploited. Consequently, the main alternative to improve the productivity of agaves is by means of the selection of elite individuals to be propagated through tissue culture for the establishment of plantations. This strategy has been used by some industries in Mexico with very good short-term results. Some *Agave* species have been domesticated and have become important plantation crops, but others are extracted from wild populations endangering the natural resource. Tissue culture and domestication have been used to rescue endangered varieties and offer a means of protection against overexploitation of the natural environment. There are several studies on the genetic variability of agaves but not enough is known to be directly applied in genetic improvement programs. This chapter reviews the current knowledge of this field and discusses advances and potential applications and uses of agaves.

Keywords Agave · Genetic resources · Germplasm conservation · Industrial products · Tissue culture · Variability

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10.1 Introduction

10.1.1 Origin and Distribution

Agave (from the Greek word αγαυή, *noble* or *admirable*), also known as *century plant* is a genus of the family Agavaceae that originated around 12 million years ago in the arid regions of what is now northern Mexico and the southern USA. It has diversified into more than 300 species some of which are endemic in their regions of origin while others have been distributed all over the world.

There is no general agreement on the taxonomic classification of these plants but according to Gentry (1982), the species of the *Agave* genus are classified as follows:

Kingdom:	Plantae
Division:	Magnoliophyta – angiosperms (flowering plants)
Class:	Monocotyledons
Order:	Asparagales
Family:	Agavaceae
Subfamily:	Agavoideae
Genus:	*Agave* L.

Depending on their floral characteristics, the genus is divided into three subgenera (*Manfreda*, *Littaea* and *Euagavae*) and these are subdivided into sections. The taxonomy of agaves has been extensively studied by Gentry (1982), Eguiarte et al. (2000) and García-Mendoza (2010); however, wild plants are difficult to identify unequivocally due to, among other things, morphological differences caused by natural variability, sexual reproduction, age and varied environmental conditions. To make things more complicated, the same type can be known by different names in different regions. The *Agave angustifolia* complex is a good example of this (Garcia-Mendoza and Chiang 2003).

Many species of the genus *Agave* are used as the source of raw material for a variety of products that include: hard fiber for ropes, sacks and mats, steroidal sapogenins for pharmaceutical purposes, inulin for the food industry; alcohol for beverages such as tequila, mezcal and bacanora and, more recently, as a potential source of biofuels. Some of these products supply large global industries, which have fast-growing international markets.

In spite of their economic importance, the agaves used for industrial purposes have rarely been genetically improved. Some of them have not even been properly domesticated and are exploited from wild populations.

Domestication and the establishment of commercial plantations of species exploited in the wild constitute an effective way of increasing yields, reducing production costs and preserving the natural resource. However, this is not an easy task in the case of perennial plant species adapted to arid and semiarid environments because their very slow growth rates and long life spans (8–20 years) make genetic improvement and domestication difficult. On the other hand, the selection of elite

individuals from the wild is also problematic due to the impossibility of knowing the age of the plants, their physiological status and the environmental conditions in which they have grown.

The use of many agaves for ornamental purposes has resulted in the establishment of private nurseries that exploit small seed producing species. The cross-pollination of such species has generated many morphological variants and hybrids that are very attractive for gardens and landscaping. Many commercial pages can be found on the web, but there is very little scientific literature regarding these activities.

Biotechnology, in particular tissue culture, is therefore, the best option for tackling the improvement of agaves used for commercial purposes. An extremely important contribution of these techniques is related to the conservation of endangered species and the maintenance of potentially useful lines for future improvement and the search for new uses of this very important but not yet fully exploited group of plants.

Probably the most important trait that agaves have to offer agriculture in the future is their capacity to capture and retain water. This is the consequence of a mixture of anatomical and physiological characteristics that include a thick wax cover on the leaves, CAM photosynthesis and a large root system, all of which combine to allow them to grow under conditions of severe water scarcity where other crops cannot thrive.

10.1.2 Domestication and Cultivation of Agaves

The term *domestication* is used in a very broad sense by some authors (Colunga-GarcíaMarín et al. 2017; García-Mendoza 2007) who consider that the conservation and minimal use of the plant signify that it falls into this category. In reality, very few species have been partially domesticated, being conserved and exploited in situ (most of the agaves employed for mezcal production) and even fewer have been established in plantations (*Agave fourcroydes, A. sisalana, A tequilana* and a few others). Genetic crossings have been negligible (See hybrid H11648).

Cultivation is carried out following standard fertilization and pest management practices, under rainfed conditions with a minimum of mechanization.

The most sui generis practices are the propagation methods through the harvesting of rhizome offshoots and, occasionally, of the bulbils that grow on the inflorescences. In most cases, the inflorescence stalks are cut when they start developing in order to prevent them from using up the nutrients stored in the boles and the water in the leaves that would make the plant unproductive and would, eventually, kill it.

New plants are established in nurseries at a density of 60,000–100,000 plants/ha for a few months to allow them to reach a minimum size (some 60 cm high) before they are transplanted to the plantations (2400–3600 plants/ha) where they will stay for a varied length of time. *Agave tequilana* will be grown for 4–6 years before the leaves are removed and the bowls taken to the distilleries to be processed. Henequen,

sisal and H11648 will grow for 3–5 years before the leaves are cut at regular periods and transported to the decorticating mills to extract the long fibers. The times and yields are very variable and depend on the species and culturing conditions.

The main problems for agave cultivation are the infestations of insects that feed from the stems and bring microbial pathogens such as *Erwinia*, *Phytophtora* that rot the bowls or damage the leaves affecting the amount or quality of the final product.

10.1.3 Economic Importance of Agaves

Over the last 20 years, changes in market trends and the need for more environmentally-friendly options, have increased the demand for agave derived products and, consequently, for their market opportunities and value. Also, the fact that agaves are tolerant to water scarcity makes them an attractive alternative for productive activities in arid lands and, possibly, as a strategy to cope with climate change. New waste management alternatives that reduce costs, are friendly to the environment and generate energy, may also be contributing to their growing economic potential. Agaves are also helpful to control soil erosion and desertification by carbon sequestration (Davis et al. 2015). Furthermore, agaves are low-input perennial crops, similar to *Miscanthus* and switchgrass that exhibit lower greenhouse gas emissions and nitrogen leaching than maize (Davis et al. 2010).

Today, the main products with commercial value are hard fibers, spirits (Table 10.1) and ornamentals, but the demand for nutritional products is rapidly increasing and could become an interesting option for agave cultivation. Less obvious is their potential as a source for biofuels, particularly bioethanol, but the demand for clean energy in the future might make them an option if the yields and growth rates can be incremented.

10.2 Anatomy and Biology of Agaves

10.2.1 Anatomy

Anatomically, agaves present a combination of traits that appear to be special adaptations for survival under unfavorable climatic conditions with long periods of drought and high temperatures (Gentry 1982; Nobel 1988). Agaves are formed by a central fibrous stem, called bole or piña, around which leaves grow forming a spiral rosette following a Fibonacci number pattern that allows some leaves to shade others, thereby reducing the temperature on their surface (Nobel and Valenzuela 1987). New leaves emerge from a central meristem, so that the youngest leaves are always at the center of the stem.

Table 10.1 Listing agave species and cultivars classified according of their uses and their cultivation location

Scientific name	Common name	Location
Hard fibers: Leaves are used as raw material to obtain carpets, ropes, handicrafts, bricks for construction, and their waste as a source of renewable energy, either as bioethanol or biogas.		
Agave fourcroydes	Henequén	Yucatan, Mexico and Cuba
A. sisalana	Sisal	Brazil and China
Hybrid 11,648	Hybrid	Tanzania, Pakistan and China
Spirits: Boles are used as feedstuff of sugars for fermentation and distillation for tequila production		
A. tequilana Weber var. *azul*	Tequila	Jalisco, Nayarit, Guanajuato, Tamaulipas, Mexico
A. angustifolia Haw	Bacanora	Sonora, Mexico
Boles are used as feedstuff of sugars for fermentation and distillation for mezcal production		
A. angustifolia	Espadín	Oaxaca, Mexico
A. rodhacantha Trel.	Maguey de campo	Oaxaca, Mexico
A. potatorum Zucc.	Tobalá	Oaxaca, Mexico
A. marmorata Roezl.	Tepextate	Oaxaca, Mexico
A. karwinkii Zucc.	Madre-cuishe	Oaxaca, Mexico
A. convallis Trel.	Maguey	Oaxaca, Mexico
A. amaericana L. var. *oaxacencis Gentry*	Maguey	Oaxaca, Mexico
A. americana L. var. *americana*	Castilla	Oaxaca, Mexico
A. chino verde,	Maguey chino verde	Oaxaca, Mexico
A. mexicano penca larga,	Maguey mexicano penca larga	Oaxaca, Mexico
A. tobasiche	Maguey tobasiche	Oaxaca, Mexico
A. pelón	Maguey pelón	Oaxaca, Mexico
A. cantala	Maguey cincoaeño	Oaxaca, Mexico
A. lumbre	Maguey lumbre	Oaxaca, Mexico
A. cuishito	Maguey cuishito	Oaxaca, Mexico
A. americana L.	Maguey	Oaxaca, Mexico
A. macrocantha	Maguey mexicano sin espinas, barril	Oaxaca, Mexico
A. caballo	Maguey caballo	Oaxaca, Mexico
A. angustifolia Haw var. *rubescens* (SalmDick) H Gentry	Maguey	Oaxaca, Mexico
A. duranguensis	Cenizo	Durango, Mexico
A. seemanniana Jacobi.	Maguey	Oaxaca, Mexico
A. aff. *karwinskii* Zucc.	Cirial o Cuishe	Oaxaca, Mexico
A. salmiana ssp. *crassispina*	Mezcal	San Luis Potosí and Zacatecas, Mexico

(continued)

Table 10.1 (continued)

Scientific name	Common name	Location
A. salmiana	Maguey pulquero, manso o verde	Tlaxcala, Puebla, Hidalgo, State of Mexico
A. mapisaga	Maguey pulquero, de manos largas o mexicano	Tlaxcala, Puebla, Hidalgo, State of Mexico
Agave	Maguey pulquero	Tlaxcala, Puebla, Hidalgo, State of Mexico
A. hookerii	Maguey ixquitécatl o pulquero	Tlaxcala, Puebla, Hidalgo, State of Mexico
A. marmorata	Maguey tepeztate	Tlaxcala, Puebla, Hidalgo, State of Mexico

Leaves The leaves are long, thick and fleshy, with an impermeable cuticle covered by waxes that help preserve water from evaporation. The development of succulence in the leaves is one of agaves' most important adaptations, since the water stored during the rainy season allows the plants to survive in the absence of water for long periods of time (Gentry 1982). To protect them from depredation, the leaves of most agaves have a thick apical spine and present marginal thorns of different shapes along their edges that are important taxonomic traits. The leaves can be flexible such as in *Agave vilmoriniana* or very rigid as is the case of most of the industrial types that will be mentioned here. The abundance of fibers in the leaf tissues maintains rigidity during the dry periods preventing the leaves from deformation and maintaining their ability to capture enough light for photosynthesis.

Roots The root system is formed by very long branched roots, without a tap- root, that spread radially near the surface to facilitate the absorption of scarce rainwater that only moistens the surface of the soil.

Rhizomes Agaves produce modified underground stems called rhizomes that grow horizontally near the surface of the soil. Eventually, the apical meristem turns upwards and emerges forming a new plant that remains attached to the mother plant until it forms a new root system and becomes photosynthetically self-sufficient. Keeping the offspring attached to the mother plants is a form of vegetative propagation particularly well suited to the adverse arid conditions in which agaves live.

Inflorescence Towards the end of the life of an agave plant, the same meristem from which the leaves were formed changes and converts into a flowering pole (O'Neill and Roberts 2002). A thick stem that grows very fast, using up all the reserves stored by the plant, to a height of several meters (2–10 m depending on the species). The stem finally forms branches that produce flowers (Fig. 10.1).

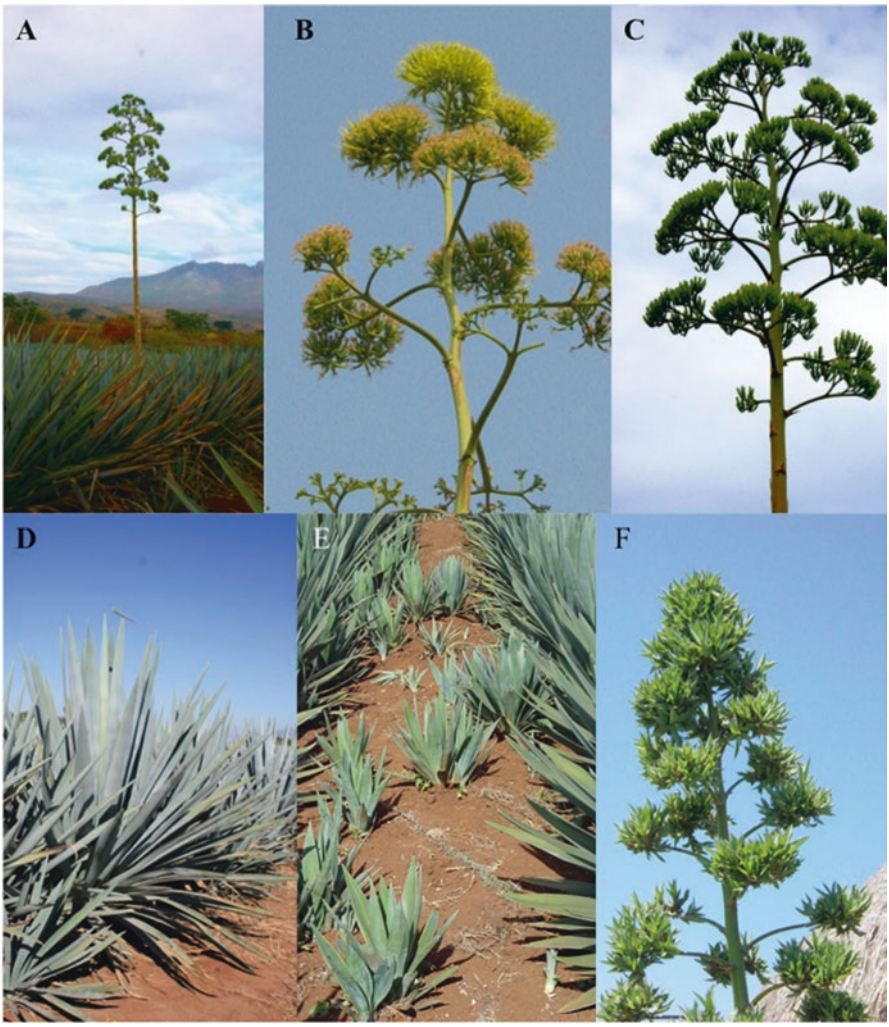

Fig. 10.1 Sexual reproduction of the agaves. (**a**) Inflorescence of *Agave tequilana*, (**b**) Inflorescence of *A. fourcroydes*, (**c**) Inflorescence of *A. angustifolia*, (**d**) Asexual reproduction by rhizomes in *A. tequilana*, (**e**) Suckers of H11648, (**f**) Bulbils of *A. angustifolia*

Bulbils These are fully-formed plants that originate from buds in the axils of the fruit stems. The bulbils eventually fall from the inflorescence and some establish themselves on the ground and carry on growing. Rhizomes and bulbils are both vegetatively generated and are frequently used as propagative material by agave growers (Fig. 10.1).

10.2.2 CAM Photosynthesis

The most remarkable adaptation of the agaves is the structure of the stomata linked to the crasulacean acid metabolism (CAM) that allows them to close during the day and open at night, once again, preventing the loss of water by evapotranspiration (Nobel 1988). The gas exchanges take place during the night when carbon dioxide is fixed in the cytoplasm of mesophyll cells by enzyme reactions, like the one in the C4 pathway, and stored in vacuoles. During the daylight period, CO_2 is released again and enters the stroma of the chloroplasts and is transferred to the Calvin cycle to be incorporated into sugars.

10.2.3 Reproductive Biology

Agaves can propagate both sexually and vegetatively. However, for reasons related to the characteristics of their habitat and their genetic nature, some species use asexual propagation as their main means of multiplication and preservation of the species.

Sexual reproduction takes place by pollination of the flowers in the inflorescence, which is paniculated in the subgenus *Euagave*. Flowers are hermaphrodite, with great floral diversity (Gentry 1982). Pollination is mediated by bats, birds and insects (Figueredo et al. 2011; Gentry 1982; Howell and Roth 1981; Molina-Freaner and Eguiarte 2003; Rocha et al. 2005, 2006; Silva-Montellano and Eguiarte 2003a) and produce tri-loculated fruits that can contain very large numbers of seeds (Howell and Roth 1981; Nobel 1977).

Some species such as *Agave cupreata*, only reproduce by seeds whose germination rate is high (Ramírez-Tobías et al. 2012), but there are also species that do not produce viable seeds and reproduce only by asexual propagation such as *A. fourcroydes* and *A. sisalana*. The low fertility of seeds in some species of agaves may be due to a variety of reasons. Piven et al. (2001) reported abnormal development of male and female gametophyte at the meiotic and post-meiotic stages in *A. fourcroydes,* which could be due to its pentaploid nature, and these alterations might be responsible for the high sterility of pollen grains of this species. Similarly, the observed meiotic irregularities in *A. tequilana* suggest that structural chromosome aberrations have occurred that promote low pollen viability (Ruvalcaba-Ruiz and Rodríguez-Garay 2002). In other species such as *A. tequilana*, an irregular development of the embryos has been observed (Ramírez Tobías et al. 2016). Furthermore, temperature and other environmental factors can play an important role in the seed germination capacity (Ramírez-Tobías et al. 2012, 2016).

Asexual propagation takes place by means of two mechanisms: the formation of rhizomes and bulbils. Offshoots from the tip of rhizomes are the main source of planting material for many cultivated agaves and cuttings are periodically made to

collect them from the plantations. The offshoots, however, are not complete plants since they lack roots and it is necessary for them to be planted in nurseries before they are transferred to the plantation.

A second asexual propagation mechanism is through the bulbils that form in the branches of the inflorescences. The number of bulbils produced can vary depending on the species, In *Agave fourcroydes*, 3255 bulbils per inflorescence were reported by Szarek et al. (1996).

10.3 Genetic Variability

10.3.1 Chromosome Numbers

Several studies revealed that the genus contains species of many ploidy levels (2x, 3x, 4x, 5x, 6x and 8x) with a basic chromosome number of n = 30 formed by two sets of 25 small and 5 large chromosomes in a bimodal karyotype, that increases in direct proportion with ploidy, i.e. *Agave tequilana* (2x = 60), hybrid H11648 (2x = 60) (Finch and Osborne 1990), *A. sisalana*, 5x = 150 (Castorena-Sánchez et al. 1991; Finch and Osborne 1990); *A. fourcroydes*, 5x = 150 (Castorena-Sánchez et al. 1991); and *A. angustifolia*, 6x = 180 (Castorena-Sánchez et al. 1991; Finch and Osborne 1990).

A study involved several species of different ploidy levels in which the c values were compared by flow cytometry using the same calibration standard, and by fluorescent in situ hybridization using 45S and 5S ribosomal DNA (rDNA) and the telomeric sequences (TTAGGG)n and (TTTAGGG)n as genetic markers. The studied species consisted of two diploids (A. *tequilana* and the hybrid H11648), a triploid (*Agave fourcroydes* var. kitam ki), a tetraploid (*A. angustifolia* var. letona), three pentaploids (*A fourcroydes* var. sac ki, var. yaax ki and *A. sisalana*) and two hexaploids (*A. angustifolia* var. chelem ki from two locations). The results showed proportional increases with ploidy level of: (1) chromosome numbers from diploid 2n = 2x = 60 to hexaploid 2n = 6x = 180, including the number of large and small chromosomes in the bimodal karyotype of *Agave*; (2) genome size, with a mean monoploid genome size (1Cx) of 7.5 pg (range of 7.36–7.61 pg) and; (3) the number and distribution of 45S and 5S rDNA loci, with one locus of each per basic, monoploid genome (Robert et al. 2008).

The high frequency of pentaploid and hexaploid *Agave* species in Yucatán, Mexico suggests a strong selection in favor of polyploids under the harsh, water-stressed environments. The few studies on sexual reproduction in *Agave* revealed very low seed germination in *A. fourcroydes* (Piven et al. 2001). Perhaps the reduced incidence of sexual reproduction in *Agave* species may contribute to a stable genome size in the polyploids.

10.3.2 Molecular Variability

The analysis of molecular markers to understand genetic variation and genetic relationships in *Agave* species can produce very valuable information for crop improvement and resource conservation strategies. In agaves, the most frequent techniques used for this purpose have been: amplified fragment length polymorphism (AFLP), isozymes, inverse sequence tagged repeat (ISTR), inter simple sequence repeats (ISSR), microsatellites, randomly amplified polymorphic DNA (RAPD) and sequence-specific amplified polymorphism (SSAP). These markers have been used mainly for studies of genetic variation, taxonomic relationship and genetic fidelity (Table 10.2).

The level of genetic diversity within *Agave* species can vary enormously depending of their level of ploidy and their reproductive efficiency. High levels of genetic diversity have been reported in *A. cupreata* (Aguirre-Dugua and Eguiarte 2013), and *A. potatorum* (Aguirre-Dugua and Eguiarte 2013; Félix-Valdez et al. 2016), possibly due to the fact that all the offspring are the product of cross-pollination and not asexual reproduction.

Species that present both sexual and vegetative reproduction have a wider range of genetic diversity as reported for *Agave parryi* (Lindsay et al. 2012) and *A. palmeri* (Lindsay et al. 2012), *A. salmiana* ssp. *crassispina* (Lara-Ávila and Alpuche-Solís 2016), *A. victoriae-reginae* T. Moore (Martínez-Palacios et al. 1999), *A. utahensis* (Byers et al. 2014) and *A. lechuguilla* Torr. (Silva-Montellano and Eguiarte 2003b). This could be the result of a synergism between a component of inheritable genetic variability (cross-pollination) and an inheritable epigenetic component (Lara-Ávila and Alpuche-Solís 2016).

There are several reports of a certain degree of variability in species whose propagation is mostly vegetative; such is the case of *Agave tequilana* (Gil-Vega et al. 2006; Rodríguez-Garay et al. 2009), *A. angustifolia* (Sánchez-Teyer et al. 2009) and *A. sisalana* (dos Santos et al. 2015). The existence of asexual genetic variability between the mother plant and its rhizome progeny has been reported in *A. tequilana* by Torres-Morán et al. (2010), using ISTR as a marker. Using AFLP, Díaz-Martínez et al. (2012), found low levels of variation between mother plants and rhizomes, but a higher level between the mother plant and its bulbils in *A. tequilana*. In *A. fourcroydes* variability between mother plants and their rhyzomes (González et al. 2003) and in wild populations of bacanora (*A. angustifolia*), some degree of genetic variation between the mother plant and their rhizomes has been observed, in spite of the fact that they are clones produced by asexual propagation (Sánchez-Teyer et al. 2009), possibly by the presence of several repetitive elements including LTR-retrotransposons (Bousios et al. 2007). This genetic variability could be very useful in the genetic improvement of agaves, especially in species whose main form of reproduction is vegetative.

Reports on *Agave tequilana* have been variable depending on the marker and the population analyzed. Using RAPDs, Rodríguez-Garay et al. (2009) observed more diversity than the one reported by Gil Vega et al. (2001). However, when Gil-Vega et al. (2006) used AFLP, they observed more diversity in a different population.

Table 10.2 Molecular markers used in *Agave* species

Species	Marker	Work objective	References
A. fourcroydes	Isozymes	Phylogenetic relationships	Colunga-GarciaMarin et al. (1999)
A. victoria-reginae	Isozymes	Genetic diversity	Martínez-Palacios et al. (1999)
A. tequilana	RAPD	Genetic diversity	Gil Vega et al. (2001)
A. fourcroydes	AFLP	Genetic variability	González et al. (2003)
A. deserti	RAPD	Genetic variation	Navarro-Quezada et al. (2003)
A. lechuguilla	Isozymes	Genetic variation	Silva-Montellano and Eguiarte (2003a)
A. fourcroydes	AFLP and ISTR	Genetic analysis	Demey et al. (2004)
A. angustifolia	AFLP	Genetic diversity	Barraza-Morales et al. (2006)
Agave sp.	AFLP	Molecular taxonomy	Gil-Vega et al. (2007)
A. tequilana	AFLP	Genetic diversity	Gil-Vega et al. (2006)
A. salmiana	RAPD	Genetic diversity	Alfaro-Rojas et al. (2007)
A. murpheyi and *A. delmateri*	Isozymes	Genetic diversity	Parker et al. (2007)
A. tequilana	SSAP	Phylogenetic relationships	Bousios et al. (2007)
A. cocui Trel., *A. angustifolia* Haw. and *A. tequilana* Weber	ISSR	Genetic variation	Dávila et al. (2007)
A. tequilana and *A. angustifolia*	RAPD	Genetic diversity	Rodríguez-Garay et al. (2009)
A. tequilana and *A. angustifolia*	ISSR	Genetic variation	Vargas-Ponce et al. (2009)
A. angustifolia	AFLP	Genetic variation	Sánchez-Teyer et al. (2009)
A. parryi	Isozymes and microsatellites	Genetic variation	Parker et al. (2010)
A. tequilana	ISTR	Genetic fidelity	Torres-Morán et al. (2010)
A. cocui	Isozymes	Genetic diversity	Figueredo and Nassar (2011)
A. cupreata	Isozymes	Genetic diversity	Martinez-Palacios et al. (2011)
A. palmeri and *A. parryi*	Microsatellites	Genetic diversity	Lindsay et al. (2012)
A. tequilana	AFLP	Genetic variation	Díaz-Martínez et al. (2012)
A. durangensis	Microsatellites	Genetic variation	Almaraz-abarca et al. (2013)
A. cupreata and *A. potatorum*	ISSR	Genetic diversity	Aguirre-Dugua and Eguiarte (2013)

(continued)

Table 10.2 (continued)

Species	Marker	Work objective	References
A. parryi var. *huachucensis*	Microsatellites	Genetic variation	Parker et al. (2014)
A. utahensis	Microsatellites	Genetic diversity	Byers et al. (2014)
A. sisalana Perrine	ISSR	Genetic variation	dos Santos et al. (2015)
A. inaequidens Koch	Microsatellites	Genetic diversity	Figueredo et al. (2015)
A. striata	ISSR	Genetic variation	Trejo et al. (2016)
A. salmiana ssp. *crassipina*	AFLP	Genetic diversity	Lara-Ávila and Alpuche-Solís (2016)
A. potatorum	Microsatellites	Genetic diversity	Félix-Valdez et al. (2016)
A. inaequidens, A. cupreata and *A. hookeri*	Microsatellites	Genetic divergence	Figueredo-Urbina et al. (2017)
A. palmeri and *A. parryi*	Microsatellites	Genetic structure	Lindsay et al. (2018)
A. fourcroydes	AFLP	Genetic variation	Monja-Mio et al. (2018)

AFLP Amplified fragment length polymorphism, *ISTR* Inverse sequence target repeat, *ISSR* Inter simple sequence repeats, *RAPD* random amplified polymorphic DNA, *SSAP* sequence-specific amplified polymorphism

Vargas-Ponce et al. (2009), on the other hand, observed low genetic diversity using ISSR as a marker. These differences may be due to the fact that each molecular marker used evaluates a different region of the genome (Torres-Morán et al. 2010).

10.4 Genetic Improvement of Agaves

10.4.1 Limitations and Difficulties in Agave Breeding

The breeding of agaves, particularly those used for industrial purposes, is a lengthy and fastidious process due to a variety of factors: (a) many of them flower only once towards the end of their long-life spans (8–20 years). (b) the large sizes and height of the inflorescences (up to 5 m tall), (c) genetic complexity, many of them such as *Agave sisalana* Trel an *A. fourcroydes* Lem. are polyploids and (d) inefficient sexual reproduction caused by polyploidy, sterility and recalcitrancy of the seeds.

10.4.2 Mlingano Sisal Research Station Program

The only comprehensive and successful program implemented to genetically improve agaves was carried out by the British in Tanzania during the first half of the twentieth century. The selection and crossing of several diploid agaves produced a

number of hybrids among which, hybrid H 11648 (Lock 1962) is the only high-yielding hybrid that has been cultivated for industrial purposes and it is still grown in the plantations for the production of hard fiber in East Africa.

Production in Tanzania reached about 112,000 mt by 1945. The world price of sisal fiber rose greatly in 1951, and accordingly, stimulated the cultivation of sisal throughout East Africa. The increased production was possible thanks to the work of George W. Lock who, between 1934 and 1959 lead a group of geneticists and agronomists based at the Sisal Research Station that he developed and ran at Mlingano, near Tanga.

All aspects of agave cultivation were studied, such as soil composition, plantation density, but the main contribution was the generation of new high-yielding varieties. The aim of the project was to develop improved types resistant to diseases and capable of producing high yields of good quality fiber under a wide range of soil and climatic conditions. Particularly desirable was the absence of marginal spines from the leaves since these make the harvesting extremely cumbersome and can cause painful injuries. The main type used until then was *Agave sisalana*, which was a satisfactory crop but had the limitation of producing a small number of leaves.

For many years Lock collaborated with George Daughty, a geneticist at the nearby East African Agricultural Research Institute at Amani, on sisal breeding and field trials of several hybrids produced by cross-pollination of various *Agave* species including *A. fourcroydes, A. sisalana, A. cantala* and *A. angustifolia* and, later on, *A. amaniensis*, a promising plant with very long blue leaves that was discovered growing under bushes at Amani.

All *Agave cantala* crosses were infertile, *A. fourcroydes* and *A. sisalana* produced very few fruits; and the few seeds produced generated an enormous variation in size and number of leaves with marginal spines.

Neither *Agave angustifolia* nor *A. amaniensis* performed satisfactorily when planted commercially, but as parental lines, their crosses generated a family of potentially important lines. *Agave angustifolia*, although small and pale, contributed the traits of rapid growth and a very large number of leaves while *A. amaniensis* contributed the long smooth leaves with a fine long fiber content. Since both species are diploids they are fertile and were used for many different crosses that produced a family of hybrids (see Germplasm collections) that were tested in field trials at Amani and Mlingano. However, only one proved to be remarkable in relation to most of the desired traits that were expected in the improved lines.

10.4.3 Hybrid H11648

This hybrid, named for the date (11 June 1948) on which it was released, is the product of a cross between *Agave angustifolia* x *A. amaniensis* Trel and Nowel, backcrossed with *A. amaniensis*. This hybrid contains more fiber per leaf and produces more leaves per year than henequen or sisal, moreover, the leaves lack marginal spines thus facilitating harvesting (Table 10.3). Unfortunately, H11648 is very

Table 10.3 Relative performance of Hybrid 11,648 compared to *Agave sisalana*

Performance criteria	Hybrid 11,648	*Agave sisalana*
Plants per ha	5000	4000
Number of leaves grown per plant per cycle	648	205
Number of leaves grown per plant per month	5.9	2.8
Average leaf weight (grams)	520	548
Total fiber yield (mt per ha)	62.8	22.6
Fiber yield per year (mt per ha)	6.8	3.7
Length of cycle from planting to poling (months)	110	74

Source: Hopkinson and Wienk (1966)

susceptible to several fungal infections: Korogwe leaf spot, zebra leaf rot and bole rot diseases.

H11648 has been since then, the main hybrid planted in thousands of hectares in Tanzania, Kenya and other parts of the world. The genetic improvement program was cancelled after Tanzania's independence and nationalization and competition from plastic fibers made sisal production fall in East Africa from 230,000 mt to 20,000 mt by the early 1980s.

10.4.4 CFC/UNIDO Project: Product and Market Development of Sisal and Henequen

Reprivatization later began reversing the downward trend and a new initiative to boost the development of the sisal industry in East Africa was implemented in Tanzania and Kenya between 1998 and 2005 under the auspices of the Common Fund for Commodities (CFC) and The United Nations Program for Industrial Development (UNIDO). The project: Product and Market Development of Sisal and Henequen, was undertaken by the Sisal Boards of both countries and private enterprises such as Katani Ltd. in Tanzania and Rea Vipingo in Kenya (CFC 2006).

The principal aim of the project was to promote the development of alternative nontraditional end uses of sisal, particularly the production of fibers to be used in the pulp and paper mills for specialty paper. The main project activities focused on the analysis of the available sisal varieties, the evaluation of best agricultural practices and plant multiplication by meristematic tissue culture to improve productivity, reduce costs and, therefore, produce competitively-priced fiber. Other uses that were also tested included the utilization of sisal wastes for biogas and electricity generation.

The Sisal Research Station, now Agricultural Research Institute (ARI) at Milingano, oversaw the development of new sisal varieties and improvement of cultivation practices, the implementation of the tissue culture laboratory and the establishment of a new germplasm collection (see Sect. 10.7.3) to rescue the varieties and hybrids previously developed there.

Table 10.4 shows some interesting data regarding important traits for genetic improvement of hard fiber production: The pentaploid agaves do not produce viable seed, they produce less but longer leaves and have marginal spines and are less susceptible to pests and diseases than the diploids. Conversely, the latter do not have marginal spines on their leaves and tend to produce more leaves, they flower and produce viable seed but are much more susceptible to damage by pests and diseases.

10.4.5 Other Research Initiatives

Although research on agaves has been an ongoing activity in several laboratories in Mexico, it is only recently that industries have started to take an interest and participate in the development and use of biotechnologies for the improvement of yield and quality. Two of the largest tequila companies have implemented micropropagation programs and, more recently, the mezcal industry has followed suit and is planting hundreds of thousands of micropropagated plants. Embrapa in Brazil also carries out research for different purposes such as biofuels, dietary carbohydrates and fiber (Embrapa 2018).

10.4.5.1 Genetic Transformation

Genetic transformation could be a feasible approach to introduce genes to help plants to tolerate diseases or to overexpress some of their own genes such as those involved in fructan biosynthesis, which could lead to a more efficient production of these highly sought after probiotic compounds (Flores-Benítez et al. 2007).

Flores-Benítez et al. (2007) reported the genetic transformation of *Agave salmiana* using two different protocols: cocultivation with *Agrobacterium tumefaciens* (a microbial vector that carries the new gene into the recipient cells) and particle bombardment (a method to introduce the genes by shear force). In both cases, the gene *uidA* (β-glucuronidase) was used as a reporter gene (That indicates that the cells have been transformed) and the *nptII* and bar genes as selectable markers for *A. tumefaciens* and biolistic transformation, respectively. The first protocol was the most effective method of transformation, producing 32 transgenic plants and giving a transformation efficiency of 2.7%. Polymerase chain reaction (PCR) detected the transgenes in 11-month-old plants. The second protocol produced transgenic calli, cultured in vitro, which tested positive with the GUS assay after 14 months on selective medium while still undergoing regeneration.

Gao et al. (2014) reported a transformation system to express hevein-like peptides in tissues (calli) of agave hybrid 11,648 cultured in vitro to enhance resistance to *Phytophthora nicotiana*. They discussed the main factors influencing plant transformation efficiency such as the time for infection (10 min), the optimum concentration of acetosyringone (200 μM), the optimum time for the in vitro culture of the

Table 10.4 Characteristics of the main varieties and hybrids available for the improvement of hard fiber producing agaves

Ploidy	Species, variety or hybrid	Origin	Leaves				Fiber content	Plant size	Inflorescence					Degree of susceptibility	
			Marginal spines	Apical spine	Length	No. of leaves			Pole	Flower	Fruit	Viable seed	Bulbil	Korogwe	Weevil
5n	*Agave foucrocydes*	Yucatan, Mexico	Large	✓	Long	Medium	High	Large	✓	✓	✓	✓	✓	S +	S +
	A. sisalana	Yucatan, Mexico	Short	✓	Long	Medium	High	Large	✓	✓	x	–	✓	S +	R
	Hildana	Kenya	Short	✓	Long	High	High	Large	✓	✓	✓	–	✓	–	R
	Sisal type collected by Grundy in Tanzania	Yucatan, Mexico	Short	✓	Long	–	High	Large	✓	–	–	–	–	–	–
	Sisal non-poling variety collected in Tanzania	Yucatan, Mexico	Short	✓	Short	–	Low	Large	Not formed	Not formed	Not formed	Not formed	Not formed	S +	–
	A. spectabilis	Mexico	Large	x	Long	Low	Low	Medium	✓	–	–	–	✓	–	–

2n	*A. amaniesis*	Amani, Tanzania	None	✓	Long	Low	Low	Large	✓	✓	✓	✓	✓	S +++	S +++
	A. amaniensis variegated	Tanzania	None	✓	Long	Low	Low	Medium	✓	✓	✓	✓	–	–	–
	A. angustifolia	Mexico	None	✓	Short	High	Low	Short	✓	✓	✓	✓	✓	S +++	–
	H.11648	Breed at Mlingano, Tanzania	None	✓	Long	High	High	Large	✓	✓	✓	✓	✓	S +++	S ++
	H.1300		None	✓	Long	High	High	Large	✓	✓	✓	✓	✓	S ++	S ++
	Mlola 1		None	✓	Long	High	High	Large	✓	✓	✓	✓	✓	S +	S ++
	Mlola 487		None	✓	Medium	High	Medium	Large	✓	✓	✓	✓	✓	S ++	–
	A. americana	Arizona, USA	Large	✓	Medium	Medium	Low	Medium	✓	✓	✓	✓	–	–	–
	A. americana x *A. amaniensis*	Mlingano, Tanzania	None	✓	Long	Medium	Medium	Large	✓	✓	✓	✓	–	–	–

Presence (✓); Absence (x); No information available (–); Resistant (R); Susceptible (S); Degree of susceptibility (+,++,+++)

agave tissues (3 days), and the optimum co-culture time for the calli and *Agrobacterium* (4 days). The integration, stability and expression level of the transgene in the H.11648 genome was analyzed by using Southern blot and reverse transcription polymerase chain reaction. They obtained 37 lines from 150 explants and demonstrated that the hevein-like gene was expressed in seven lines.

Gutiérrez Aguilar and Gil-Vega (2016) developed a methodology for genetic transformation of *Agave tequilana* and *A. desmettiana* mediated by *Agrobacterium tumefaciens* using the meristems of bulbils as explants from which new plants are formed by organogenesis. The vector used contains the reporter genes GUS and GFP. The strains of *Agrobacterium* used were LBA4404 and GV2260, and transformation was carried out by coculture with the bacterium, the selective agent was phosphinothricin (PPT). They found that bulbils could be a good source for regeneration and genetic transformation protocols.

Although these techniques offer the possibility of producing new improved plants with useful traits, a negative public perception of them has severely limited their development and commercialization. In Mexico, the use of GMOs is plagued by legal problems (LBOGM DOF 18-03-2005) and the tequila industry will not hear about GMOs in spite of the fact that this technology could solve their most serious problems (diseases caused by pathogenic microorganisms), and that the trans gene would not be passed to other plants since no sexual reproduction takes place in the plantations.

10.4.5.2 Aneuploidy and Polyploidy Generation

Polyploids have been reported to be more vigorous plants than their diploid counterparts and they can be related to adaptations to stressful environmental conditions (Te Beest et al. 2012). Polyploidy can be artificially induced by means of mitostatic compounds, such as colchicin and oryzalin, that inhibit the separation of chromosomes during the mitotic cell division, (Gallone et al. 2014; Miguel and Leonhardt 2011). The induction of aneuploid and polyploid plants could, therefore, be an interesting strategy for the improvement of certain crops. Aneuploid induction has been reported in *Alocasia* (Thao et al. 2003), grape (Sarikhani and Wakana 2006); and polyploid induction has been reported in different species such as *Humulus lupulus* (Roy et al. 2001) and *Vitis vinifera* (Yang et al. 2006). In agaves, both techniques have been reported; it can be useful for genetic studies and genetic improvement program.

Using para-fluorophenylalanine (PFP), an amino acid analog that alters and/or inhibits the formation of the mitotic spindle (Thao et al. 2003). Ruvalcaba-Ruíz et al. (2012), obtained trisomic plants of *Agave tequilana* in somatic embryos cultures. They found significant morphological differences between the normal and trisomic karyotypes, and that the terminal spines are longer and the leaves wider in the trisomic than in the wild plants.

Huijara Vasconselos et al. (2015) obtained the induction of polyploidy in vitro using micropropagated plants of *Agave angustifolia* and Oryzalin as mitostatic agent. The meristematic tissue was exposed to the mitostatic during 4 weeks during

the multiplication phase. The best response was with 5.0 μM, which produced 10% of shoots with increased nuclei sizes and four fluorescent signals, observed by FISH that suggest the formation of putative tetraploids. This is not a simple technique and a lot of work will be required before some viable tetraploid plants are produced.

10.5 Other Methods for Genetic Improvement

Given the difficulties mentioned above, the alternatives for the genetic improvement of agaves depend very much on the efficiency of tissue culture methods to mass propagate elite individuals, whether selected from wild or cultivated populations or genetically modified by means of mutation or genetic engineering.

Methods for in vitro culturing have been reported for several *Agave* species (Table 10.5) and, although the multiplication efficiency varies greatly from one species to another, it has been shown that most *Agave* species can be cultured in vitro and that the methods make it feasible to produce clonal lines of, at least, a few hundred plants for genetic studies and to rescue and conserve germplasm.

10.5.1 Clonal Selection of Elite Mother Plants

The difficult part, when starting a cloning program from wild populations, is the selection of the best individuals to be used as mother plants. Selecting from nature on the basis of comparative vigor or morphology presents several problems such as the impossibility of knowing the age of the plants, their physiological and health status or the environmental (such as rainfall and temperature) and ecological (such as soil and topography) conditions in which they have developed.

In the case of some agaves selection must be made at remote and, sometimes, inaccessible locations where the number of individuals might be very small. For certain types of mezcal agaves only one individual has been found after several sorties.

Selection must be based on the performance of mature individuals that manifest the trait that is sought, since young plants have not had the opportunity to show their characteristics. However, mature plants are not suitable starting material for tissue culture, since they are very hard and fibrous, their tissues tend to phenolize easily and are more likely to be infested by microorganisms and, above all, their meristematic tissues are not very responsive. In consequence, what is needed are young rhizomes attached to good mature mother plants. Whatever the situation, the best, larger and healthiest plants must be used to start lines from which second and third selection rounds can be carried out (Fig. 10.2). Another option, whenever possible, is to start from seeds grown in plots for selection purposes. The vitroplants, from several clonal lines, produced from a first generation must be planted under homogeneous conditions for a true first round of selection. None of the above are short-term strategies but they are efficient and shorter than a crossbreeding program (Arce-Montoya et al. 2006, 2007).

Table 10.5 Plant tissue culture protocols for *Agave* species

Species	Response	Explant	Medium and growth regulators	Propagation rate	References
Agave sp.	IO	Seed fragments	Callus induction: LS medium, 4.53 μM 2,4-D and 23.25 μM KIN	Not reported	Groenewald et al. (1976)
			Shoot regeneration: LS medium, 0.91 μM 2,4-D and 4.65 μM KIN		
A. sisalana	DO	Meristematic Tissue of bulbils	MS medium and 4.4 μM BA	5.4 shoots per explant	Frydrych (1982)
A. fourcroydes	DO	Stem Tissue from 2-year old field- grown plants	MSM, 0.11 μM 2,4-D and 44.4 μM BA	1–6. shoots per explant	Robert et al. (1987)
A. arizonica	IO	Bulbil explants	*Callus induction:* Basal medium MS and 1.4 μM 2,4-D.	5–10 new shoots	Powers and Backhaus (1989)
			Shoot regeneration: Basal medium MS, 5.4 μM ANA and 44.4 μM BA.		
A. cantala Roxb., *A. fourcroydes* Lem., *A. sisalana* Perrine	DO	Stolon plantlets	MS basal medium, 0.40 μM ANA, 0.49 μM IBA and 2.33 μM KIN	4.86 Multiplication index	Binh et al. (1990)
A. sisalana	DO	Rhizome	SH medium and 22.2 μM BA	12 buds per culture	Das (1992)
A. tequilana and *A. fourcroydes*	DO	Stem segments	*Shoot initiation and Multiplication:* MSM, 0.11 μM 2,4-D and 44.4 μM BA	0–30 shoots per explant	Robert et al. (1992)
A. victoriae-reginae Moore	DSE	Leaf blade explants from *in vitro* propagated plants	*Callus induction:* MS medium, L2 vitamins 1.4 μM 2,4-D		Rodríguez-Garay et al. (1996)
			Maturation or germination: Half-strength MS medium without growth regulators		
A. sisalana	I.O	Stem of bulbils	*Callus induction:* MS, BA, KIN, IAA o ANA (dates no reported)	27.7 shoots per culture	Nikam (1997)
			Shoot regeneration: MS 2.33 μM KIN		

A. parrasana	DO	Plantlets obtained from the *in vitro* germinated seed	MS medium 53.2 μM BA	48.6 shoots per explant	Santacruz-Ruvalcaba et al. (1999)
A. sisalana	IO	Rhizome and Immature leaf	*Callus induction*: MS medium, 9.05 μM 2,4-D, 4.6 μM kinetin. *Shoot regeneration:* MS medium 26.6 μM BA	25.3 shoot buds per g callus	Hazra et al. (2002)
A. *fourcroydes* Lem.	ISE	Apices of bulbils	*Callus induction:* MS with the nitrogen sources modified and 1.12 μM 2,4-D *Maturation or germination:* MS modified, 0.11 μM 2,4-D and 22.2 μM BA	17.14 embryogenic aggregates per treatment	González Oramas et al. (2002)
A. victoriae reginae	ISE	Seedling stem segments	*Callus induction:* MS medium 2.26 μM 2,4-D *Maturation or germination:* MS medium without growth regulators	50% of explants with embryogenic masses	Martínez-Palacios et al. (2003)
A. sisalana Perr. ex. Engelm.	ISE	Young shoots raised in vitro from the stem portion of the bulbil	*Callus induction:* MS medium 1.13 μM 2,4-D and 4.44 μM BA *Maturation or germination:* MS medium and 0.47 μM KIN	9.5 embryos per responding callus	Nikam et al. (2003)
A. fourcroydes, A. tequilana, A. angustifolia, A. sisalana	DO	Stem segments	*Shoot initiation and Multiplication*: MSM, 0.11 μM 2,4-D and 44.4 μM BA	Not reported	Robert et al. (2004)
A. angustifolia	DO	Stem segments	MS medium, 4.44 μM BA	Not reported	Enríquez del Valle et al. (2005)

(continued)

Table 10.5 (continued)

Species	Response	Explant	Medium and growth regulators	Propagation rate	References
A. tequilana	IO	Meristem explant	*Callus induction*: MS, 5.2 μM ANA	14.5 buds per explant	Valenzuela-Sánchez et al. (2006)
			Shoot regeneration: MS medium 1.1 μM 2,4-D, 44 μM BA		
A. tequilana Weber var. *azul*	ISE	Roots of 1 cm length	*Callus induction:* MS medium, L2 vitamins 13.6 μM 2,4-D and 1.3 μM BA	101.73 embryoid by repetition (four root pieces)	Portillo and Santacruz-Ruvalcaba (2006)
			Maturation or germination: MS medium, 500 mg L − 1 L-glutamine and 250 mg L − 1 casein hydrolysate		
A. fourcroydes var. *kitam ki*, *A. fourcroydes* var. *yaax ki*, *A. tequilana*, *A. letonae*, *A. angustifolia* var. *marginata*, *A. angustifolia* var. *macanora*, *A. sisalana*, *A. amaniensis*, hybrid H11648	DO	Stem segments	Shoot initiation and Multiplication: MSM, 0.11 μM 2,4-D and 44.4 μM BA	Not reported	Robert et al. (2006)
A. salmiana	DO	Stem explants	MS medium, 8.88 μM 6-BA and 1.43 μM IAA	20.3 shoots per explant	Silos-Espino et al. (2007)
A. vera-cruz Mill.	ISE	Leaf of seedlings	*Callus induction:* MS medium, L2 vitamins, 4.52 μM 2,4-D	46 embryos per explant	Tejavathi et al. (2007)
			Maturation or germination: MS medium, L2 vitamins, 5.37 μM NAA and 0.91 μM Zeatin		
A. tequilana Weber var. *azul*	ISE	Leaf segments of *in vitro*-maintained plantlets	*Callus induction*: Modified MS medium, L2 vitamins, 9.0 μM 2,4-D and 1.3 μM BA	557 embryos per dish. Each dish contained 4 segments of leaf.	Portillo et al. (2007)
A. cupreata, *A. difformis*, *A. karwinskii*, *A. obscura y A. potatorum.*	DO	Meristematic tissues from in vitro germinated seedlings	MS medium with different concentrations of regulators depending on the species	6.1–11 shoots per explant depending of species	Domínguez Rosales et al. (2008)

A. grijalvensis	DO	Plantlets obtained from the in vitro germinated seed	MS medium with 38.2 μM BA	Not reported	Sánchez-Urbina et al. (2008)
A. inaequidens Koch	DO	Stem sections of plants	*Shoot initiation*: 1.47 μM IBA, 44.4 μM BA *Multiplication:* MS with 13.32 μM BA	72 shoots	Aureoles-Rodríguez et al. (2008)
A. salmiana ssp. *crassispina, A. duranguensis, A. oscura, A. pigmaea* and *A. victoria-reginae*	DO	Offsets (approximately 1-year-old) collected from adult plants	IBA/BA combination	Varied depending on the IBA to BA ratio,	Ramírez-Malagón et al. (2008)
A. tequilana Weber var. *azul*	ISE	In vitro plants	MS medium L2 vitamins, 9 μM 2,4-D and 1.3 μM BA	2 ml of cellular suspension produced up to 556 somatic embryos	Santacruz-Ruvalcaba and Portillo (2009)
A. fourcroydes	DO	Rhizome shoots	*Shoot initiation:* MS medium with 4.9 μM IBA and 3.3 μM BAP *Multiplication:* MS medium with 4.9 μM IBA, 4.44 μM BAP and 3.41 μM TDZ	3.45 shoots per explant	Garriga Caraballo et al. (2010)
A. tequilana Weber var. *azul*	ISE	Leaf segments of in vitro propagated plants	*Callus induction*: MS medium 13.6 μM 2,4-D and 1.3 μM BA *Maturation or germination:* modified MS medium, 500 mg L − 1 L-glutamine and 250 mg L − 1 casein hydrolysate	18 germinated embryos per explant	Rodríguez-Sahagún et al. (2010)

(continued)

Table 10.5 (continued)

Species	Response	Explant	Medium and growth regulators	Propagation rate	References
A. angustifolia Haw.	ISE	Zygotic embryos	*Callus induction:* Quarter-strength MS medium, L2 vitamins 13.6 μM 2,4-D and 4.44 μM BA	182 embryos per embryonic axes	Arzate-Fernández and Mejía-Franco (2011)
			Maturation or germination: Half strength MS medium without growth regulators		
A. fourcroydes Lem.	DSE	tTCLs stem in vitro propagated plants	MS medium, L2 vitamins 2.26 μM dicamba or 2.07 μM picloram	92 embryos per stem (dicamba) and 81.72 (picloram)	Monja-Mio and Robert (2013)
A. hybrid 11,648	IO	Shoot tip of plantlets	*Callus induction*: MS medium, 8.88 μM BA and 1.07 μM NAA.	13.19 shoots per callus	Zhang et al. (2013)
			Shoot regeneration: SH medium 22.2 μM BA, 0.54 μM NAA and 0.49 μM IBA		
A. americana var. *oaxacensis*	DO	Stem tissues	MS	4–10 adventitious shoots	Miguel Luna et al. (2013)
A. americana	DO	Basal stems	MSI 13.32 μM BA	18.5 shoots per explant	Chen et al. (2014)
A. sisalana Perrine	ISE	Bulblets	*Callus induction*: Half-strength MS medium 13.6 μM 2,4-D, 88.8 μM BA	26.16 embryos per 250 mg embryogenic callus	Dos Santos Carneiro et al. (2014)
			Maturation or germination: Half strength MS medium, 39.96 μM BA		
Agave hybrid No. 11648	IO	Bases of the young leaves were	*Callus induction:* SH medium 13.32 μM BA 2.69 μM NAA and 0.45 μM 2,4-D	85 budding rate	Gao et al. (2014)
			Budding medium: SH medium, 6.66 μM BA 2.69 μM NAA		

A. salmiana	DO	Plantlets obtained from the in vitro germinated seed	MS culture medium, L2 vitamins, 0.18 μM 2,4-D, 44.4 μM BA	14 axillary shoot	Puente-Garza et al. (2015)
A. americana	IO	Apical meristem	*Callus induction*: MS medium 2.26 μM 2,4-D and 38.2 μM BA	74 shoots per explant	Reyes-Zambrano et al. (2016)
			Shoot regeneration: MS medium without regulators		
A. americana	IO	Meristematic tissue	*Callus induction:* MS medium, 0.11 μM 2,4-D and 44 μM BA	194.0 shoots per explant	Lecona-Guzmán et al. (2017)
			Shoot regeneration: MS hormone-free medium		
A. angustifolia	DO	Stem Tissue of selected plants	MS mineral salts, 6.66 μM BA and 5.71 μM AIA	32.7 total shoots per explant	Ríos-Ramírez et al. (2018)

2,4-D 2,4-dichlorophenoxyacetic acid, *IAA* indol-3-acetic acid, *IBA* indole- 3-butyric acid, *NAA* α naphthalene acetic acid, *KIN* Kinetin, *BA* 6 – bencylamino-purine, *MS* Murashige and Skoog (1962), *MSM* Murashigue Skoog Modificated (5 mM, KNO_3; 18 mM, NH_4NO_3), *SH* Schenk and Hildebrandt medium, *LS* Lismaier and Skoog (1965), *IO* Indirect organogenesis, *DO* Direct organogenesis, *ISE* Indirect somatic embryogenesis, *DSE* Direct somatic embryogenesis

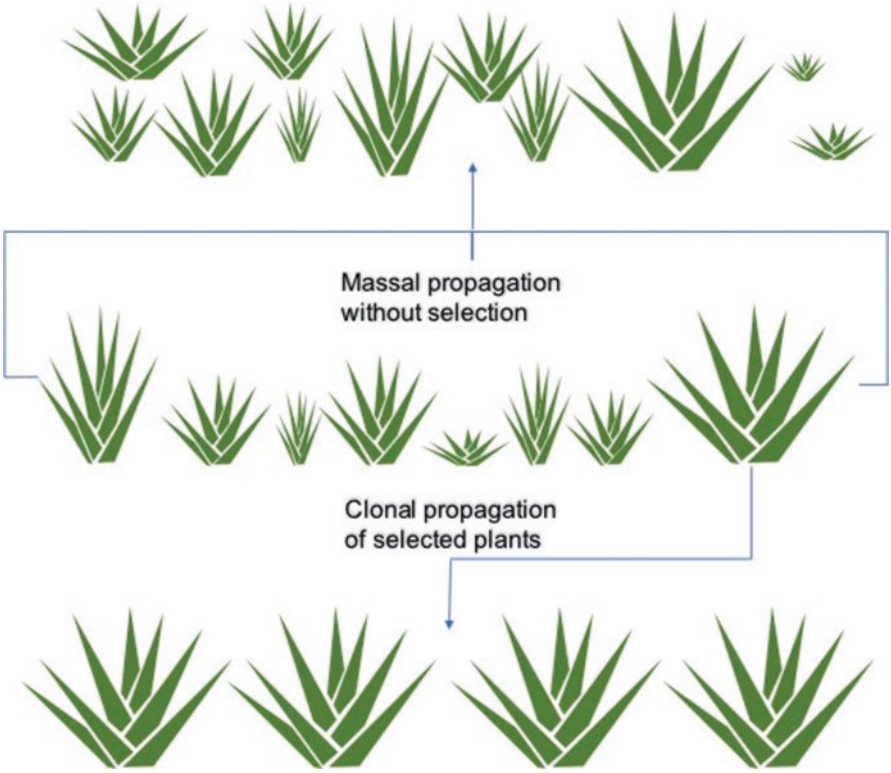

Fig. 10.2 A representative scheme that demonstrates how the selection of elite cultivars allows a greater production of biomass than if we opt for massal propagation without selection of the cultivars in the field

Starting from plantation materials is much easier and faster than starting from wild populations because there are large numbers of plants growing under relatively homogeneous conditions, but in such cases, selection must be implemented with the direct involvement of the agronomists who can identify the best plants in their parcels of land.

It must be remembered that genetic improvement is a recurrent selection process and that it must be directed to obtaining specific traits.

10.6 Micropropagation

Micropropagation is the induction and development of new plants from the cells or tissues of selected mother plants cultured in vitro. Under the right conditions, thousands of clonal plants, genetically identical (or very similar) to the mother plant can be produced in relatively short periods of time. Another advantage of micropropaga-

tion is that it can be used to eliminate pathogenic microorganisms and propagate healthy lines. Micropropagation is, therefore, used for practical aspects related to the productivity of crops. Micropropagation makes use of some PTC (plant tissue culture) techniques, mainly the culture of meristematic tissues for the generation of new plants and, in some cases, of somatic embryogenesis.

There are many reports of PTC/micropropagation protocols for a variety of *Agave* species (Table 10.5) that show that there are many ways to culture agaves and that there is ample room for applying PTC to these species. However, some protocols are more efficient than others, some lengthier than others and, probably, some are more expensive than others. Most of them do not mention the problems they might face, such as microbial contamination or morphological abnormalities and are, therefore, not very useful for large-scale micropropagation. The reports cover different *Agave* species (like *A. tequilana*, *A. fourcroydes*, *A. angustifolia* and *A. sisalana*) from many different explant tissues (such as bulbils, rhyzomes, seeds and roots) and include the use of different culture media [like MS: Murashige and Skoog (1962), SH: Schenk and Hildebrandt medium and LS: Linsmaier and Skoog (1965)], wide ranges of concentration of different auxins (including 2,4-dichlorophenoxyacetic acid, indol-3-acetic acid, indole-3-butyric acid and α naphthalene acetic acid), cytokinins (including 6-bencylaminopurine and kinetin), sugars and vitamins (such as MS vitamins and LS vitamins). Many of these protocols are specifically designed for certain species and can be the basis for large-scale protocols or used for in vitro germplasm conservation.

10.6.1 Large-Scale Propagation of Agaves

The production of a few hundred, even thousands of plants can be done in any reasonably well-equipped laboratory through standard techniques. However, the scaling up to tens and hundreds of thousands of plants requires very fine-tuned procedures, very well-trained people, special laboratory facilities to avoid contamination and, above all, a well-designed production system to avoid bottlenecks and to minimize costs and loses. Plant quality and health are essential considerations when thousands of plants are being produced for plantations.

Large-scale propagation is, basically, a commercial enterprise carried out by industrial companies in specialized labs, popularly known as bio-factories, which are specially designed for this purpose and, therefore, require well-defined protocols for every species. Standardized Operational Procedures (SOP) must describe with precision every manipulation, sterilization and disinfestation, preparation of the culture media (concentration of mineral salts, vitamins, growth regulators and sucrose), and the process (number of cultivation cycles, multiplication rates, time required for each stage), aspects of plant quality (no generation of somaclonal variants and healthy plants), costs (reagents, number of hours worked, number of hours used per cabin, use of autoclave, liters of medium required per lot of production, number of plants produced weekly, monthly and annually). Furthermore, the propa-

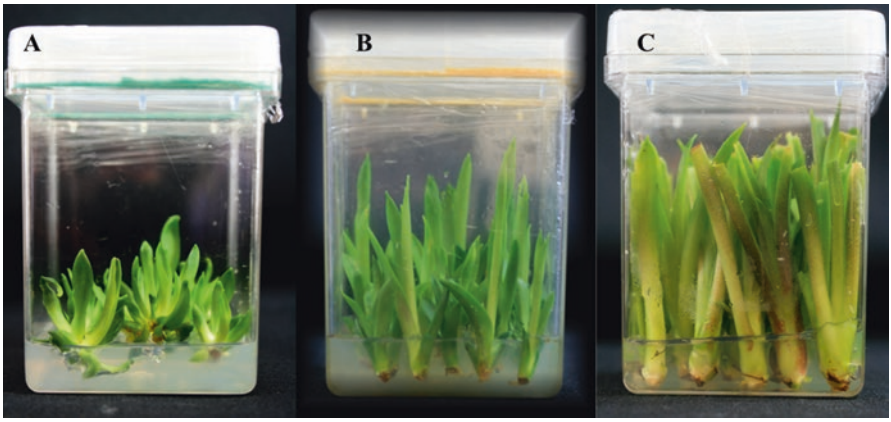

Fig. 10.3 Different stages of the micropropagation process of *Agave*. (**a**) Stage of multiplication, (**b**) Stage of maturation, (**c**) Stage of rooting

gation protocols are species-specific, making it necessary to consider the critical aspects of each stage and species (Fig. 10.3).

10.6.2 Genetic Variation in In Vitro Cultured Plants

Using AFLP as a molecular marker, González et al. (2003) detected genetic differences between mother plants and their somatic embryogenesis-derived plantlets of *Agave fourcroydes* cultured in vitro. However, Díaz-Martínez et al. (2012) using AFLP, did not observe variations between the original explant and plants of the four next generations of in vitro cultured plants of *A. tequilana*. Torres-Morán et al. (2010) using ISTR as marker found that *A. tequilana* plants can be grouped according to the propagation method employed (somatic embryogenesis or proliferation of axillary buds), and that plants were not genetically identical within each group.

10.6.3 Mezcal as an Example

Plantations of agaves used for mezcal beverage production have only recently begun to be established from micropropagated materials. In 2016, a collaboration program was established, between industries and research institutions, in order to rescue, select and micropropagate as many varieties as possible. Nearly 600,000 plants from all collected types have been propagated, although with different degrees of efficiency, and adapted to soil in greenhouses before being established in nurseries and, finally, in experimental plantations (Fig. 10.4) from which a second round of selection is being carried out.

Fig. 10.4 (**a**) Selection of an elite cultivar of mezcal Arroqueño, (**b**) In vitro tissue culture of mezcal plants, (**c**) Establishment of nurseries of mezcal obtained from in vitro culture, (**d**) Field mezcal plants originating from micropropagation

10.7 Rescue of Endangered Species and Germplasm Conservation

Many agave species are endemic to the mountains and arid regions of Mesoamerica and North America, particularly Mexico where 75% of all known *Agave* species are found (Gentry 1982; Martinez-Palacios et al. 1999). Because of their long-life spans, slow growth, low reproduction rates and economic value as raw material for industrial products and ornamental uses, these species are more susceptible to suffer genetic erosion due to overexploitation. There are many studies that show that a worrying loss of species and diversity has occurred in Mexico over the last 20–30 years. The development of alternative ways of exploiting commercially valuable species without affecting the natural resource need to be implemented as do programs for conservation in situ (national laws forbidding the exploitation of the endangered species), ex situ (botanic gardens and nurseries as the examples given below) and in vitro (slow growth or cryopreservation techniques). Some efforts to rescue and establish agave germplasm collections are described below.

10.7.1 Germplasm Collections

There are important general collections of *Agave* species in Mexico such as the Ethno Botanical Garden in Oaxaca, The Botanical Garden at The National Autonomous University in Mexico City and The Regional Botanical Garden Roger Orellana at CICY in Merida, Yucatan.

Other International collections worth pointing out are: the Desert Botanical Garden in Phoenix, Arizona, USA and the Royal Botanic Gardens at Kew in London, England and, as already mentioned elsewhere, there are many commercial nurseries that conserve many species of ornamental agaves (https://www.ranchotissue.com/category/agave/).

10.7.2 Germplasm Collections of Agaves used for Mezcal Production

In recent years there has been a large increase in international demand for mescal beverages, which has, in turn, greatly increased the need for raw material. Since producers have traditionally used plants from the wild the resource has been over-exploited producing a scarcity of plants for the whole industry.

The need for raw material has led Destileria Los Danzantes, a Oaxaca-based mezcal producer, to initiate a micropropagation program to mass propagate selected elite individuals, not only to satisfy the need for plants but to improve yields, make the whole process more efficient and less costly and to help in the conservation of some of the species used for mezcal production.

The most important and most difficult part of the strategy is the adequate selection of mother plants from the wild. An additional complication is that some types are extremely rare and, in some cases, only one individual has been recovered. A few individuals of all collected varieties in the Danzantes project are being maintained ex situ at the Universidad Autonoma de Chapingo (UACH), Campus Oaxaca and in vitro at CICY.

INIFAP keeps 25 ex situ introductions of 8 species at the Banco de Germoplasma de Maguey Mezcalero at Campo Experimental Valles Centrales de Oaxaca and UACH has established another collection in the State of Tlaxcala (Agencia Quadratín 2014) that will keep maguey Manso, Púa Larga, Chalqueño y Ayoteco, for the extraction of fructose syrups and agua miel (sap). In situ conservation of Jalisco's agaves is a current concern of ethnobotanists (Colunga-GarcíaMarín and Zizumbo-Villarreal 2007).

The establishment of in vitro storage techniques for agaves has been carried out by Pérez Molphe Balch et al. (2012). The use of mannitol and sorbitol as osmotic agents have made it possible to keep the cultures of several species for a period of 10 months without any significant loss of viability or regenerating capacity.

10.7.3 East Africa Germplasm Banks

Many of the hybrid lines generated at Mlingano during Lock's years were lost when sisal research came to a halt in East Africa after Tanzanian independence. However, farmers in Kenya kept some promising lines and a large, though unattended, collection, was kept, at ARI Mlingano (CFC 2006). During the CFC/UNIDO project, two large blocks of hybrids, mainly from the *Agave angustifolia* X *A. amaniensis* crosses and a third block of different species were cleaned and fertilized. Rhyzomes, when available, were transplanted to a fourth block (Fig. 10.5) behind the tissue culture lab where they have been maintained. Table 10.6 shows the experimental parameters of some of the 85 varieties and hybrids that were in Block D at the end of the project in 2005: H. 9I and *Agave amaniensis* had taller plants with longer leaves when compared to the other varieties. Average leaf production however was low for *A. amaniensis*. Regarding leaf production, *A. verschafeltii* showed the highest number of leaves per plant followed by H. 91 and H.7l.

Another small collection that included the most promising types was established at KEPHIS Muguga near Nairobi in Kenya (Fig. 10.5) but agaves are not a priority for the inspectorate and no information was available about its present status.

Mlingano still keeps some 80 accessions in its collection and is therefore the main source of existing germplasm for hard fiber producing agaves. However, there is a risk of further losses due to the lack of rhizomes to replant the lines and tissue culture might be, once again, the only a means of maintaining this invaluable asset. There have been recent calls for action in this respect from research and industry headquarters in order to support the plans announced by the Tanzanian Sisal Board to increase Tanzania's production to 100,000 mt by the year 2021 (Sembony 2017; Tanzania Invest.com 2016).

10.7.4 Embrapa

The Brazilian Agricultural Research Corporation (Embrapa) has an *Agave* collection containing 37 *A. sisalana* and *A. fourcroydes* accessions, maintained in vivo in a semiarid environment, in the Cariri region of Paraíba, Brazil. Phenotypic descriptors, based on morphological and agronomic traits are periodically recorded. Knowledge of the genetic diversity of the collection is limited, although such information is essential to estimate the potential of the genotypes for later use in any breeding program (Chaves de Souza et al. 2018).

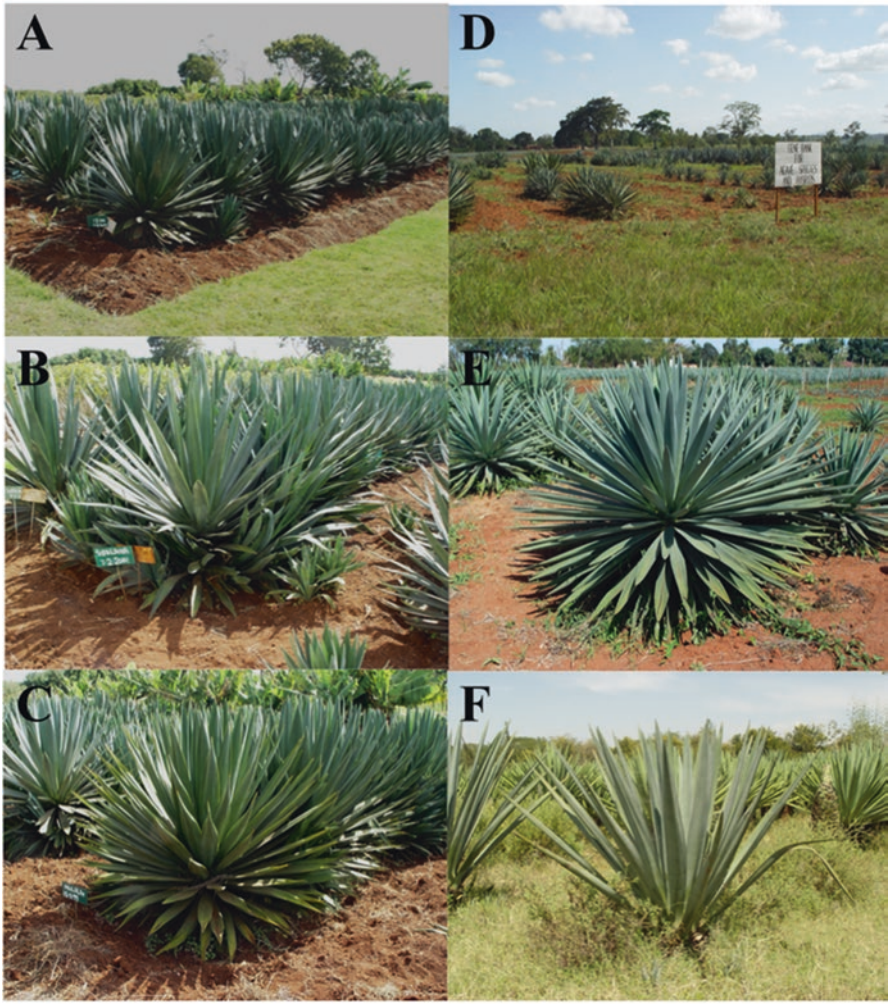

Fig. 10.5 (**a**) Germplasm bank at KEPHIS Muguga, Kenya, (**b**) *Agave sisalana*, (**c**) *A. mulolah*, (**d**) ARI germplasm bank at Milingano, Tanzania, (**e**) *A. verschafelti*, (**f**) *A. amanuensis*

10.7.5 Impact of Micropropagation on Variability

There is a great deal of criticism about the impact of micropropagation on the populations of wild agaves, arguing that clonal lines will severely reduce their variability. We would like to emphasize, however, that plantations planted with micropropagated plants include, not one, but many different clonal lines and that these plantations remove the strain from the overexploitation of natural populations. Additionally, micropropagation is being used to generate lines of a few hundreds of plants from individuals that are very scarce.

Table 10.6 Early assessment of selected clones from the gene bank

Variety	Mean leaf number	Leaf length (cm)	Leaf width (cm)	Plant height (cm)
H. 11,648	5	89.9	10.8	127.8
H 83366	7	97.4	n.a.	134.0
Mlola 1	4	77.8	9.0	106.6
H 91	8	112.6	12.1	155.6
H 62025	7	107.7	10.25	139.5
Agave verschafeltii	11	80.9	9.0	121.2
H 71	8	103.5	10.0	144.9
A. amaniensis	2	119.9	11.5	153.8

10.8 Conclusions and Prospects

Because of several anatomical, physiological and genetic characteristics, agaves are very difficult to improve genetically by traditional crossbreeding methods and that is why other methods, such as mutation breeding, polyploidization and genetic engineering need to be incorporated. The use of genetic engineering could be particularly useful for the production of disease- resistant varieties, However, the social perception of GMOs, at least in Mexico, has prevented its use to try to combat the bacterial and fungal diseases that periodically ravage the tequila plantations and it is likely that it will continue to find strong oppositions from certain sectors in the near future.

This leaves selection of elite individuals and cloning through tissue culture as the main alternative for genetic improvement. This strategy has proven to be very effective when applied to some of the agaves used for hard fiber and alcohol production in Mexico. There are many reports on agave tissue culture that show that these techniques can be applied to many different *Agave* species but the main problem for their adoption has been the slightly higher price of the micropropagated plants compared to the naturally generated ones in the plantation. This is without any consideration of the economic benefits generated by the higher yields at the end of the production cycle because these will only come many years later.

More efficient methods are required but, even more importantly; it is necessary to have a broader germplasm base to select from. The latter makes it essential to rescue and conserve in situ, in vivo and in vitro the variability of the natural populations, as well as the scarce breeding lines that exist.

The future economic importance of some agaves is highlighted by the fact that they are a source of natural biodegradable products such as cellulose for various uses (cordage, mats or paper) and even for clean biofuel production. Because of their capacity to cope with limiting water conditions, agaves are potentially useful crops for the future either as an alternative to cultivate on arid lands or to combat climate change and soil erosion.

Until now, the use of a few *Agave* species as raw material sources for hard fibers (henequen, H11648) and alcoholic beverages (tequila, mescal and pulque) have been of economic importance to only a few countries, particularly Mexico, Brazil,

Kenya, Tanzania and, more recently China. However, as water becomes scarcer for agriculture and there is an increased likelihood of climate change producing drastic and unpredictable global environmental changes, agaves should be considered as an option for cultivation on arid lands in many different parts of the world.

Appendices

Appendix I: A List of Major Institutes Engaged in Agave Research

Institution	Specialization and research activities	Contact information and website
Centro de Investigación Científica de Yucatán, A.C.	Research and Teaching. Biotechnology and Molecular Biology of Agaves	Manuel L. Robert Calle 43 N° 130, Unidad de Biotecnología, Chuburná de Hidalgo, Mérida, Yucatán 97,200, México robert@cicy.mx
Universidad de Matanzas "Camilo Cienfuegos".	Research and Teaching. Studies in Plant Biotechnology and Agriculture.	Silvia Alemán García Centro de Estudios Biotecnológicos (CEBIO). Autopista a Varadero Km 3.5. Matanzas. CP 40100. Cuba. silvia@cdict.umtz.edu.cu
Universidad Michoacana de San Nicolás de Hidalgo	Research and Teaching. Ecology, Biotechnology, Evolution in Plants.	Alejandro Martınez-Palacios Instituto de Investigaciones sobre Recursos Naturales. A.P. 12, Morelia, Michoacán 58,041, México marpal@zeus.ccu.umich.mx
Universidad Nacional Autónoma de México (UNAM)	Research and Teaching. Studies in Conservation, Ecology, Botany and Climate change.	Abisaí Josué García Mendoza Instituto de Biología Circuito exterior s/n, Ciudad Universitaria, Copilco, Coyoacán A.P. 70-233 México, Distrito Federal. C.P. 04510 abisai@ib.unam.mx
Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco, A.C. Mexico	Research and Teaching Institute. Plant Biotechnology	Benjamín Rodríguez-Garay Camino el Arenero Núm. 1227, Colonia El Bajío del Arenal, C.P. 45019, Zapopan Jalisco, México brodriguez@ciatej.mx

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Institution	Specialization and research activities	Contact information and website
Universidad Nacional Autónoma de México (UNAM)	Research and Teaching Institute. Plant evolution	Luis Enrique Eguiarte Fruns Laboratorio de Evolución Molecular y Experimental Departamento de Ecologia Evolutiva, UNAM, México. Teléfono: 5622-9006 fruns@unam.mx http://web.ecologia.unam.mx/laboratorios/evolucionmolecular/index.php/es
Centro Universitario de Ciencias Biológicas y Agropecuarias, Universidad de Guadalajara	Research and Teaching Institute. Biotechnology and Agriculture	Liberato Portillo Martínez Apartado Postal 1-139, Zapopan, Jalisco 45101, México. Teléfono y fax (52-33) 3777-1192, portillo@cencar.udg.mx
Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional CINVESTAV-Irapuato, Gto.	Research and Teaching Institute Areas of research: Alcoholic beverages, Agave as a crop, Fructans and Inulins	Mercedes Guadalupe López Pérez Km. 9.6 Libramiento NorteCarr. Irapuato-León 36,824 Irapuato Gto. México mercedes.lopez@cinvestav.mx
Centro de Investigación en Alimentación y Desarrollo, A.C	Research and Teaching Institute. Agave Biotechnology	Martín Esqueda Valle Carretera a La Victoria km 0.6. Col. Ejido La Victoria Hermosillo, CP. 83,304, Sonora Tel 662-2892400 ext. 326, esqueda@ciad.mx
Bangalore University	Research and Teaching Institute. Agave Biotechnology	D. H. Tejavathi Department of Botany, Bangalore 560,056, India, e-mail: Tejavathi_hanu@yahoo.com
Instituto Potosino de Investigación Científica y Tecnológica, AC, División de Ciencias Ambienltales	Research And Teaching Institute. Agaves as Biofuels	Elías Razo Flores Camino a la Presa San José 2055, Lomas 4a. Sección, San Luis Potosí, San Luis Potosí C.P. 78216, México erazo@ipicyt.edu.mx
Chinese Academy of Tropical Agricultural Sciences	Research Institute. Tropical agricultural technology developments	Lingling Lv South Subtropical Crops Research Institute. Zhanjiang 524,091 2 China lianzi9381@yahoo.com.cn
Central Research Institute for Jute & Allied Fibers	Research Institute. ICAR-CRIJAF is a premier institute on jute & allied fiber crops	S.K. Hazra Crop Improvement Division, Barrackpore-743,101 24- Parganas (N), West Bengal, India crijaf@ca12.vsnl.net.in

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Institution	Specialization and research activities	Contact information and website
University of California, Los Angeles UCLA	Research and Teaching Institute. Physicochemical Biology and Plant Physiological Ecology	Park S. Nobel Department of Ecology and Evolutionary Biology 621 Charles E. Young Drive, South Los Angeles, Ca 90,095-1606 USA psnobel@biology.ucla.edu
Universidad Autónoma del Estado de México-Campus "El Cerrillo"	Research and Teaching Institute. Agave Biotechnology	Amaury M. Arzate-Fernández Laboratorio de Biología Molecular Vegetal, Facultad de Ciencias Agrícolas, Carretera Toluca-Ixtlahuaca, km 11.5 entronque al Cerrillo Piedras Blancas. 50,200, Toluca, Estado de México amaury1963@yahoo.com.mx
The Samuel Roberts Noble Foundation.	Research Institute. Agaves as bioenergy crops	L. L. Escamilla-Treviño Plant Biology Division 2510 Sam Noble Parkway, Ardmore, OK 73401, USA llescamilla@noble.org
James Cook University	Research and Teaching Institute. Agave as biofuel	Joseph A. M. Holtum Tropical Plant and Agricultural Science School of Marine and Tropical Biology,, Townsville, Qld 4811, Australia joseph.holtum@jcu.edu.au
Universidad Autónoma Agraria "Antonio Narro". Unidad Saltillo.	Reseach and Teaching Institure. Biotechnology and Agriculture	F. Aureaoles Rodriguez Departamento de Horticultura Buenavista, Saltillo, Coahuila. México. C. P. 25315. fabyaureoles@yahoo.com.mx
University of Illinois	Research and Teaching Institute. Bioenergy crops	Sarah C. Davis Energy Biosciences Institute and Department of Plant Biology University of Illinois at Urbana-Champaign, Urbana, IL, USA, davissc@illinois.edu
Agricultural Research Institute	Research Institute. Agriculture and Biotechnology	Shabani Hamisi Tissue Culture Lab. Mlingano P.O. Box 5051,Tanga, Tanzania. Cell Phone: +255,754,387,593 e.mail: matimIingano@hotmail.com

Appendix II: Genetic Resources Maintained in Agave Germplasm Collections

Species and varieties	Uses	Germplasm collection location
Botanic Gardens		
Wild and ornamental species	Genetic conservation, research and Leisure	Desert Botanic Garden, University of Arizona, Phoenix, Arizona, USA.
Agave angustifolia Haw.	Conservation, research and education	Jardín Botánico Roger Orellana, Centro de Investigación Científica de Yucatán (CICY), Mérida, Yucatán, México
A. fourcroydes, A. tequilana, Hybrid 11,648, *A. sisalana, A. angustifolia* var. *Letonae*		
A collection of more than 100 *Agave* species representing 75% of Mexico	Conservation and education	Jardín Botánico, Universidad Nacional Autónoma de México (UNAM), Cd. México, México
Wild and ornamental species	Education and leisure	Jardín Etnobotánico. Oaxaca, México
Wild and ornamental species	Conservation, research and leisure	The Royal Botanic Gardens, Kew, UK
Germplasm collections at Research Institutions		
Agave rodhacantha Trel.	Conservation and propagation of varieties employed for mezcal production	Banco de germoplasma de maguey mezcalero, Instituto Nacional de Investigaciones Agricolas Forestales y Pecuarias (INIFAP), Campo Experimental Valles Centrales de Oaxaca, México.
A. potatorum Zucc.		
A. marmorata Roezl.		
A. karwinkii Zucc.		
A. convallis Trel.		
A. angustifolia Haw.		
A. americana L. var. *oaxacencis Gentry,* var. *chino verde,* var. *Mexicano penca larga, var tobasiche,* var. *madrecuishe,* var. *cincoaeño,* var. *lumbre,* var. *cuishito,* var. *mexicano sin espinas,* var. *caballo,* var. *mexicano penca angosta,* var. *rubescens* (SalmDick*) H Gentry, A. seemanniana Jacobi*		
A. angustifolia Haw., var. *espadin; A. americana* L. var. *mexicano penca larga*, var. *tobasiche*, var. *madrecuishe*, A. cincoaeño,	Conservation and propagation of agaves employed for mezcal production	Universidad Autónoma de Chapingo (UACH), Campus Oaxaca, México
A. salmiana, var. *maguey manso*, var. *pua larga*, var. *chalqueño*, var. *ayoteco*	Agaves employed to extract *aguamiel* and for the production of fructose syrups	Universidad Autónoma de Chapingo (UACH), Campus Tlaxcala, México

(continued)

Species and varieties	Uses	Germplasm collection location
Hard fiber producing agaves		
A. americana x *A. amaniensis*	This Site maintains, for conservation and research purposes, the remaining species and hybrids produced at Amani and Mlingano by G.W. Lock and G. Daughty	Gene Bank (Block D) Agricultural Research Institute (ARI), Mlingano, Tanzania
A. ameniensis		
A. amaniesis variegated		
A. angustifolia		
A. cantala		
A. lespenassei		
A. lespinassei x *A. cantala*		
A. sisalana		
A. spectabils		
A. verschafeltii		
Bubu Ex- Kulasi Iraddiated sisal no.2015 Irradiated sisal no.3011 Non-flowering sisal No. 32 Ex Thika Non-flowering sisal No. 7 Balam		
69 Hybrids: Outstanding: H1300, H11648 4 Mlola hybrids		

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Chapter 11
Sugarcane (*Saccharum* spp.): Breeding and Genomics

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Abstract Sugarcane (*Saccharum* spp.) is cultivated and credited worldwide for its ability to synthesize and store exceptionally high concentration of sucrose. Since prehistoric times sugarcane cultivation has undergone many transformations into present-day noble cane. Initially, selection of desirable clones and interspecific hybrids brought many agronomically-useful traits into the cultivated species. Wild related species played a major role as the donor for most of the desirable traits through gene introgression. Pre-breeding strategies and intergeneric hybridization have played a crucial role in development of noble high-yielding canes. Cultivated sugarcane has been further enriched with other valuable traits such as high fiber, high fermentable sugar and biotic and abiotic stress tolerance. Despite its genomic complexity, crossability barriers within the genus, long breeding and selection cycles, etc., remarkable progress has been achieved to develop a wide range of cultivars, hybrids and mutants suitable for different agroclimatic conditions. Germplasm collections, preservation and their utilization for development of an ideotype bearing desirable traits has become a research priority. For this purpose, molecular-marker tools are acting as potential drivers during pre-breeding and selection of

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desirable progenies. Supplementary tools such as in vitro culture, isolation of somaclones, induced mutagenesis and transgenics have played an essential role in the generation of novel genetic variability. In recent decades much emphasis has been given to enrich sugarcane breeding strategies; however, future avenues need to be focused on the utilization of inexhaustible and wealthy sugarcane genomic resources and advancements made through biotechnological interventions.

Keywords *Saccharum* · Genetic · Ideotype breeding · Mutagenesis · Interspecific hybrids · Somaclonal variation · Synteny · Transgenics

11.1 Introduction

Sugarcane (*Saccharum* spp.) is cultivated mostly in tropical and subtropical regions of the world, primarily for its ability to synthesize and store exceptionally high concentrations of sucrose in its stem or stalk. Since prehistoric times, sugarcane has been used as the main source of sugar, and lately for biofuel and cogeneration purposes. There is increased interest by sugarcane breeders and researchers to target genetic improvement for sucrose content using conventional and modern approaches to produce more sugar per unit land area. The world's cane sugar industries are driven solely by plants of interspecific hybrids of *Saccharum* species, which have almost entirely replaced the noble canes, *Saccharum officinarum* L. Sugarcane crop improvement began with years of extensive and rigorous cross-hybridizations and selections to obtain intergeneric and interspecific hybrids, ultimately resulted in a manyfold increase in global sugar production. Sucrose derived from sugarcane juice contributes to more than 70% of the world's sugar consumption; the rest comes from the sugar beet (Lakshmanan et al. 2005). Hence, in the context of global trade and economy, sugarcane remains an important food commodity, as well as a valuable industrial and bioenergy resource (Mirajkar et al. 2017).

Present-day commercial sugarcane cultivars are the result of exploitation of interspecific hybridization, primarily pioneered in Java (Price 1963; Stevenson 1965), which has transformed the sugarcane crop improvement across the globe. Recently the rate of genetic gain has been reduced mainly due to the decreasing genetic base. In this context, Hawkes (1977) stated that a broader genetic base can be introgressed from wild species and distinct primitive cultivars for crop improvement. It is therefore imperative to overcome the limitations of conventional crop improvement approaches in order to expand the genetic base by investing in underutilized clones of cultivated plants and distinct wild species in breeding programs (Arceneaux 1965).

11.1.1 Evolution and Distribution

The origin of *Saccharum officinarum* L. is believed to be in New Guinea, the Malayan Archipelago or the Melanesian and Polynesian islands (Mukherjee 1957). Basically, sugarcane is considered an ancient crop dating back to around 2500 B.C., as a garden plant. Among the *Saccharum* species, *S. spontaneum* L. led to the origin of other *Saccharum* species (*S. officinarum*, *S. robustum* Brandes & Jesw. ex Grassi, *S. sinense* Roxb. and *S. barberi* Jesw.) through introgression with associated grass genera. Also *S. spontaneum* has been utilized as a donor for several desirable traits in breeding sugarcane varieties adaptive to various adverse environmental conditions. The species *S. robustum* and *S. officinarum* share natural distribution areas and are restricted to the Papua New Guinea, Indonesia and the Pacific region. Before the introduction of interspecific hybrids, sugarcane cultivars native to the North Indian subtropical zone have been classified as *S. barberi*. During the course of evolution, genes responsible for diverse environmental adaptability, and to an extent resistance against diseases and pests, have been accumulated in these primitive cultivars (Sreenivasan 2004). Now sugarcane is grown in numerous countries over an area of 26.5 million ha, accounting for approximately 0.54% of the world's total cultivated land area (FAOSTAT 2013). Presently, commercial cultivation of sugarcane has spread across the continents, predominantly in South America (Brazil), Asia (India, Pakistan, China, Thailand), North/Central America (USA, Mexico), the West Indies and Australia.

11.1.2 Taxonomy

The taxonomic classification of the *Saccharum* genus is challenging, and has been revised several times (Amalraj and Balasundaram 2006a; Bor 1960; Hackel 1883; Hitchcock 1951; Pilger 1940). Cultivated sugarcanes originate from extensive hybridizations and have a high mutation rate. Therefore, cultivated sugarcane has its unique taxonomic status due to the presence of *synthetic species* that often complicate its taxonomic identity and the delimitation of species (Amalraj and Balasundaram 2006a). Taxonomically, all modern cultivated sugarcanes are placed under Saccharinae (subtribe), along with their 9 progenitor genera (*Imperata*, *Eccoilopus*, *Eriochrysis*, *Spodiopogon*, *Erianthus*, *Miscanthidium*, *Narenga, Miscanthus*, *Sclerostachya*). Saccharinae belongs to the tribe Andropogoneae, which in turn is placed under the subfamily Panicodeae and family Poaceae (Gramineae) (Amalraj and Balasundaram 2006a).

The term *Saccharum complex* was introduced by Mukherjee (1957) to represent the closely-related interbreeding group comprised of the genera *Saccharum*, *Erianthus*, *Sclerostachya* and *Narenga*. Genus *Miscanthus* sect. *Diandra* Keng was then added by Daniels et al. (1975). Several other classifications are also often followed, however, the genera *Saccharum*, *Sclerostachya, Narenga*, *Erianthus* and *Miscanthus* are important based on their morphological similarity, breeding behavior, hybridization compatibility and evolutionary relationships. Most of present-day cultivated sugarcane hybrids are derived primarily from crossings between *S. officinarum* and *S. spontaneum*; however, *S. barberi, S. robustum* and *S. sinense* have also contributed to the development of some of modern sugarcane varieties (Dillon et al. 2007).

11.1.3 Economic Importance and Nutritional Attributes

Sugarcane is an important global cash crop occupying more than 28.4 million ha of arable land, with an annual production of about 2013.7 million mt (FAOSTAT 2016) (Table 11.1). This makes sugarcane the world's largest biomass-producing crop plant, with an average yield potential of 70.91 mt/ha, surpassing the major cereal crops which occupy several folds more arable land (FAOSTAT 2016). Brazil is the world's largest producer of sugarcane followed by India, China, Thailand and Pakistan. In India, the livelihood of over 35 million farmers, and an equal number of agricultural laborers, depends on sugarcane cultivation and associated farm practices. Hence, the sugar industry is the second largest agro-based industry in India, second only to textiles. There are around 493 sugar factories in operation to crush 303.6 million mt of cane (ISMA Statistics 2016). The sugar industry generates multimillions of government taxes in terms of excise duty and revenues annually that aids India's economic growth. Sugarcane also provides scope and inputs for small-scale industries such as *gur* (*jaggery*) and *khandsari* industries which contribute to meet the needs within the territory of the country or the states. Thus sugarcane is the only raw material for all the major sweeteners produced in India. Besides consumption of sugarcane juice and raw sugar, there are several foods

Table 11.1 Comparison of sugarcane production, area harvested and yield in top five countries in the world, 2016

Region	Production (million mt)	Production share (%)	Area harvested (million ha)	Yield (mt/ha)
Brazil	768.7	38.2	10.2	75.2
India	348.4	17.3	5.0	70.4
China	245.7	12.2	3.3	73.5
Thailand	87.5	4.3	1.3	65.4
Pakistan	65.5	3.3	1.1	57.9
Total	2013.7	–	28.4	–

Source: FAOSTAT (2016)

items, beverages and confectioner that use sugar derived from sugarcane as a sweetener. In many parts of the cane-growing countries, sugarcane is also used a source of animal feed and forage.

Nutritionally, sugarcane juice contains carbohydrates (27.51 g), protein (0.27 g), calcium (11.23 mg), iron (0.37 mg), potassium (41.96 mg) and sodium (17.01 mg). In the Ayurvedic system of Indian medicine, both roots and stems of sugarcane are used in the treatment of infections related to the skin and urinary tract, besides bronchitis, anemia, constipation and general weakness. Sugarcane juice has also been shown to have several biological effects which include, immunostimulation (El-Abasy et al. 2003), anti-thrombosis activity (Molina et al. 2000), anti-inflammatory activity (Ledon et al. 2003), vaccine adjuvant, modulation of acetylcholine release (Barocci et al. 1999) and anti-stress effects (Brekhman et al. 1978) and raising of innate immunity levels (Lo et al. 2005). Sugarcane juice has been shown to have high antioxidants and protection of plasmid DNA as well as enhanced survival of *Escherichia coli* cultures exposed to gamma irradiation (Kadam et al. 2008). It is suggested that sugarcane juice could be as useful as a radio-protector.

11.2 Genetic Resources and Conservation

11.2.1 Genetic Complexity of Sugarcane

Saccharum species present wide variations in their ploidy levels (Table 11.2). *Saccharum officinarum* is basically an octoploid (2n = 80) with x = 10 chromosomes, a representative of the basic chromosome number of members of the Andropogoneae tribe (Cesnik and Miocque 2004; D'Hont et al. 1995). However, *S. spontaneum* has x = 8 chromosomes (D'Hont et al. 1996) but represents 5 different cytotypes (2n = 62, 80, 96, 112 or 128) (Daniels and Roach 1987; Sreenivasan et al. 1987). Modern cultivated sugarcane cultivars are allopolyploid hybrids (Daniels and Roach 1987) that have a 2n + n genomic constitution mainly because of 2 sets of the *S. officinarum* genome and a single set of the *S. spontaneum* genome (Cesnik and Miocque 2004). The *Saccharum complex* hypothesis is that there has been intercrossing between members of the genera *Saccharum*, *Narenga, Sclerostachya* and *Miscanthus* sect. *Diandra* established the basis of modern sugarcane varieties (Daniels and Daniels 1975; Mukherjee 1957; Roach and Daniels 1987). *Saccharum officinarum* was likely derived from crosses involving *S. spontaneum*, *S. arundinaceus, S. robustum* and *Miscanthus* (Roach and Daniels 1987). However, later molecular investigations of the genera in the *Saccharum* complex provided clues for the taxonomy rearrangements as many of the divisions appear to be polyphyletic (Hodkinson et al. 2002).

Before the introduction of interspecific hybridization, sugarcane improvement was solely based on the selection of naturally-occurring variants of *Saccharum officinarum,* often as a result of collection during expeditions at its

Table 11.2 Diversity of genomic constitution of *Saccharum* species and their distinct characteristics

Species	Chromosome number (2n)	Distinct characteristics
S. officinarum (noble canes)	80	Source of genes for sucrose accumulation, all milling qualities such as low fiber, juiciness, easy detrashing, thick and erect stem etc.
S. barberi (North Indian canes)	111–120	Cultivated canes of subtropical India; well adapted to adverse growth conditions such as drought, waterlogging, low temperature; suitable for jaggery making
S. sinense (Chinese canes)	81–124	Have all the characteristics of *S. barberi*, cultivated in China
S. robustum	60, 80	The closest wild relatives of *S. officinarum*, very low sugar and high fiber, use to breed the sugarcane varieties with high fiber
S. spontaneum	40–128	Wild species, widely distributed in Asia and Africa, adapted to stress situation, many clones are resistant to major diseases and pests, use as a parent in sugarcane breeding program as source of genes for adaptability
S. edule Hassk	60–80	Similar to *S. robustum* but with aborted inflorescence

center of origin in New Guinea. Later, during the nineteenth century, *S. officinarum* was one of the variety substitutions primarily because of susceptibility to diseases which resulted in tremendous yield losses and that restricted crop adaptation. The discovery of fertility in sugarcane, pioneered in Java and Barbados, led to the beginning of the nobilization program (Stevenson 1965). The process of crossing wild species with *Saccharum officinarum* and their subsequent improvement through backcrossing with *S. officinarum* is referred to as nobilization (Parthasarathy 1948). Although this nobilization program was initially restricted to improving disease-resistance traits, it continued with the development of better novel varieties, enriched with the available genetic pool for future breeding programs. Sugarcane breeding initiatives underwent several spectacular transformations with incorporation of the wild species *S. spontaneum* during late 1800s and early 1900s, in order to combine the desirable disease-resistance traits into the derived hybrids and cultivars.

The necessity to expand the genetic base, which threatened to stagnate yield, raised interest in pre-breeding, the early phase of any breeding program that utilizes exotic germplasm for introgression of desirable characters from wild and closely-related genera (Frankel 1989). The importance of pre-breeding to obtain high yield, wide adaptability against different biotic and abiotic stresses, along with other biological and economic constraints, were emphasized during the 1980s (Ladizinsky 1989; Stalker 1980) (Fig. 11.1).

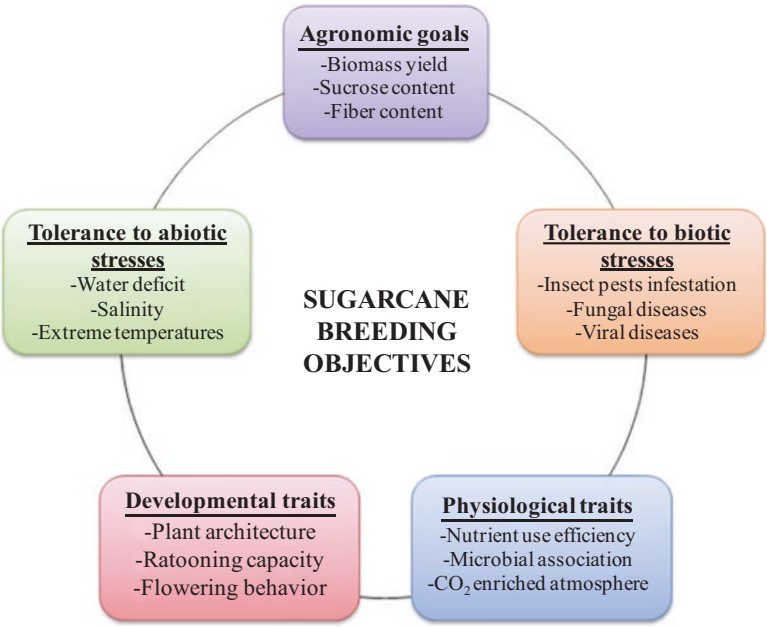

Fig. 11.1 Sugarcane breeding objectives for introgression of desirable traits

11.2.2 Genetic Resources

Sugarcane genetic resources are primarily stored at two world repositories, the Sugarcane Breeding Institute (SBI) in India and the World Collection of Sugarcane and Related Grasses (WCSRG) in Miami, Florida, USA. The WCSRG has more than 1002 accessions of valuable alleles for biomass, important agronomic traits and stress resistance (Nayak et al. 2014). The SBI is regarded as the world's largest *in situ* germplasm collection center which functions independently and/or in joint collaboration with other national agencies for expeditions and collection of different species of *Saccharum* and allied genera. At SBI, the genetic resources are conserved by three different approaches:

(a) Field gene bank: most *Saccharum* species (*S. officinarum, S. barberi, S. sinense, S. robustum, S. edule*) are being maintained at the SBI Research Centre, Kannur, Kerala, while the wild species such as *S. spontaneum, Erianthus* spp. and associated genera are maintained at Coimbatore, Tamil Nadu. Some of the clones collected from high elevations (*S. spontaneum* clones from Arunachal Pradesh, *E. fulvus* and *Miscanthus nepalensis* collected from Meghalaya), are being maintained at the Indian Agricultural Research Institute (IARI) Regional Station, Wellington, Tamil Nadu (Table 11.3).

Table 11.3 Sugarcane germplasm and improved genetic stocks maintained at SBI, India

(A) Sugarcane germplasm holding at SBI, India			
Location	Species/genera	No. of Indian accessions	No. of ISSCT accessions
SBI, Coimbatore	*Saccharum spontaneum*	878	–
	Erianthus spp.	313	–
	Allied genera	41	–
SBI, Kannur	*S. officinarum*	–	757
	S. robustum	–	145
	S. barberi	–	42
	S. sinense	–	30
	S. spontaneum	305	79
	Erianthus spp.	–	132
	Allied genera	88	20
SBI, Agali	*S. officinarum*	130	–
IARI, Wellington	*S. spontaneum*	47	–
	Erianthus rufipilus	5	–
	Miscanthus nepalensis	3	–
Total		1810	1205
(B) Improved genetic stocks of sugarcane maintained at SBI, India			
Location	Details		No. of clones
SBI Research Center, Kannur	Indian hybrids		1031
	ISSCT[a] hybrids and subsequent introductions		614
	Indo-American (IA) clones		130
SBI Research Center, Agali	Improved clones of *S. spontaneum*		97
	Improved clones of *S. barberi*		21
	Interspecific hybrids		94
	NDHF[b] (hybrid clones)		141
Total			2128

[a]*ISSCT* International Society of Sugar Cane Technologists
[b]*NDHF* National Distant Hybridization Facility

(b) Seed bank: SBI has a designated center, the National Active Germplasm Site (NAGS), where genetic stocks are registered and released/reported as commercial varieties are conserved as active collections for reference and distribution to bonafide researchers. As of April 2016, 226 registered germplasm and reported varieties are being maintained in the field. Every year polycross true seeds of *S. spontaneum* and National Active Germplasm (NAG) varieties are collected, tested for germination and viable seeds stored in the National Gene Bank at National Bureau of Plant Genetic Resources (NBPGR), New Delhi.

(c) In vitro conservation: An in vitro culture facility has been established at SBI's Kannur Research Centre for conserving germplasm.

11.2.3 Conservation

Global sugarcane germplasm collection is organized and maintained at the Miami world collection center and at Kannur in India. It is believed that the Miami collection is not optimally located in a stress-free environment, whereas the Kannur center is better situated (Berding et al. 2004); the Indian world collection is located at the Sugarcane Breeding Institute (SBI) Research Centre, in the field gene bank. This center has developed more than 2600 Co (Coimbatore) selections, which occupy the majority of the sugarcane cultivation areas in India (Amalraj and Balasundaram 2006b) (Fig. 11.2).

There is a greater risk of biotic and abiotic stress factors with field-based conservation methods and hence, in vitro conservation methods have been suggested as the preferred method for sugarcane germplasm conservation (Chandran 2010; Glaszman et al. 1996; Taylor and Dukic 1993). In vitro germplasm storage has been demonstrated using different long- and short-term options using whole plantlets, or cryostorage of in vitro shoot apices (Banasiak and Snyman 2017; Martínez-Montero et al. 2008). Whole tissue cultured plantlets maintained under a slow-growth regime of 18 °C and 24 °C on media supplemented with different levels of sucrose, abscisic acid for over 4 years, allowed recovery and multiplication of shoots (Banasiak and Snyman 2017). These approaches also employed cryopreservation as a method of preservation of in vitro shoot meristems with good survival rates (41.7–69.4%).

11.3 Utilization of Wild Related Species in Sugarcane Improvement

11.3.1 Sugarcane Breeding

The success of sugarcane breeding relies on the generation of segregating-progeny populations, obtained by crossing two or more contrasting individuals, followed by continuous selection and multiplication of superior clones. Since prehistoric times, clones of *Saccharum sinense* and *S. barberi* have been used for sugar production in China and India, respectively. These include clones well-adapted to subtropical areas and characteristically tolerant to extreme temperatures, drought and waterlogging; ensured their use in those areas until recently. Several investigations have clearly showed that related wild plant species are important reservoirs of genes for resistance against several biotic and abiotic stress factors accumulated during evolution and natural selection (Ming et al. 2010; Shrivastava and Srivastava 2012). Several useful genes for resistance to diseases or pests have been identified in the wild-related species of sugarcane that can be introduced into cultivated varieties by conventional plant breeding methods. Despite its complex polyploidal genomic

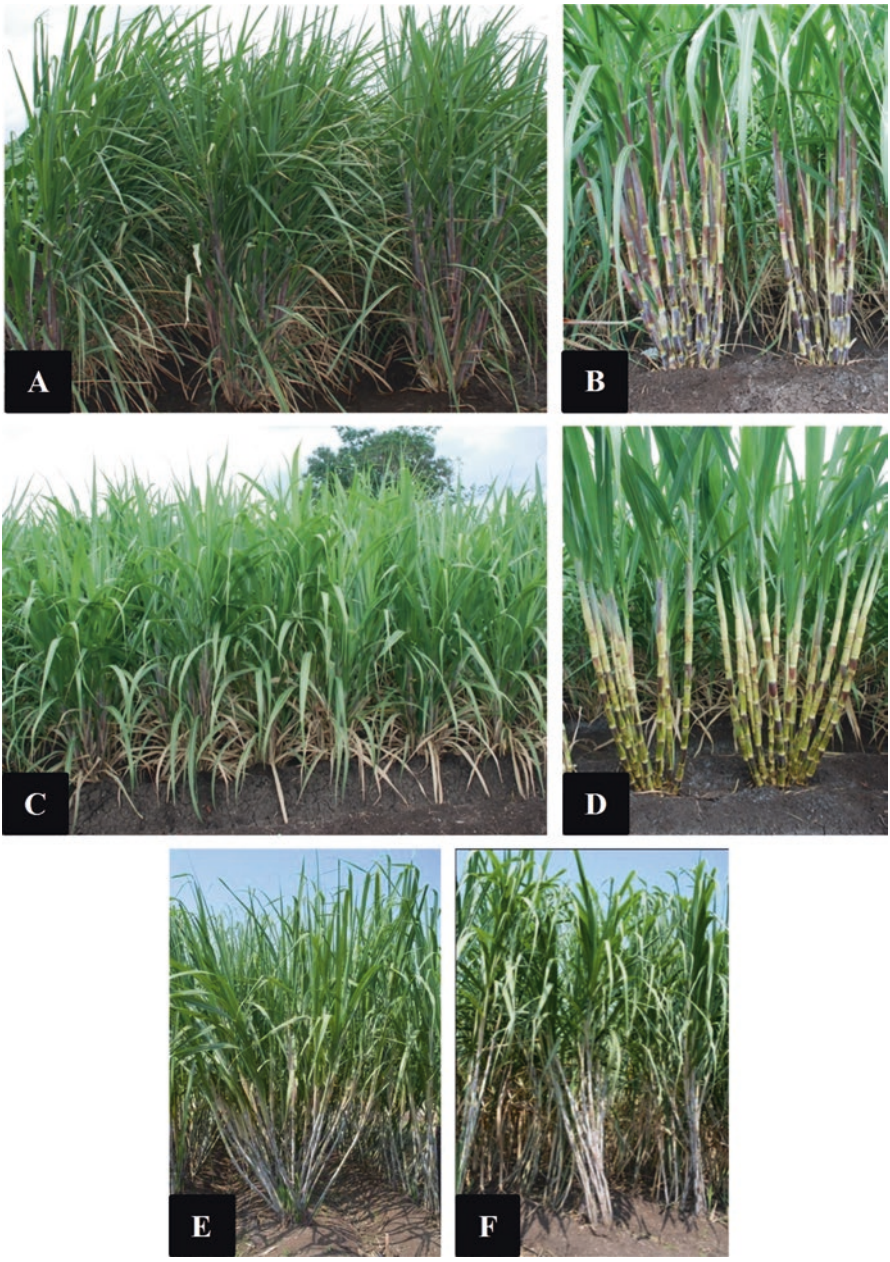

Fig. 11.2 Some of the commercially-grown Indian sugarcane varieties. (**a–b**) Co 86032, (**c–d**) CoC 671, (**e–f**) VSI 08005

configuration, the sugarcane genome is favorable to the transfer of useful genes from related wild species into the cultivated varieties. The transfer of desirable traits from the wild *Saccharum* species and related genera to acceptable commercial interspecific hybrids (ISH) involves multiple cycles of crossing, selection, backcrossing and evaluation of progeny populations (Srivastava et al. 1999).

A major breakthrough in sugarcane improvement was achieved by the use of the wild species such as *Saccharum spontaneum* in breeding. Modern sugarcane varieties are complex interspecific hybrids (ISH) derived from the combination of two or more *Saccharum* species. They have high productivity and adaptability mainly conferred by the presence of traits from *S. spontaneum* (Ram and Sahi 2000). Wild germplasm available in India has been characterized over the years for various attributes and potential sources for important traits have been identified. *Saccharum spontaneum* and *Erianthus arundinaceus* are considered the most potent wild sources for the varietal improvement of sugarcane; *S. spontaneum* is a primary source for high productivity, adaptability and tolerance against pests and diseases. It is also endowed with the natural ability to withstand severe adverse conditions including cold, salinity, drought and waterlogging. *Saccharum robustum,* the immediate progenitor of *S. officinarum*, is a source for fiber, yield and waterlogging-resistance traits. *Erianthus arundinaceus* is also an important source for fiber and is now being used as an alternative for wood pulp in the paper industry (Amalraj et al. 2008).

11.3.2 Utilization of Saccharum Germplasm

The earliest attempts to utilize the wild germplasm of sugarcane were undertaken in Java and India. Initially, interspecific hybridization between *Saccharum officinarum*, *S. spontaneum* and to some extent *S. barberi* was adopted (Roach 1977; Stevenson 1965). Subsequent backcrossing of these interspecific hybrids to *S. officinarum* and thereafter the intercrossing among the nobilized progenies led to the generation of outperforming and promising commercial varieties. Interest in interspecific hybridization was revived in the 1960s, following the realization that the cultivated varieties have a narrow genetic base drawn from a limited number of original species and clones. Interspecific hybridization remained the backbone of breeding programs to improve and sustain sugarcane productivity (Roach 1972).

The nobilization program of sugarcane carried out in India, the USA, Australia, the West Indies and other countries suggested that maintenance of higher genetic divergence within the breeding pool through pre-breeding is necessary to drive positive gains through breeding programs (Babu and Ethirajan 1962; Hapase et al. 2010; Walker 1971). In many countries significant progress was achieved through the use of wild species, particularly *S. spontaneum*, to impart higher productivity, resistance to diseases and pests, and tolerance against abiotic stresses, into commercial cultivars. In India, breeding programs to expand the genetic base were initiated during the 1980s, wherein new cycles of interspecific and intergeneric crosses were made by utilizing hitherto underutilized germplasm and the hybrids were screened for

their yield and quality potential (Amalraj and Balasundaram 2006b). The success of the base broadening programs in India is reflected in the release of a number of Co series varieties like Co 95017, Co 97010, Co 97015, Co 97016, Co 97017, Co 98003, Co 99007, Co 99016 and Co 2000-05, with ISH parentage. Similar work on utilization of interspecific hybrids (ISH) in the crossing program were initiated at the Sugarcane Breeding Centre, Vasantdada Sugar Institute (VSI), Amboli, Maharashtra, India and found ISH 154 as a potential donor for drought and salinity along with significant yield improvement (Hapase et al. 2010).

The use of genetic resources in sugarcane breeding has been largely confined to *Saccharum officinarum* and *S. spontaneum*. Indigenous *S. barberi* canes of India which have been grown for centuries in the country are highly adapted to the adverse climatic conditions of subtropical India (Nair 2011). The use of *S. barberi* proved successful during the early years of breeding research in India that led to the development of several early varieties like Co 213, Co 244, Co 312 and Co 313. These varieties have a *S. barberi*-derived genetic background and their success can be attributed to the contribution of highly acceptable *S. barberi* to their genetic makeup. Utilization of this species was revived during the 1980s after proper characterization at different locations in India (Nair 2011). Potential clones of *S. barberi* were crossed to *S. officinarum* and commercial varieties. The hybrids showed potential as commercial varieties and better adaptability to the abiotic stresses.

Saccharum robustum has been extensively used in the Hawaiian breeding program and many commercial varieties like H37-1933 have *S. robustum* genealogy. However, utilization of this species in other countries had been limited. The species has been evaluated in India for agronomic and disease/pest tolerance. It showed better tolerance to waterlogging, besides being resistant to major pests and thus holds promise as potential germplasm. In India, interspecific hybridization and selection within the species resulted in the development of *S. robustum* clones with better sucrose (Nair 2008). These clones were crossed to *S. officinarum* and commercial varieties and the progenies expressed advantage in terms of sucrose and other agronomic traits (Yu et al. 2018).

11.3.3 Other Trait-Specific Germplasm

Available germplasm has been screened for agronomic traits and tolerance to certain biotic and abiotic stress conditions and as potential sources for desirable agronomically-important traits have been identified (Table 11.4). Apparently, the sugarcane germplasm available today is highly variable (Ming et al. 2010). The genetic variability required for the improvement of the crop in terms of productivity, stress tolerance and product diversification is readily available in the germplasm represented by *Saccharum* species, derived hybrids and related genera (Nair 2011; Srivastava and Srivastava 2000). In the absence of any crossing barrier among the

Table 11.4 Sugarcane germplasm identified as potential sources for different agronomically important traits

Trait	Germplasm	
	Species	Hybrids
Yield/productivity	*Saccharum officinarum*: NG 77-171, 51NG 0957, 57 NG 116, 51 NG 115G, 28 NG 266, NG 77-42	H 382915, Q50, B43337, B 45116, H 507000, B 45116, CP 49-50
Sucrose content	*S. officinarum*: Creoula rayada, 57 NG 174, Saipan G, Seleri, Selemi, Bali, Striped Mauritius, Chrystalina, Chittan	PR 1095, PR 1085, PR 905, PR 1056, B 45285, CP 62251, CP 63384, CoC 671, Co 94012, CoJ 64, Co 62126, Co 888, Co 887, Co 62198
Early sucrose (7 months)	*S. officinarum*: 57 NG 174, Azul De Caza, Oramboo, 57NG 155	Co 62175, Co 785, Co 513, Co 805, Co 958, Co 1290, Co 8231, Co 62100, Co 8371, Co 99006, B54-142, H63-361, CP 49-50, CP 63-361, H63-361, H50-7209, H52-3689, H53-263, H59-3775, H49-3553, PT 4352
	S. barberi/*S. sinense*: Lalri, Kansor, Kavangiri	
Drought tolerance	*S. officinarum*: Gungera, 57 NG 73, IJ 76-412, IS 76-564, Caledonia ribbon	Co 7336, Co 8367, Co 99008, Co 94008, Co 8368, Co 8371, Co 8372, Co 87016, Co 955, Co 99004, ISH 100
	S. barberi/*S. sinense*: Nargori, Lalri, Mangwa sic, Matna Shaj, Pararia Shaj, Mcilkrum Reha, Lalkhadi, Kalkya, Kheli	
	S. robustum: NG 77-79, 57 NG 19, NG 77-146, NG 77-23, 57 NG 27	
Cold tolerance	*S. barberi*/*S. sinense*: Kalkya, Manjuria, Pararia Shaj	Q 63, PR 1016, B44-130, Co 8339, Co 1148
	S. spontaneum: SES 114, SES234A, IND 81-144, IND 81-165, IND 81-80	
Red rot resistance	*S. officinarum*: Baragua, Seleri, Saipan G	Bo 91, Co 62175, Co 7314, Co 94008, Co 62198, Co 86249
	S. spontaneum: many clones	
	Erianthus spp.: IK 76-78, IK 76-88, IK 76-99, IJ 76-383, IJ 76-365, IJ 76-383, IJ 76-384, IJ 76-400	
Smut resistance	*S. officinarum*: Awela Green Sport, Bamboo, Fiji 64, HItam Broewang, Timor Riet, Vellai, NC 17, NC 92, 28NG 57, 57 NG 186, 57 NG 251, IJ 76-504	Several clones
Resistance to multiple pests	*S. barberi*/*S. sinense*: Katara barah, Mangwa sic, Rekha, Hemja, Maneria	B44-130, Cp 29-116, Cp 33-409, TUC 472, CP 33-425, LF 65-4233

members of the *Saccharum complex*, interspecific and intergeneric gene transfer is a distinct possibility in conventional breeding of sugarcane. Precise characterization of the germplasm is essential to optimize its utilization. The world collection has been largely characterized for agro-morphological traits in India and the information in this respect has been well documented and disseminated (Shrivastava and Srivastava 2016). Within the context of the latest development in plant molecular biology, it is appropriate to carry out allele mining in *Saccharum* species and related wild genera with respect to important traits like high productivity, biotic and abiotic stress tolerance, biomass production and sucrose accumulation (Balsalobre et al. 2017; Nair et al. 2002; Selvi et al. 2003). Wild germplasm also holds promise for locating sugarcane specific genes and promoters for developing transgenic sugarcane (Shrivastava and Srivastava 2016).

11.3.4 Utility of Related Genera

Over the years, several hybrids have been derived by intercrossing different *Saccharum* species and allied genera (Jankiammal 1938, 1941; Jankiammal and Singh 1936; Kandasami 1964; Li et al. 1948; Nair et al. 1999). Serious efforts to utilize *Erianthus* spp., which is an important source for higher biomass production as well as for pest and disease resistance, are currently underway in several countries including India. *Erianthus* spp. was crossed with *S. officinarum*, *S. robustum* and *S. spontaneum* and hybrids were obtained and characterized for their agronomic potential. Crosses were made with *Erianthus* spp. as male and female parents. Juice sucrose was found to be significantly higher in *S. officinarum* X *Erianthus* crosses, compared to the reciprocal combination. The hybrids were backcrossed to commercial varieties and there was progressive improvement in sucrose content in the backcross. Molecular markers were found useful in following the introgression of the *Erianthus* spp. genome in the hybrids and their backcrosses (Nair et al. 2006). Among the related genera, *Miscanthus* holds promise as a source for high biomass, disease resistance and cold tolerance.

Erianthus spp. are wild perennial grasses (Poaceae) related of sugarcane. Their biomass production is high and tolerance to drought and infertile soil are superior because of their large and deep root systems. Furthermore, sprouting in ratoon after harvesting is superior and multiple ratoon cultivation is possible. Therefore, *Erianthus* spp. represent important breeding material for further improvement and for the development of new types of sugarcane which can increase food and energy production under inferior environmental conditions. Intergeneric hybridization has made a breakthrough in sugarcane that favored incorporating commercially-important traits and to impart hybrid vigor to commercial cultivars. *Erianthus arundinaceus* is a species with desirable characters such as good vigor, rationing ability, disease resistance and adaptability to stress conditions. Production of true intergeneric hybrids is hindered by limitations imposed by asynchronous flowering times and anthesis, and incompatibilities associated with wide hybridization.

Earlier researchers crossed *Saccharum* X *Erianthus* and also used *S. spontaneum* as a bridge species. The Vasantdada Sugar Institute (VSI), Pune, India has performed some crosses involving *Saccharum* X *Erianthus* as well as reciprocal *Erianthus* X *Saccharum*. The intergeneric hybrids with *Erianthus* X *Saccharum* observed with n + 2n chromosome transmission with improvement in cane diameter and sucrose content as compared to *Saccharum* X *Erianthus* crosses (Ann Rep VSI 2013–14).

11.4 Scope and Prerequisites of Sugarcane Ideotype Breeding

The varieties normally evolved to suit sugar production are of high cane-yielding potential with high sugar content, possessing resistance to important diseases and pests so as to satisfy the need of both farmers and sugar mills. However, if sugarcane varieties have to be identified for other special purposes like production of ethanol, the breeding and selection strategies will have to be directed towards clones having a high amount of total fermentable solids (TFS) i.e. high Brix. If the objective is to breed varieties suitable for cogeneration, then clones with high fiber content are required (Alexander 1985). In the program to identify energy cane in Puerto Rico, Alexander has selected high-fiber cane, which also gave high total dry matter yield of about 80 mt/ha. The variation in fiber and sugar content of different *Saccharum* species has given ample scope for the production of cane suited to specific uses. In order to support cogeneration and ethanol production, there is a need for breeding varieties capable of high biomass with high fiber content and high total sugars. The varieties also need to have wide adaptation to broad range of environments.

11.4.1 Breeding for High Biomass Production

Saccharum spontaneum and the related *Erianthus arundinaceus* have high fiber content and low sucrose; *E. arundinaceus* occurs in wild forms and produces a high amount of biomass in a short period, has multi-ratooning potential and is resistant to pests and diseases. It has a Brix of 2.3–10, sucrose of 1.4–7.5% (extraction of 39.1–63.1%), fiber of 21.3–31.9%, stalk yield of 15–51 kg/plant, total biomass of 25–62 kg/plant and total biomass of 173 mt/ha. A mt of bagasse generates about 2.5 mt of steam. On average *Erianthus* spp. can yield up to 125 mt/ha of canes and 173 mt of biomass production. It is evident that breeding varieties for high biomass production is possible with parent genetic stock available within sugarcane hybrids; interspecific hybrids and progenies involving *E. arundinaceus* (Heinz 1988). In Cuba, the first generation sugarcane interspecific hybrids (ISH) between *S. officinarum* and *S. spontaneum* are cultivated as energy gardens which provide raw material to boilers during the start of crushing season.

11.4.2 Breeding for High Fiber Content

In general, clones of *S. spontaneum, S. robustum* and *Erianthus* spp. have high fiber content. While the varieties cultivated for sugar production possess around 12–14% fiber, clones of *S. spontaneum* have about 25–30% fiber. Interspecific hybrids involving *S. spontaneum* and cultivated varieties have around 20–25% fiber. High to moderate heritability has been reported for fiber content by a number of researchers. Fiber has a strong negative correlation with juice extraction percentage (Senthil Kumar et al. 2015).

Sugarcane represents one of the most efficient cropping systems for conversion of solar energy into biomass, which can provide both solid fuel for combustion to produce heat, steam or electricity and easily fermentable juice which can yield ethanol for use as fuel (Ohara et al. 2005; Senthil Kumar et al. 2015). Hence, the future of sugar industry depends on the use of sugarcane for not only sugar, but other products such as alcohol, electricity, etc. Hence sugarcane breeders have challenges before them to develop varieties suitable for cogeneration and ethanol production and adapted to marginal land and low-input conditions. Probably this could be achieved through the use of new genetic stocks possessing high biomass, total sugars and high fiber content in breeding programs. In the past, sugarcane breeding programs were carried out with an emphasis on enhancing extractable sucrose yields vis-a-vis retaining adequate levels of fiber content, resistance to diseases and pests, and several other desirable agronomic traits. However, high fiber content was often considered disadvantageous because it increases costs for transport and mill processing. Although, compared to wild relatives, sugarcane ancestral species and related grasses, fiber content of cultivated sugarcane varieties is much lower. Seemingly, the approaches to achieve high fiber content and biomass yield along with other attributes are feasible through existing modern sugarcane breeding programs (Jayabose et al. 2017). Substantial genetic resources for sucrose and fiber content exist in the parental species that served as the basis for the present-day cultivars (Table 11.5).

Table 11.5 Diversity among *Saccharum* species, hybrid cultivars and related genera for sucrose and fiber contents

Species	Common name	Sucrose content (% f. wt)	Fiber content (% f. wt)
S. officinarum	Noble	High (18–25)	Low (5–15)
Saccharum spp.	Cultivars	High (15–20)	Low (10–15)
S. barberi	Indian	Medium (13–17)	High (10–15)
S. sinense	Chinese	Medium (12–15)	High (10–15)
S. robustum	Wild species	Low (3–7)	Very high (20–35)
S. spontaneum	Wild species	Very low (1–4)	Very high (25–40)
Saccharum spp. hybrids	Hybrids	Low	High
Erianthus spp.	*Erianthus*	Very low	Very high

Source: Moore and Botha (2014)

11.4.3 Breeding for Energy Cane

The concept of *energy cane* proposed by Alexander (1985) allows distinguishing sugarcane management systems and production purposes. Ordinary sugarcane cultivation system focuses on producing cane for sugar production alone; however, the energy cane concept favors cultivation and producing cane for energy production (Matsuoka et al. 2014). Crop breeding strategies to achieve this goal are certainly different and can be categorized as Types I and II (Tew and Cobil 2008) (Table 11.6). Based on this, breeders can choose suitable genetic diversity to develop energy-cane varieties. Type I energy-cane varieties have both sugar and fiber content, and can be processed in existing sugarcane mills. However, Type II energy canes have higher fiber content and are difficult to process with existing sugar-mill operations but are suitable for electricity generation and for cellulosic fermentations to produce biofuels (Tew and Cobil 2008).

Utilization of bagasse for generation of electricity as a renewable resource is widely adopted by many countries. Cogeneration based on bagasse and cane biomass is a safe, viable and self-sustainable alternative to supplement the electricity requirements of a country. Currently, India has cogeneration capacity of about 2200 MW and this is likely to be increased to 5000 MW to meet growing demand (Vision 2030, SBI Coimbatore). Simultaneously, cogeneration can also generate around 50 million carbon credits. Therefore, to meet these challenges cane production and productivity should be enhanced (Amalraj et al. 2008). In this context, the focus of sugarcane crop improvement strategies should place more emphasis on increasing biomass yield, fiber and sucrose content *per se*. However, the fact is that both cogeneration and ethanol production solely rely on sugar production, as the respective feedstocks are generated as by-products of sugar production. This means that every unit increase in sugar production corresponds to an increase in cogeneration and ethanol production (Soloman 2014).

11.4.4 Utilization of Wild Species as Potential Source for Biomass and Bioenergy

Genetic improvement in sugarcane has been chiefly focused on improving sugar yield and stress tolerance (Ming et al. 2010). However, the potential of sugarcane and related grasses as feedstock for biofuel and energy generation is under serious

Table 11.6 Diversity in composition among sugarcane and Types I and II energy canes

Composition	Sugarcane (%)	Type I energy cane (%)	Type II energy cane (%)
Water	75	70	65
Fiber	12	17	30
Sugar	13	13	5

consideration now in view of the high biomass potential of sugarcane and related grasses (Somerville et al. 2010). Sugarcane can produce nearly 100 mt/ha biomass, which is twice or more than any other crop can produce. The capacity of related grasses like *Erianthus* spp. and *Saccharum spontaneum* to produce high biomass is equally high. *Erianthus* spp. can be grown under suboptimal conditions and is adapted to drought and waterlogging conditions, where its biomass yield can reach 100 t/ha. *Erianthus* spp. has 25–30% fiber content along with wet bagasse yield of about 60 mt/ha, that is almost double of bagasse yield of sugarcane (32 mt/ha). Moreover, *Erianthus* spp. can be regarded as ideal candidates for energy crop that has multi-ratoonability, tolerance to biotic and abiotic stress and high fiber content (Hattori and Morita 2010). *Miscanthus* spp. are already grown in Europe as an energy crop while switch grass is popular in USA for energy generation. Being a tropical grass, *Erianthus* spp. have a significantly higher potential as an energy crop as it yields large biomass and has better adaptability (Amalraj et al. 2012). The *Erianthus* spp. germplasm at the Sugarcane Breeding Institute (SBI), Coimbatore, has been introduced for large-scale cultivation as a raw material for the paper industry. Attempts were also made to further improve the fiber content of *Erianthus* spp. by selfing the high fiber *Erianthus* spp. clones and progenies with 30% fiber which were identified. Energy canes with high biomass potential also have been developed by introgressing *S. spontaneum* and *Erianthus* spp. into commercial varieties. Type I energy canes with >20% fiber and >15% sucrose and Type II energy canes with >25% fiber and <15% sucrose have been developed and currently are under trials in factory locations for feasibility to be grown as energy plantations. The high biomass potential of *Erianthus* spp. also renders them as ideal candidates for the production of cellulosic ethanol (Jayabose et al. 2017).

11.5 Biotechnology in Sugarcane Improvement

Biotechnology applications for sugarcane improvement were initiated in the 1960s with the establishment and refinement of in vitro culture and regeneration systems (Heinz and Mee 1969; Nickell 1964). Subsequently, developments were made in the areas of molecular-marker technology, structural and functional genomics, as well as genetic engineering to introgress novel traits. There have been substantial advancements in sugarcane biotechnology pertaining to crop breeding and improvement, mainly to establish in vitro culture and regeneration, transgenics, molecular diagnostics, development of genetic maps and understanding of the molecular basis of sucrose accumulation (Lakshmanan et al. 2005; Suprasanna et al. 2011). These technologies have become useful to circumvent the limitations of conventional sugarcane breeding strategies (Dal-Bianco et al. 2012; Devarumath et al. 2013a, b; Sengar et al. 2011; Suprasanna 2010; Suprasanna et al. 2011).

11.5.1 In Vitro Plant Regeneration

To effectively utilize biotechnological tools, an efficient and reliable in vitro plant regeneration system is necessary (Guiderdoni et al. 1995; Lakshmanan 2006; Suprasanna and Bapat 2005). In vitro culture systems have been developed for the isolation of somaclones (Heinz et al. 1977; Larkin and Scowcroft 1981), in vitro micropropagation (Lee 1987), production of disease-free plants (Irvine and Benda 1987) and genetic transformation (Arencibia et al. 1997; Bower et al. 1996). Micropropagation using apical meristems has been utilized in sugarcane breeding programs to reduce the time required to multiply promising varieties and clones, as well as to restrict the spread of pathogens. The major goals of somatic embryogenesis in sugarcane are: (i) development of cost-effective and efficient regeneration methods for mass propagation of true-to-type planting material in a short time and (ii) development of a cell culture and regeneration system amenable to genetic transformation. Somatic embryogenesis can be achieved in embryogenic callus cultures derived from young leaves (Brisibe et al. 1994; Ho and Vasil 1983), immature floral inflorescences (Liu 1993) and direct organogenesis from young leaves (Fitch and Moore 1990). Embryogenic cultures have been utilized for the production of virus-resistant plants through somaclonal variation (Oropeza et al. 1995), induced mutagenesis, in vitro selection (Patade et al. 2006; Suprasanna et al. 2006), and transgenic plants (Arencibia et al. 1998; Bower et al. 1996).

In vitro regeneration in sugarcane can be accomplished through direct or indirect organogenesis (Lakshmanan 2006). Direct organogenesis can be achieved by shoot-tip culture (Lee 1987) or leaf discs (Irvine and Benda 1987). Refinements of in vitro regeneration protocols have been made in order to optimize conditions for an efficient in vitro morphogenesis that can accelerate large-scale micropropagation and genetic transformation (Snyman et al. 2000). A large-scale embryogenesis system can greatly benefit sugarcane by way of maintenance and conservation of long-term cultures, cell suspension cultures with enhanced regeneration ability and control over undesirable somaclonal variations (Suprasanna et al. 2005).

11.5.2 Genetic Variability Through Induced Mutations

The scope of somaclonal variation in sugarcane crop improvement has increased in recent decades with the availability of multiple alternatives to establish efficient plant-regeneration protocols and methods to identify and characterize genetically-variant lines (Lakshmanan et al. 2005; Patade and Suprasanna 2008). For the first time, Larkin and Scowcroft (1983) isolated sugarcane somaclones resistant to eye spot disease and, subsequently, Sreenivasan et al. (1987) isolated somaclones bearing rust resistance. Somaclones susceptible to sugarcane mosaic virus were also obtained through somatic embryogenesis (Oropeza et al. 1995). The scope of

induced genetic variability arising from in vitro regeneration and stability of variants in subsequent generations has been extensively studied, e.g. somaclones resistant to eye spot (Larkin and Scowcroft 1983; Leal et al. 1996) and rust diseases (Sreenivasan and Jalaja 1998). Detection of somaclonal variation at an early stage of growth is useful for quality assurance in tissue culture, transgenic plant production and rapid clonal propagation. Somaclonal variation along with in vitro mutagenesis followed by in vitro selection has been successfully employed to isolate salinity and drought tolerant sugarcane mutants in a short duration (Suprasanna et al. 2006).

Induced mutagenesis has played a key role in the incorporation of desirable genetic variability, which is otherwise difficult to achieve through other conventional tools (Suprasanna et al. 2015). Induced mutagenesis can overcome the constraints of conventional breeding e.g. narrow gene pool, loss of vigor, poor fertility, complex genome and the long breeding/selection cycle that are collectively impeding further improvements (Suprasanna et al. 2011). To date, there are over 3200 registered mutant varieties of different crops, released worldwide for cultivation (Suprasanna et al. 2014, 2015). Although only 13 mutant varieties were developed through induced mutagenesis in sugarcane (Mutant Variety Database FAO-IAEA 2018), this demonstrates the successful use of induced mutagenesis and the need for sustained efforts for its use in sugarcane improvement.

In vitro cell, tissue culture and regeneration systems are widely useful in crop improvement. Besides their potential application for rapid clonal propagation, these have been beneficial for obtaining disease-free planting material, germplasm collection and storage, etc. Spontaneous occurrence of somaclonal variation was reported for the first time in sugarcane (Heinz and Mee 1969; Larkin and Scowcroft 1981), which has the potential for broadening genetic variability. In order to enhance the frequency of induced genetic variation and to obtain beneficial modifications in cultivars, physical and chemical mutagens have been applied to in vitro cultures (Suprasanna 2010). Embryogenic callus cultures are now widely adopted as a good system, both for mutagenesis and in vitro selection aimed at development of desirable mutants (Patade and Suprasanna 2008; Fig. 11.3).

In vitro mutagenesis provides an alternative and feasible means for sugarcane crop improvement (Patade and Suprasanna 2008). A holistic scheme of in vitro mutagenesis and downstream screening strategies to be utilized for sugarcane crop improvement is shown in Fig. 11.4. Some of the mutant varieties developed using gamma ray-induced mutagenesis on vegetative cuttings and seeds are presented in Table 11.7. In the past decade, this method has been applied to the isolation of several agronomically-useful mutant germplasm. Aiming to utilize both technical and scientific knowledge, attempts were made to combine in vitro regeneration and induced mutagenesis to improve elite sugarcane cultivars for various agronomic traits, cane quality, as well as enhanced biotic and abiotic stress tolerance attributes (Dalvi et al. 2012; Mirajkar et al. 2018; Nikam et al. 2014; Suprasanna 2010; Vaidya et al. 2018).

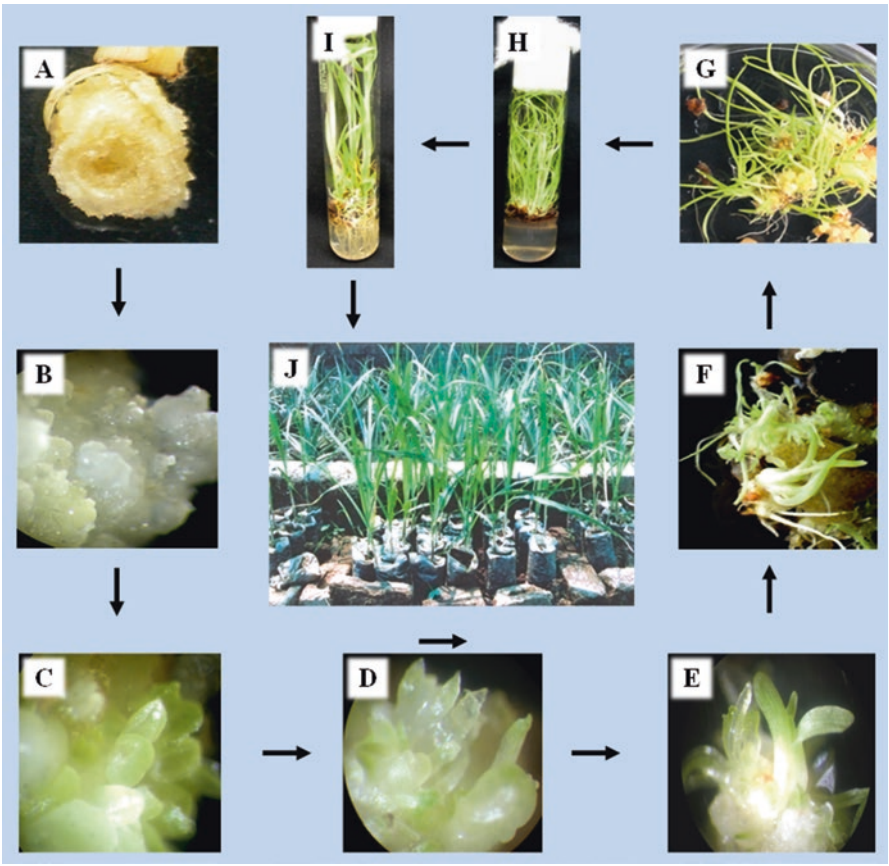

Fig. 11.3 Propagation through somatic embryogenesis and regeneration in sugarcane. (**a**) Culture of explant (viz. young leaf roll), (**b**) Callus initiation, (**c–d**) Stages of somatic embryogenesis, (**e**) Differentiation, (**f–g**) Stages in regeneration of plantlets, (**h**) Shoot elongation and multiplication, (**i**) Rooting of plantlets, (**j**) Hardened plants in the greenhouse

11.5.3 Molecular-Marker Applications

Extensive breeding and selection of beneficial clones out of thousands of progeny requires an efficient and reliable screening tool. Morphological descriptors were initially used, but were later replaced with the development of biochemical descriptors and molecular markers. Compared to morphological descriptors, molecular markers have properties such as inherent simplicity, often resulting from the effects of a single gene; and have high heritability and specificity to certain genomic region (Table 11.8). Therefore, molecular markers are a much more reliable, unbiased means offering the maximum power of discrimination. Biochemical descriptors can be certain flavonoids or isozymes, whereas molecular markers can be further classified as restriction fragment length polymorphisms (RFLP), random amplified

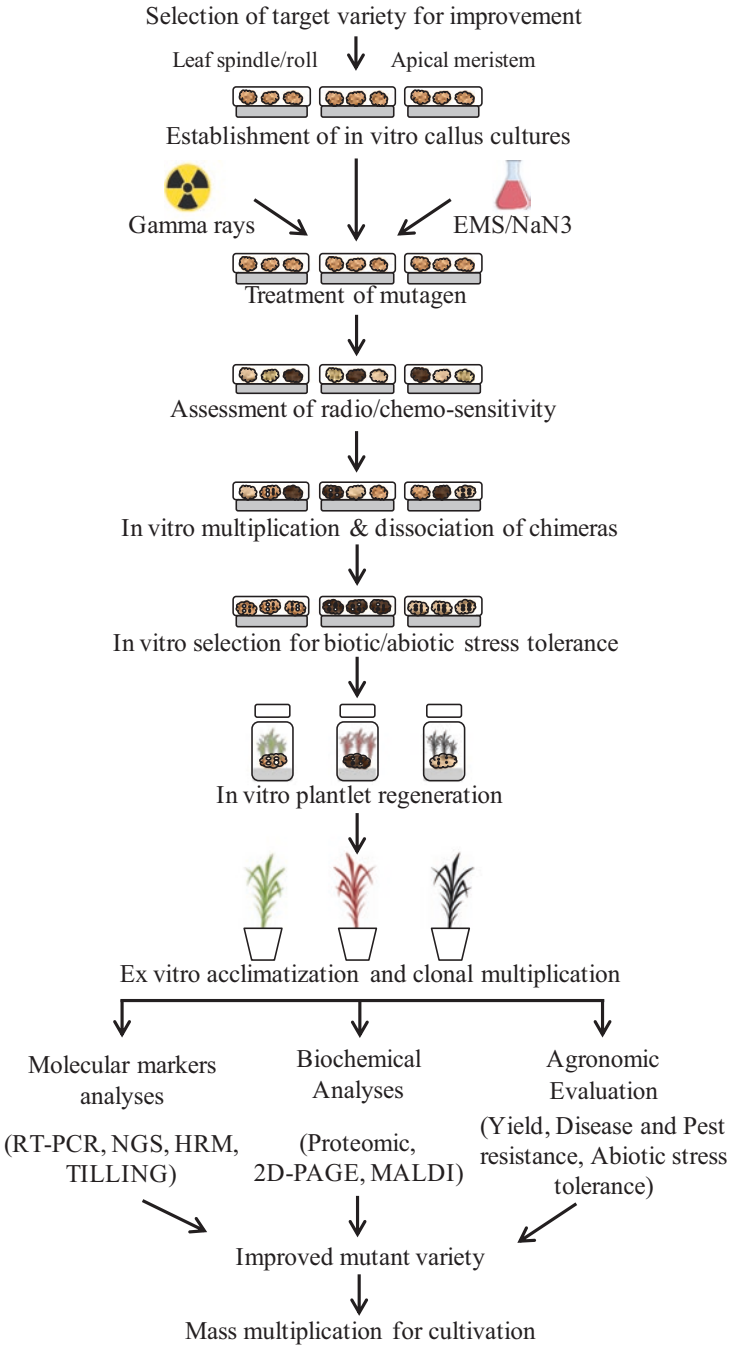

Fig. 11.4 In vitro mutagenesis and downstream screening strategies in sugarcane crop improvement

Table 11.7 Gamma ray-induced mutagenesis and development of mutant varieties in sugarcane

Mutant variety	Country	Year	Dosage (Gy)	Traits improved
Co 6608	India	1966	30–50	Resistance to red rot
Co 997	India	1967	30–50	Resistance to red rot
Co 8153	India	1981	150	Improved juice quality and high yield
Nanei	Japan	1981	420	Longer, thicker stalk, better tillering, higher yield of cane and sugar
Co 85017	India	1985	150	Improved adaptability, resistance to *Ustilago scitaminea,* high cane yield and high sucrose %
Co 85035	India	1985	150	Resistance to *Ustilago scitaminea*, high cane yield and high sucrose percentage
Guifu 80-29	China	1989	80	Late maturity, high sugar content, small stem
CCe 10582	Cuba	1990	–	Improved cane yield
Yuetangfu 83-5	China	1992	–	–
CCe 183, CCe 283, CCe 483	Cuba	1993	–	Resistance to eye spot
Guitang 22	China	2005	80 Gy	Increased biomass, energy cane

Source: Mutant Variety Database FAO/IAEA (2018)

Table 11.8 Examples of morphological descriptors and molecular makers used in sugarcane

Descriptors	References
(A) Morphological Descriptors	
Discrete Characters	Artschwager and Brandes (1958), Balakrishnan et al. (2000), Shadmehr et al. (2017) and Shahi (2000)
Stool habit (spreading and non-spreading)	
Stem color unexposed (green to purple)	
Ivory marks on internodes (present/absent)	
Bud shape (triangular-pointed/oval/obovate/pentagonal/rhomboid/round/ovate/rectangular beaked)	
Germ pore position (apical/sub-apical/medium)	
Bud cushion (present/absent)	
Leaf color (green/light green/light yellow)	
Ligule shape at leaf-sheath joint (crescent/deltoid/strap/riangular)	
Auricle shape at leaf sheath margin	
Lodging resistance (tolerant/moderately tolerant)	
Continuous Characters	
Color of exposed stem	
Stem height (numerical value indicated)	
Stem girth (diameter in cm)	
Corky patches on internodes (present/absent)	
Wax bloom on internodes (light/medium/heavy)	
Node swelling (swollen/not swollen)	

(continued)

Table 11.8 (continued)

Descriptors	References
Root zone swelling (swollen/not swollen)	
Root zone color (green/yellow/greenish yellow/light	
green/light yellow/yellowish green)	
Growth ring color (green/yellow/greenish yellow/light green/light yellow/yellowish green)	
Leaf blade length (fully expanded green leaf)	
Leaf width (at the widest portion of the lamina)	
Leaf color (green/light green/greenish yellow)	
Leaf sheath color (green/greenish yellow/light green/ green with purple blotches/green with purple tinge/ purple)	
Leaf sheath spines (present/absent)	
Leaf sheath waxiness (medium/light)	
Dewlap color (green/yellow/greenish yellow/light green/ light yellow/yellowish green/dark brown/green with purple tinge/light-greenish purple/purple)	
Flowering/low (<10%)/medium (10–40%)/heavy (100%)	
Lodging (lodging/non lodging)	
Morphological Descriptors (Combined Characters)	
Internode shape (bobbin/conidial/cylindrical/oval)	
Internode alignment (straight/zigzag)	
Internode diameter	
Pith in the interior of stem (present/absent)	
Splits or growth cracks on internodes	
Number of root eyes rows at the node	
Root eyes arrangement (regular/irregular)	
Bud size (large, medium, small)	
Bud groove (present/absent)	
Leaf carriage (erect/semi-drooping/drooping)	
Leaf sheath spines (glabrous/dense/sparse)	
Leaf sheath clasping (loose/tight/self-trashing)	
(B) Molecular Markers	
Random amplification of polymorphic DNA (RAPD)	Govindaraj et al. (2011) and Nair et al. (1999)
Inter-simple sequence repeat (ISSR)	Devarumath et al. (2012) and Shrivastava and Gupta (2008)
Restriction fragment length polymorphism (RFLP)	Burnquist et al. (1992) and Grivet et al. (1996)
Amplified fragment length polymorphism (AFLP)	Besse et al. (1998) and Selvi et al. (2005)
Single nucleotide polymorphism (SNP)	Cordeiro et al. (2006) and Devarumath et al. (2013a, b)
Simple sequence repeats (SSR)	Cordeiro et al. (2001), Pan et al. (2003) and Singh et al. (2008)

(continued)

Table 11.8 (continued)

Descriptors	References
Sequence-related amplified polymorphism (SRAP)	Chang et al. (2012), Mirajkar et al. (2017) and Suman et al. (2012)
Target region amplification polymorphism (TRAP)	Arro (2005), Mirajkar et al. (2017) and Suman et al. (2008)
Single-strand conformation polymorphism (SSCP)	Kalwade and Devarumath (2014)
Expressed sequence tag-simple sequence repeats (EST-SSRs)	Pinto et al. (2004) and Singh et al. (2015)

polymorphic DNA (RAPD) and many more molecular markers, all of them having unique implications due to their ability to discriminate among sugarcane clones. Genetic linkage maps were constructed using different molecular markers such as RAPD (Al-Janabi et al. 1993; Mudge et al. 1996), RFLP (Da Silva et al. 1995; Ming et al. 2002), amplified fragment length polymorphism (AFLP) (Andru et al. 2011; Hoarau et al. 2001) and simple sequence repeats (SSR) (Oliveira et al. 2007), although many of these approaches failed to cover one-half of the sugarcane genome.

11.5.4 Development of Transgenic Sugarcane

Commercial sugarcane cultivars have greatly benefited from biotechnological tools, especially genetic transformation in order to delimit the boundaries of conventional breeding approaches. Different methods such as electroporation of cell suspension cultures (Arencibia et al. 2000) and *Agrobacterium*-mediated genetic transformation (Arencibia et al. 1998; Elliot et al. 1998; Enriquez-Obregon et al. 1998; Manickavasagam et al. 2004; Santosa et al. 2004), have successfully been used to develop transgenic sugarcane lines. Somatic embryogenesis via embryogenic callus or cell suspension cultures has become a routine and useful method for genetic transformation (Bower and Birch 1992). Indeed, this necessitates optimization of in vitro regeneration protocols amenable to genetic transformation to minimize occurrence of somaclonal variation during in vitro culture steps (Carmona et al. 2000; Gilbert et al. 2005). Direct organogenesis or direct somatic embryogenesis can circumvent this problem, which may carry the pre-existing variability, and avoid introduction of new variability. Another hurdle to constitutive expression of a transgene in sugarcane is the mechanism of gene silencing (Wei et al. 2003). This is known to occur in polyploid crop plants; to address this limitation requires a deeper understanding of the gene silencing mechanism in polyploids like sugarcane (Leitch and Bennet 1997).

With the advances in plant genetic engineering, there has been tremendous success with genetic transformation in sugarcane. A few of the successful examples are: insect resistance via the δ-endotoxin gene (Arencibia et al. 1997), mannose-binding lectins (Irvine and Mirkov 1997) and proteinase inhibitor genes (Allsopp and Manners 1997; Nutt et al. 1999); also for disease resistance via genes encoding antimicrobial peptides (Zhang and Birch 1996) and resistance to sugarcane mosaic

virus (SCMV) (Ingelbrecht et al. 1999; Joyce et al. 1998). Transgenic sugarcane plants were developed with modified biosynthetic pathways such as for modified sucrose content via down-regulation of pyrophosphate-dependent phosphofructokinase (Groenewald and Botha 2001); for lignin modification using antisense COMT (caffeic acid 3-O methyltransferase) (Selman-Housein et al. 1999); for enhanced sucrose accumulation using antisense of soluble acid invertase gene (Ma et al. 2000); and herbicide resistance via the phosphoinothricin-N-acetyltransferase gene (Enriquez-Obregon et al. 1998; Falco et al. 2000; Gallo-Meagher and Irvine 1996; Leibbrandt and Snyman 2001; Manickavasagam et al. 2004).

Several transgenic sugarcane lines have been evaluated and tested under confined field trials to study the transgene expression and stability, along with other crop parameters. The first field trial of bialaphos-resistant NCo310 clones produced by microprojectile-bombardment was conducted in the early 1990s (Gallo-Meagher and Irvine 1996). Sugarcane (*Saccharum* hybrid cv. Ja 60-5) plants transformed with truncated *Cry* I A(b) were successfully evaluated under artificial infestation of the borer (*Diatraea saccharalis*) (Arencibia et al. 1999). Effects of the expression of the PAT gene on the agronomic performance of transgenic line were studied by Leibbrandta and Snyman (2003). Field testing of transgenic plants carrying the PAT gene showed stable expression during three successive cycles of vegetative propagation. In another study, Vickers et al. (2005) evaluated transgenic sugarcane cv. Q 117 transformed by the biolistic method with polyphenol oxidase (PPO) and sucrose phosphate synthase (SPS).

Studies on transgene segregation in sugarcane showed stable inheritance of transgenes which can be effectively utilized in hybrid breeding (Butterfield et al. 2002). Being a vegetatively-propagated crop, there is little chance of pollen-mediated transgene flow, and therefore sugarcane can become an ideal crop for mass production of valuable biochemicals and nutraceuticals (Wang et al. 2004). For example, *Acetobacter diazotrophicus* levansucase (*lsdA*) is a key enzyme of 1-ketose, a fructo-oligosaccharide biosynthesis that is essential for nutrition in humans and animals, has been expressed in transgenic sugarcane (Enriquez et al. 2000). A transgenic sugarcane producing polyhydroxy-butyrate (PHB), a biodegradable thermoplastic compound, was developed at the Bureau of Sugar Experiment Stations (BSES) and University of Queensland (Brumbley et al. 2004). Likewise, transgenic sugarcane producing para-hydroxybenzoic acid was also developed by using genes for bacterial enzymes (chorismate pyruvate-lyase and 4-hydroxycinnamoyl CoA hydratase) (McQualter et al. 2004). Expression of the human granulocyte macrophage colony stimulating factor (GM-CSF) in transgenic sugarcane plants was also demonstrated with 0.02% GM-CSF out of the total soluble protein under field conditions (Wang et al. 2004).

11.5.5 Sugarcane Genomics and Synteny with Other Grasses

Genetic studies of sugarcane have been challenging due to its polyploidy and the highly-heterozygous constitution of the genome. With the advent of high throughput genome sequencing tools, comparative analysis can be made to understand the

evolution of species and conserved genomic regions. Having a typical polyploid genome, sugarcane genomic studies lagged behind, due to non-availability of processes and tools for analyzing and understanding its genome complexity. Sugarcane comprises a huge genome (~10 Gb) and its genome complexity is mainly due to the existence of euploid and aneuploid chromosome sets (Souza et al. 2011). Although the estimated monoploid sugarcane genome size is about 750–930 Mb, it is slightly larger than the sorghum (~730 Mb) and about twice the size of rice (~380 Mb) (D'Hont and Glaszmann 2001). Despite its complex and polyploid nature, the sugarcane genome displays collinearity and synteny with other grasses, such as sorghum and maize (Grivet and Arruda 2002). Several genomic resources for sugarcane and related species are now available (Table 11.9). Comparative mapping within the tribe Andropogoneae has led to the development of mapped maize genomic probes that can be used for sorghum and sugarcane genomes (Grivet et al. 1994). Comparative mapping of 17 of 31 sugarcane RGAs in sorghum resulted in synteny of several RGAs that includes 3 brown rust associated sugarcane RGAs being mapped to the same linkage group in sorghum (McIntyre et al. 2005). In an another attempt, comparison of 20 BACs (bacterial artificial chromosomes) of sugarcane with sorghum sequences was made that revealed a mean of 95.2% sequence identity and about 53.1% of them were aligned with the sorghum sequence (Wang et al. 2010).

With the progress in development of new sequencing technologies, several genome sequencing initiatives were carried out in the last couple of years. The approaches such as the BAC by BAC and whole genome shotgun sequencing have

Table 11.9 Publicly available genomic resources for sugarcane and related species

Database	Web link	Species	Information details
Sugarcane transcription factor database	http://planttfdb.cbi.pku.edu.cn/index.php?sp=Sof	*Saccharum officinarum*	Interactions between Transcription factors (TFs) and target genes primarily to assign functional annotation to TFs
SUCEST-FUN	http://sucest-fun.org	Sugarcane	Comprehensive access to integrate transcripts, molecular markers, gene categories, gene expression, data mining tools
TropGENE	http://tropgenedb.cirad.fr/tropgene/JSP/index.jsp	Tropical crops including sugarcane	Genetic resources, molecular markers, physical and genetic maps, gene sequences, QTLs, allelic diversity
Grassius	http://grassius.org/	*Brachypodium* maize, sugarcane, sorghum, and rice	Computational and experimental resources related to control of gene expression in grasses and associated agronomic traits
Phytozome	https://phytozome.jgi.doe.gov/pz/portal.html	Many plant species	Platform for plant gene evolutionary history, gene structure, gene family, genome organization, and functional annotations of complete plant genomes

Source: Modified from Kandel et al. (2018)

shown little progress in sugarcane compared to other crop species. The technological challenges still exist in understanding and analyzing the sugarcane genome. With the advancements in robust genome sequencing tools and genome assembly strategies, availability of reference genomes (sorghum and maize), sugarcane whole genome sequencing is now becoming more realistic (Thirugnanasambandam et al. 2018). The genome sequencing of two progenitor sub-genomes, *Saccharum officinarum* and *S. spontaneum,* is currently underway. So far, sequencing of BAC clones of the *Saccharum* hybrid genome has provided a monoploid coverage that has showed close synteny with the sorghum genome. In the near future a complete polyploid genome may be sequenced and assembled that will ultimately allow comparison with closely-related genomes and deeper understanding of the functioning of polyploidy in the sugarcane genome.

11.5.6 Sugarcane Genomic Resources for Biomass and Bioenergy

Hybrids of *Saccharum* species are potential candidates for biomass production due to their unique abilities to carry out rapid photosynthesis, higher biomass accumulation, faster growth, higher tillering and ratooning, as well as perennial plant behavior. Besides being utilized as a prime source for sucrose production, sugarcane exhibits potential valuable resource for lignocellulosic biomass which has great possibility to be utilized as an emerging feedstock for second-generation bioethanol production (Kandel et al. 2018). On average, sugarcane produces lignocellulosic biomass of about 22.9 mt/ha (dry weight basis) in a year (van Der Weijde et al. 2013) against the theoretical yield potential of >100 mt/ha/year (Jakob et al. 2009; Moore 2009). As biomass yield is a complex trait, it is necessary to simultaneously improve overall biomass yield, biomass quality and adaptations to changing environment. To reach these goals, sugarcane breeders need to circumvent challenges such as a narrow gene pool, genomic complexity, poor synchronization and fertility of flowers, and long breeding and selection cycles (Lakshmanan et al. 2005; Manickavasagam et al. 2004). However, the knowledge gap between sugarcane crop physiology and biochemistry, with respect to its huge genome, necessitates placing more emphasis on photosynthesis and source-sink relationships, that ultimately empower generation of modern bioenergy resource. Besides the basic understanding about the source-sink relationships, alternatives to improve bioethanol production through utilization of whole fraction of the total crop biomass, i.e., sugars present in the cell wall along with the soluble sugars (de Souza et al. 2014). In this context, genomic resources play a major role by enabling easy dissemination of information deciphered from genetic investigations in *Saccharum* spp. and allied genera. Such genomic databases are acting as reservoirs for molecular breeders and geneticists to explore and mine the candidate genes or alleles and to assist in molecular-marker based breeding strategies (Kandel et al. 2018). With the aim to

Table 11.10 Sugarcane genomic resource databases for biomass and cell wall specific traits

Database with web link	Political unit	Purpose
Biomass Energy Centre [www.biomassenergycentre.org.uk/portal/page?_pageid=73,1&_dad=portal&_schema=PORTAL]	United Kingdom	Bioenergy
European Biomass Industry Association [www.eubia.org/]	European Union	Bioenergy
Louisiana Biomass Resources Database [www2.lsuagcenter.com/biomass/about.aspx]	United States	Bioenergy
Biomass Power Association [www.biomasspowerassociation.com/]	United States	Electricity
[http://sugarcane.org/]	Brazil	Bioenergy
SAHYOG Project [www.sahyog-europa-india.eu/]	European Union and India	Bioenergy
BioEnergy Science Center [www.bioenergycenter.org/besc/]	United States	Cellulosic biofuels
Russian Biofuel Association [www.biofuels.ru/]	Russia	Bioethanol, biodiesel
Cell wall genomics (CWG) [cellwall.genomics.purdue.edu]	United States	Cell wall-related genes in rice and maize
Plant cell walls (PCW) [cell.ccrc.uga.edu/~mao/cellwall/main.htm]	United States	Role of the cell wall in plant growth and development
CAZy database [www.cazy.org]	United States	Carbohydrate-active enzymes (CAZymes)

Source: Modified from Kandel et al. (2018)

provide the critical information necessary to increase the efficacy of lignocellulose into ethanol conversion, cell wall and biomass-related databases (Table 11.10) have been constructed to serve as excellent resources for comparative genomics to identify and study candidate genes and their potential pathways. Based on their contents, these databases may be regarded as species- or family-specific and general plant cell wall and biomass-related databases (Cao et al. 2010; Kandel et al. 2018).

11.5.7 Recent Advancements and Future Genomic Approaches

Recent genomics advancements have made it feasible to perform precise and specific genome editing (GE), rather than inducing random mutations. These GE approaches rely on the use of engineered sequence-specific nucleases and DNA repair mechanism existing in the host cell (Kadam et al. 2018; Kumar and Jain 2015). These genome editing approaches such as zinc finger nucleases (ZNFs), clustered regularly interspaced short palindromic repeat associated Cas9 nuclease

(CRISPR/Cas9) and transcription activator-like effector nucleases (TALENs) systems, are considered proficient tools for crop improvement programs to generate mutant alleles (Kumar and Jain 2015). In a crop like sugarcane which is highly polyploidal, the modification of desired traits through conventional breeding in an elite cultivar is tedious and time-consuming. Several genes can be edited to create mutant resource using GE tools (Mao et al. 2013).

GE using the TALEN approach enables precise genomic modifications, such as targeted mutagenesis, gene replacement and/or insertion (Gurushidze et al. 2014; Li et al. 2012; Zhang et al. 2013). Such TALEN-mediated targeted mutagenesis has been adopted for the development of knock out genotypes in a number of crop plants (Baltes and Voytas 2015; Weeks et al. 2016).

For the first time the utility of TALEN has been demonstrated in sugarcane to reduce lignin content for enhanced biofuel production (Jung and Altpeter 2016). Recently, Kannan et al. (2018) reported the first successful evaluation of TALEN-mediated mutant lines of sugarcane targeted to a lignin biosynthetic gene-caffeic acid O-methyltransferase (COMT). Resultant field grown COMT mutant lines had 19.7% less lignin content and about 43.8% improvement in saccharification efficiency. However, CRISPR/Cas9 and ZFNs approaches will have to be fine-tuned to bring about a revolutionary change in sugarcane (Chakravarthi 2016; Ferreira et al. 2017). Chakravarthi (2016) outlined that the complex genome, high ploidy, repetitive DNA and poorly defined genomics resources are some of the challenges to be overcome in sugarcane. Tools and technological advancements will have to be made available in order to enable feasibility of genome editing in a complex polyploidy crop like sugarcane to overcome limitations of conventional breeding strategies.

11.6 Conclusions and Prospects

Available sugarcane germplasm is large, diverse and represents abundant variability present in the native habitats. The scope for breeding and genomics in sugarcane is represented by inexhaustible rich genetic resources, but their utilization is restricted due to its hybrid nature, heterozygosity and highly polyploidal genomic content. Utilization of available germplasm needs to be accelerated to ensure a broader genetic base and to improve productivity and adaptability of future varieties. The large genetic variability represented across *Saccharum* species and related genera and the absence of any serious intercrossing ability barriers within the *Saccharum* complex, offer enormous possibilities to create genetic recombination that will meet future varietal needs for both the sugar and energy sectors. With the advancements in cellular and molecular approaches, sugarcane crop breeding and biotechnology hold a gamut of opportunities and challenges.

Currently, research needs to focus on developing novel in vitro culture and regeneration systems and transgenic plants with desirable traits. Transgenic research has moved from laboratory experimentation to field trials. Advances in sugarcane biotechnology could become remarkable in the coming years, both in terms of

improving plant productivity as well as commercial gains. In addition, the unique advantages of sugarcane as a renewable resource for the production of biofuels can address the challenges and opportunities associated with energy-cane breeding within existing sugarcane breeding programs.

Appendices

Appendix I: Research Institutes Relevant to Sugarcane

Institution	Specialization and research activities	Contact information and website
Sugarcane Breeding Institute (SBI), Coimbatore, India	Research and development pertaining to sugarcane crop improvement	ICAR-Sugarcane Breeding Institute Coimbatore – 641,007 India. E-mail: director@sugarcane.res.in Website: http://sugarcane.icar.gov.in
Indian Institute of Sugarcane Research (IISR), Lucknow, India	Researches and development on fundamental and applied aspects of sugarcane cultivation	Indian Institute of Sugarcane Research Raibareli Road, P.O. Dilkusha, Lucknow – 226,002 India. Email: director.sugarcane@icar.gov.in Website: http://www.iisr.nic.in
Vasantdada Sugar Institute (VSI), Pune, India	Scientific, technical and educational functions relevant to the sugarcane cultivation and sugar industry	Vasantdada Sugar Institute Manjari Budruk, Pune – 412,307, India. Email: vsilib@vsnl.com, webmaster@vsisugar.com Website: http://www.vsisugar.com
Sugar Research Australia (SRA), Queensland, Australia	Invests and manages portfolios of research, development and adoption projects that drive productivity, profitability and sustainability for the Australian sugarcane industry	Sugar Research Australia PO Box 86, 50 Meiers Road, Indooroopilly, Queensland 4068, Australia Email: sra@sugarresearch.com.au Website: https://sugarresearch.com.au/
South African Sugarcane Research Institute (SASRI), South Africa	Research and extension work pertaining to varietal improvement, crop protection, crop performance and management, and systems design and optimization	170 Flanders Drive, Mount Edgecombe 4300, South Africa Email: sasri@sugar.org.za Website: https://sasri.org.za/

(continued)

Institution	Specialization and research activities	Contact information and website
Centro de Tecnologia Canavieira (CTC), Brazil	Sugarcane breeding, rigorous testing and selection for identification of disease resistant varieties. Also employs advanced biotechnology techniques to develop insect-resistant transgenic sugarcane varieties	Fazenda Santo Antônio, S/N, Bairro Santo Antônio Piracicaba – SP – CEP 13400-970 Caixa Postal 162, Brazil Website: http://new.ctc.com.br/en/ Email: comunicacao@ctc.com.br
Philippine Sugar Research Institute Foundation (PHILSURIN), Inc., Philippines	Sugarcane research, development and extension activities with the aims to promote the growth and development of the sugar industry through greater and significant participation of the private sector	Rm. 1403, 14th Security Bank Centre, 6776, Ayala Avenue, Makati City, PHILSURIN Experiment Station, VICMICO Cmpd, Victorias City, Makati City/ Victorias City, 1226/6119, Philippines Website: http://philsurin.org.ph/ Email: info@philsurin.org.ph
Sugar Processing Research Institute (SPRI), Inc., USA	Aims to develop processes and analytical methods to increase yield and productivity, to develop innovative processes or technologies and to provide trainings for its members and the international sugar industry	1100 Robert E. Lee Blvd. New Orleans LA, 70124-4305, USA Website: http://spriinc.org/ Email: charley@sugarjournal.com

Appendix II: Genetic Resources of Indian Sugarcane

Cultivar	Important traits	Cultivation location
Co 86249 (Bhavani)	Mid-late variety of tropical India Moderately resistant to red rot, smut and rust Tolerant to drought and pests Erect, medium thick canes with good ratooning Suitable for growing during October and January/February Cane yield: 104.25 mt/ha Sucrose content: 18.71% CCS yield: 14.3 mt/ha Fiber content: 14.27% Parentage: CoJ 64 x CoA 7601 Year of identification: 1997 Year of release and notification: 2000	East-coast zone of India (Tamil Nadu, Andhra Pradesh, Orrisa)

(continued)

Cultivar	Important traits	Cultivation location
Co 2001-13 (Sulabh)	Mid-late variety High tillering with good rationing Good for jaggery quality Resistant to red rot and moderately resistant to smut Tolerant to drought and salinity Cane yield: 108.6 mt/ha Sucrose content: 19.03% CCS yield: 14.73 mt/ha Fiber content: 13.43% Parentage: Co 7806 PC Year of identification: 2008 Year of release and notification: 2009	Recommended for peninsular zone (Gujarat, Maharashtra, Madhya Pradesh, Karnataka, interior of Tamil Nadu, Kerala and Andhra Pradesh)
Co 238 (Karan 4)	An early maturing variety of subtropical zone Moderately resistant to red rot disease Tolerant to low temperature, water stress and waterlogging Good ratoonability in winter season Cane yield: 81.1 mt/ha Sucrose content: 18.0% CCS yield: 9.95 mt/ha Fiber content: 13.05% Parentage: CoLk 8102 x Co 775 Year of identification: 2008 Year of release and notification: 2009	Recommended for cultivation in North western zones of India (Punjab, Haryana, Rajasthan, Uttarakhand, Western and central Uttar Pradesh)
Co 98014 (Karan-1)	An early variety (spring planting) Resistant or moderately resistant to red rot disease Tolerant to drought and water logging, suitable for co-generation Cane yield: 76.29 mt/ha Sucrose content: 17.59% CCS yield: 9.26 mt/ha Parentage: Co 8316 x Co 8213 Year of identification: 2006 Year of release and notification: 2007	Suited to north western zone of India (Punjab, Haryana, Rajasthan, Central and western Uttar Pradesh and Uttarakhand)

(continued)

Cultivar	Important traits	Cultivation location
Co 0118 (Karan 112)	Early maturing variety of subtropical zone Non-logging and non-flowering variety Tolerant to water stress and water logging Resistant to red rot and wilt Medium cane thickness Cane yield: 78.20 mt/ha Sucrose content: 18.45% CCS yield: 9.88 mt/ha Fiber content: 12.78% Parentage: Co 8347 x Co 86011 Year of identification: 2008 Year of release and notification: 2009	Recommended for cultivation in North western zones of India (Punjab, Haryana, Rajasthan, Uttarakhand, Western and central Uttar Pradesh)
Co 99004 (Damodar)	A mid-late variety with shy flowering habit Resistant to red rot and wilt diseases Tolerant to drought and salinity conditions Tolerant to internode borer Suitable for good quality jaggery making Cane yield: 116.69 mt/ha Sucrose at 12th month: 16.83% CCS yield: 18.76 mt/ha Fiber content: 14% Parentage: Co 62175 and Co 86250 Year of identification: 2006 Year of release and notification: 2007	Suited to peninsular India (Gujarat, Maharashtra, interior Andhra Pradesh, Tamil Nadu, Karnataka and Kerala)
CoM 265 (Phule 265)	Mid-late variety suitable for areas with less availability irrigation water Moderately susceptible to red rot and wilt diseases, Resistant to smut disease High ratoon yield potential Cane yield: 199.80 mt/ha Sucrose at 12th month: 19.33% CCS yield: 21.79 mt/ha Fiber content: 14% Parentage: Co 87044 GC Year of identification: 2009	Recommended for peninsular zone of India (Gujarat, Maharashtra, Kerala, Karnataka, interior of Tamil Nadu and Andhra Pradesh, Madhya Pradesh and Chhattisgarh)

(continued)

Cultivar	Important traits	Cultivation location
CoC 671 (Vasant-1)	Early maturity High quality cane, suitable for jaggery making Susceptible to red rot disease Cane yield: 135 mt/ha Sucrose yield: 19.50% CCS yield: 17.50 mt/ha Parentage: Q 63 x Co 775 Year of identification: 1982	Recommended in the states of east coast and peninsular zones of India (Maharashtra, Kerala, Karnataka, Interior of Tamil Nadu and Andhra Pradesh and Madhya Pradesh)
Co 86032 (Nayana)	Mid-late maturity Resistant to smut, field tolerant to red rot and moderately resistant wilt diseases Tolerant to drought conditions Cane yield: 102 mt/ha Sucrose yield: 20.10% CCS yield: 14.44 mt/ha Parentage: Co 62198 X CoC 671 Year of identification: 1994 Year of release and notification: 2000	Recommended for peninsular zone of India (Gujarat, Maharashtra, Kerala, Karnataka, interior of Tamil Nadu and Andhra Pradesh, Madhya Pradesh and Chhattisgarh)
Co 09004 (Amritha)	Early maturity Tolerant to drought and salinity Moderately resistant to red rot, resistant to smut and yellow leaf disease Cane yield: 109.85 mt/ha Sucrose yield: 18.94% CCS yield: 14.56 mt/ha Parentage: CoC 671 X CoT 8201 Year of identification: 2017 Year of release and notification: 2017	Recommended for peninsular zone of India (Gujarat, Maharashtra, Kerala, Karnataka, interior of Tamil Nadu and Andhra Pradesh, Madhya Pradesh and Chhattisgarh)

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Part II
Food Crops

Chapter 12
Cacao (*Theobroma cacao* L.) Breeding

Frances Bekele and Wilbert Phillips-Mora

Abstract The suboptimal productivity in cocoa farmers' fields, particularly those of small-holders who produce over 80% of the global supply, and the demand for cocoa that meets stringent quality and flavor criteria necessitate enhanced breeding methods and outcomes. Progress in cacao breeding has been hindered by a long-generation cycle, limitations in land availability for large-scale breeding trials, and challenging abiotic and biotic stress factors, including several major diseases. Cacao tends to be outbreeding and cocoa production is often reduced by the incompatibility status of planting material and pollination inefficiency. The complex breeding mechanisms in cacao and difficulty in predicting the performance of promising selections as parents also pose challenges to breeders. Reciprocal recurrent selection schemes have been most successful to date. The advent of breeding with genomics and the unravelling of the cacao genome portend unprecedented advancements in cocoa breeding. This chapter explores the past, present and future prospects of cacao breeding, and describes how the use of traditional breeding allied with molecular and genomic approaches can empower cocoa breeders to meet the need for improved planting material with high productivity and yield efficiency, disease resistance, climate change adaptations, nutraceutical value and superior flavor and quality attributes.

Keywords Diversity · Genetic gain · Genomics · Genotype × environment interaction · Hybrids · Molecular breeding

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12.1 Introduction

Cacao (*Theobroma cacao* L.), of the family Malvaceae sensu lato, subfamily Byttnerioideae (Alverson et al. 1999), is a perennial, Neotropical plant on which the thriving global cocoa and chocolate industry is based. *Cocoa* is the dried and usually fermented fatty seed of the cacao tree from which chocolate is made. The crop was domesticated in Central America in pre-Columbian times, and the native Indians considered it to be of divine origin. Consequently, in 1753, Linnaeus designated its scientific name, *Theobroma,* meaning *food of the gods*.

The cacao tree only grows in humid, tropical climates, and cocoa is currently an important agricultural commodity in many developing countries in West Africa, which produces over 70% of the world's supply, Southeast Asia, Latin America and the Caribbean. It is cultivated by over 5 million growers in 50 countries, and 40–50 million people derive their livelihood from it (World Cocoa Foundation 2012). The global production of cocoa beans for 2013/2014 was estimated as 4.552 million mt (ICCO 2017). The world grindings of beans in 2015/2016 was estimated as 4.24 million mt. The global sale of beans for 2011–2012 was worth USD 10 billion (Anga 2014). Consumption of cocoa products occurs mainly in developed countries such as Switzerland, Germany, Ireland, UK, Norway, Sweden, Australia, the Netherlands, the USA and France. However, Asian markets were expected to hold a 20% share of the global market by 2016. The global chocolate market was estimated to reach a value of USD 98.3 billion in 2016, an increase of 2.7% from 2011 (marketsandmarkets.com 2011).

Genetic improvement of cacao has not advanced as rapidly as that of many annual and biennial crops and other tropical perennial crops such as rubber (*Hevea brasiliensis*) (Lockwood 2003; Simmonds 1993; Warren and Kennedy 1991). Progress in cacao breeding has been hindered by a long-generation cycle, and limitations in land availability and other necessary resources (Kennedy et al. 1987; Toxopeus 1972). It has also been hampered by insufficient knowledge of the genetic diversity of cacao (Lanaud et al. 2003b), among other constraints. Indeed, accelerated progress in cocoa breeding is still required to meet cocoa farmers' needs for high-yielding, disease-resistant, drought and other abiotic stress tolerant genotypes with good flavor potential. Another drawback is that the genetic base of parental types used in cacao breeding globally has been rather limited (Eskes and Lanaud 2001; Kennedy et al. 1987). Breeding efforts globally have been based on fewer than 80 genotypes from the Scavina (SCA), POUND, Nanay (NA), Parinari (PA), United Fruit (UF), Iquitos Mixed Calabacillo (IMC) and the Imperial College Selection (ICS) groups (Appendix I). The extensive genetic diversity within cacao germplasm collections (ex situ conservation) (Fig. 12.1) has not been properly exploited (CacaoNet 2012; Eskes and Lanaud 2001; Lopes et al. 2011). The diversity that exists in situ (in the wild) is yet to be comprehensively tapped even with the significant progress made at the various cacao research institutes (Appendix II).

The role of cacao breeders is becoming more complex. The globalization of cacao diseases and pests and the impact of climate change impose new challenges

Fig. 12.1 (**a**) Diversity in fruit morphology of *Theobroma cacao* L. at CATIE, Costa Rica (some related wild types, *Herrania* spp., included), (**b**) ICGT, Trinidad. (Photo is credited to Terry Sampson)

for the generation of new varieties adapted to the emerging scenarios. In addition, manufacturers of cocoa-based products are now seeking cocoa with enhanced nutraceutical (functional food) value. Breeders will soon be seeking varieties with genetic predisposition for low uptake of heavy metals such as cadmium (Cd) (Ramtahal et al. 2016), among other desirable traits. The traditional cocoa breeder must now collaborate with the analytical chemist, biotechnologist, molecular biologist, physiologist, pharmacologist and other specialists in order to fulfil his/her rigorous mandate. This chapter deals with the traditional role of cacao breeding, early selection and improvement of cacao planting material and constraints to cacao breeding as well as the advances underway with the advent of biotechnological tools and the genomic era.

12.1.1 Origin, Domestication, Distribution and Cultivation

Cacao is native to the understory of the Amazon and Orinoco rainforest. Its accepted center of origin and diversity is near the headwaters of the Amazon River Basin near Iquitos, Peru at the confluence of the Caquetá, Napo and Putumayo rivers (Cheesman 1944; Pound 1945). The greatest known variation in morphological and physiological characters of cacao occurs in this region (Cheesman 1944; Motamayor et al. 2002).

The native range of cacao is designated as southwestern Amazonia, including Peru, Ecuador, Colombia and Bolivia (Motamayor et al. 2002; Sereno et al. 2006; Thomas et al. 2012; Whitkus et al. 1998). Pound (1938, 1945) described a continuous range of phenotypic variability in cacao between Pará, Brazil and Ecuador. These are presumed *end points* of diverging segregation of cacao from the northern tributaries of the Amazon. The eastern Cordillera of the Andes is the boundary between the early plantings of Criollo (*native*) and Forastero (*exotic*) cacao. Amerindian settlers from the Amazon region are believed to have migrated from South to Central America, taking pale-coloured, plump cacao beans (least astringent) with them (Cheesman 1944). With this movement of cacao, the Isthmus of Panama probably served as a secondary barrier, allowing spatial isolation of the cacao introduced into Central America and further differentiation of distinct Central American Criollo cacao (Cuatrecasas 1964). Soria (1970) and Motamayor et al. (2002, 2003) described the various cacao varieties subsequently cultivated in Tropical America.

Cacao cultivation was extended southwards from Central America by the Spaniards, who promoted the consumption of cocoa in the sixteenth century (Badrie et al. 2015; Van Hall 1932). From mid-seventeenth century and onwards, cacao was introduced into Peninsular Malaysia, Indonesia, Ceylon (now Sri Lanka), and the Philippines from South America (Brazil and Venezuela) and Central America and Trinidad (Van Hall 1932). It is documented as reaching Nigeria in 1874, Cameroun in 1876 and Ghana in 1878, as *Amelonado* cacao from Brazil (Lockwood and Gyamfi 1979; Opoku et al. 2007), and Côte d'Ivoire in 1919 (Wood and Lass 1985). Subsequently, it was distributed to other parts of Southeast Asia such as Papua New Guinea (Wood and Lass 1985) (Fig. 12.2).

Upper Amazonian Forastero cacao was transferred from Trinidad to Ghana and established at Tafo in 1945 (Posnette 1948). This significant germplasm transfer was the foundation of cacao improvement and the development of new cacao plantings in Ghana, Nigeria and other West African cocoa-producing countries. It also facilitated similar developments in other cocoa-growing countries outside of the center of origin and domestication of cacao.

The genetic diversity of cacao that exists in the wild (in situ) is not exhaustively represented in the areas of domestication (Anon. 1981). This may be attributed to the *founder effect* (Mayr 1954), and a consequence of the limited diversity taken into cultivation when cacao was collected in the wild (Wood and Lass 1985). Much of the diversity in the wild is therefore still untapped (Anon. 1981). Currently, the

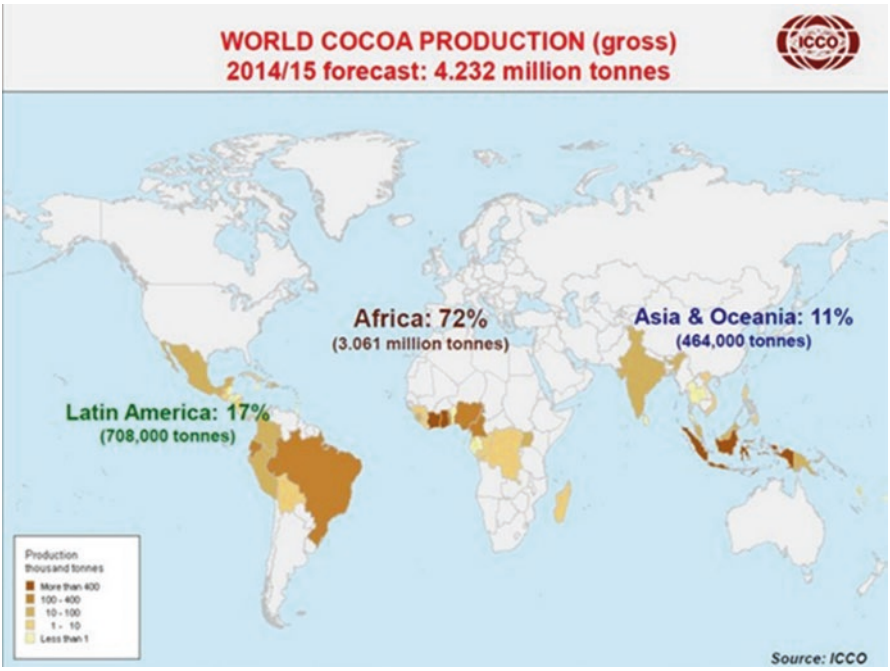

Fig. 12.2 Map of cocoa producing countries

majority of cacao cultivation is based on varieties assembled since before 1950. Approximately only one-third of cacao plantings globally are derived from hybridization and crop improvement (CacaoNet 2012). One consequence of this narrow genetic base of commercial varieties is their vulnerability to devastating diseases, as occurred, for example, with the appearance of witches' broom disease in Brazil, in 1989, and frosty pod rot in Mexico, in 2005 (Phillips et al. 2013).

12.1.2 Botanical Classification

Three major classes of cacao are traditionally recognized in the cocoa trade: Forastero (Upper and Lower Amazon, Amelonado and Guianese), Criollo, and hybrids of Criollo and Forastero referred to as Trinitario (Cheesman 1944; Lachenaud and Oliver 2005; Wood and Lass 1985). This classification is based on morphological traits, particularly those of the fruit (pod) and seed (bean). All of the existing wild, semiwild and cultivated cacaos form one inter-breeding population.

Amazon Forastero trees comprise approximately 70% of cacao cultivation globally (Eskes and Lanaud 2001). The term *Forastero* (*exotic*) was originally used in Venezuela to describe cacao introduced from Trinidad. Subsequently, the term *Amazon Forastero* was used to classify hardy and robust cacaos from Ecuador,

Brazil and those introduced into West Africa. Amazon Forastero seeds are typically small in size and flattened. The cotyledons (seeds without the coats or testae) are purple in color due to the presence of high concentrations of the pigment, anthocyanin. They possess a strong *cocoa* flavor and are therefore preferred for the manufacture of milk chocolate. Forastero beans tend to have higher butterfat contents than Criollo cocoa (Khan et al. 2008; Pires et al. 1998). This increases their value in the cocoa trade because cocoa butterfat attracts an attractive price. Forastero cacao trees currently yield 95% of traded cocoa, referred to as *bulk* cocoa in the market (Wood and Lass 1985). Iwaro et al. (2003) observed that a relatively larger proportion of individuals from this group are resistant to diseases, such as black pod disease, compared to other groups.

Criollo cacao is currently grown throughout Central America, in countries such as Mexico and in South America. Venezuelan Criollos are highly regarded. Authentic or pure Criollo trees are rare globally because Criollo varieties are typically susceptible to diseases and display low vigor. Criollo hybrids are slightly more common. Motamayor et al. (2002) and Motilal et al. (2010) reported on the low genetic diversity and heterozygosity found among the Criollo germplasm they studied.

Trinitario cacao is a cross between Criollo and Forastero (Cheesman 1944). Hybridization that produced Trinitario cacao is believed to have first occurred in Trinidad hence the designation. The Trinitario group currently comprises a wide range of hybrids between Criollo and Amazon Forastero cacao found in the Caribbean region, South and Central America, Papua New Guinea and wherever there has been a mixture of Criollo and Amazon Forastero cacao (Bekele et al. 2009; Johnson et al. 2009; Motamayor et al. 2003; Wood and Lass 1985). This is thus a very heterogeneous group, displaying extreme phenotypic and genetic diversity. Trinitario and Criollo cacao produce *Fine or Flavor* cocoa.

Refractario cacao is another recognised group of cacao, and has a distinctive genetic background when compared to Nacional cacao from Ecuador, Amazon Forasteros from Peru and Trinitarios (Zhang et al. 2008). This group of cacao was selected from trees, which showed no symptoms of witches' broom disease under high disease pressure in Ecuador (Pound 1938). It is conserved within the Trinidad genebank (ICGT), and Refractario accessions with favorable traits, such as good yield potential, have been used for germplasm enhancement along with other promising accessions from the main classes of cacao conserved there (Bekele and Bekele 1996; Bekele et al. 2006; Iwaro et al. 2003, 2010).

Research findings of Motamayor et al. (2008) revolutionized the classification of cacao in that they revealed ten genetic clusters of cacao. These genetic groups are Amelonado, Contamana, Criollo, Curaray, Guiana, Iquitos, Marañon, Nacional, Nanay and Purús. The study did not include cultivated types such as Trinitarios, but 1241 wild types. This genetic classification of cacao is expected to enhance the management of cacao germplasm. Ongoing genetic studies, using molecular markers, are likely to further elucidate the genetic diversity and phylogeny within cultivated cacao and that conserved ex situ.

12.2 Germplasm Selection, Diversity and Conservation

Selection for improved yield and other favorable traits in cacao germplasm was initiated in the early twentieth century in several cocoa producing countries. Among the most significant genetic resources are Upper Amazon Forastero (UAF) cacao clones collected by the late Dr F.J. Pound in Ecuador and Peru between 1937 and 1943 (Bartley 2005; Bekele et al. 2008a, b; Efron et al. 2005a; Eskes et al. 1993; Lockwood and End 1993; Lockwood and Pang 1993). The utilization of cacao genetic resources in some cocoa-producing countries is highlighted in Appendix I and Table 12.1.

UAF cacao genotypes account for approximately 60% of cacao cultivation globally (Wood and Lass 1985). IMC (Iquitos Mixed Calabacillo) 47, 60, 67; NA (Nanay) 31, 32, 33, 34; POUND 7, 12, 18; PA (Parinari) 7, 35, 150 and SCA (Scavina) 6, 9 and 12 were among the most widely distributed and utilized UAF parental types used in cacao breeding globally (particularly in Southeast Asia, West Africa and Latin America and the Caribbean (CacaoNet 2012)). In Ecuador, the highly productive and disease resistant CCN 51 (Boza et al. 2014) was the result of crossing the UAF clone, IMC 67, with the Trinitario, ICS 95 and then the hybrid, CCN 1, in the next generation.

12.2.1 Conservation of Cacao Ex Situ and via In Vitro Techniques

In 1981, the International Board for Plant Genetic Resources, now Bioversity International, designated *Theobroma cacao* L. as a priority for conservation and characterization. This was prompted by the threat of loss of genetic diversity in wild cacao (in situ) from the upper Amazon region due to deforestation and the changing agricultural practices (Anon 1981). Consequently, ex situ conservation was prioritized to complement the conservation of cacao genetic resources in situ.

Since the cacao seed is recalcitrant (it cannot be stored for long periods due to its sensitivity to dehydration and temperature variation), it is necessary to conserve cacao in field collections rather than seed banks. The Cocoa Research Centre (CRC), Trinidad, manages the ICGT, which is one of two international field collections of cacao germplasm in the public domain. The other universal collection, maintained at the Centro Agronómico Tropical de Investigación y Enseñanza (CATIE), is in Costa Rica (Phillips-Mora et al. 2013). The cacao germplasm conserved at the ICGT and CATIE is in the public domain and distributed to all bona fide users via quarantine. There are several national collections of cacao germplasm in various cocoa producing countries (Appendix II; CacaoNet 2012). However, sharing of the varieties from national collections is not common.

ICGT conserves roughly 2300 cacao accessions. They include Upper and Lower Amazon Forastero clones (Bekele et al. 2006; 2008a, b) and Imperial College

Table 12.1 The 32 most promising accessions among 1586 studied at the International Cocoa Genebank Trinidad based on pod index, mean cotyledon weight and mean bean number

Accession	Group	Cotyledon weight	Bean number	Pod index[a]
UF 11	Trinitario	1.84	39	13.94
CC10	Unclassified	1.75	39	14.65
UF 12	Trinitario	1.77	38	14.87
CRU 116	Unclassified	1.54	43	15.10
CRU 147	Unclassified	1.43	46	15.20
CRU 153	Unclassified	1.60	41	15.24
TRD 35	Trinitario	1.59	41	15.34
CRU 122	Unclassified	1.47	44	15.46
JA 5/36	Refractario	1.40	46	15.53
CRU 34	Unclassified	1.37	47	15.53
ICS 60	Trinitario	1.64	39	15.63
CRU 4A/4	Unclassified	1.48	43	15.71
CRU 138	Unclassified	1.35	47	15.76
JA 5/7	Refractario	1.41	45	15.76
CRU 4A/11	Unclassified	1.51	42	15.77
ICS 68	Trinitario	1.26	50	15.87
ICS 43	Trinitario	1.64	38	16.05
CRU 51	Unclassified	1.41	44	16.12
CRU 5B/25	Unclassified	1.51	41	16.15
CRU 38	Unclassified	1.26	49	16.20
CRU 154	Unclassified	1.54	40	16.23
SILECIA 8 (EET 395)	Trinitario	1.40	44	16.23
CRU 73	Unclassified	1.23	50	16.26
CRU 35	Unclassified	1.43	43	16.26
LCT EEN 261/S-4	Forastero	1.41	43	16.49
PA 205	Forastero	1.41	43	16.49
AM 1/85	Refractario	1.54	39	16.65
CLM 59	Refractario	1.50	40	16.67
MOQ 2/29	Refractario	1.38	43	16.85
IMC 10	Forastero	1.02	58	16.90
ICS 5	Trinitario	1.37	43	16.98
JA 5/35	Refractario	1.37	43	16.98

[a]The number of pods required to produce 1 kg of dried cocoa, a component of yield
Source: Bekele et al. (2014)

Selections (ICS) (Bekele et al. 1994; Johnson et al. 2004). There is a poor representation of Criollo cacao (Bekele and Bekele 1996), and attempts are being made to rectify this through collection initiatives. Over the past 30 years, new introductions include Trinitarios from the Caribbean and Central America, Lower Amazon selections from French Guiana and Venezuela, and Belizean wild Criollo (Motilal et al. 2010). Many clones at the ICGT have been found to combine favorable traits such as high yield potential (Table 12.1) and resistance to BP and WB (Iwaro et al. 2003).

The ICGT thus conserves a broad and valuable genetic base for cacao breeding and improvement (Kennedy and Mooleedhar 1993).

Roughly 1200 primary germplasm accessions, many of them Criollos and Trinitarios, are conserved at the CATIE cacao collection (Phillips-Mora et al. 2013). Utilization of Criollo varieties was earmarked as a priority in the 1980s (Morera 1996). Breeding for disease resistance, particularly to *Moniliophthora roreri* (moniliasis or frosty pod disease (FP)), and *Phytophthora palmivora* (black pod rot) have been another priority (Phillips-Mora 1999; Phillips-Mora et al. 2005, 2011, 2012), and screening of the CATIE cacao germplasm collection for FP resistance has revealed some tolerant clones (Fig. 12.3).

12.2.1.1 Tissue Culture

Research on cryopreservation, tissue culture and somatic embryogenesis (SE) in cacao has been undertaken for decades (Figueira and Yanick 1995; Guiltinan 2007; Maximova et al. 2008; Pence 1995; Sena Gomes et al. 2015; Shires et al. 2017; Sondahl et al. 1993) and is still in progress (Ramirez et al. 2018). Tissue culture and somatic embryogenesis have been tested as methods to facilitate propagation of cacao (Adu-Gyamfi et al. 2016; Alemanno et al. 1996; Goenaga et al. 2015; Guiltinan 2007; Guiltinan et al. 2008; Maximova et al. 2005). The advantages of plant tissue culture for propagation of cacao have been elaborated by Adu-Gyamfi et al. (2016); Guillou et al. (2014); Juárez Gámez (2012) and Maximova et al. (2005, 2008). One is the avoidance of the dissemination of diseases, which can be

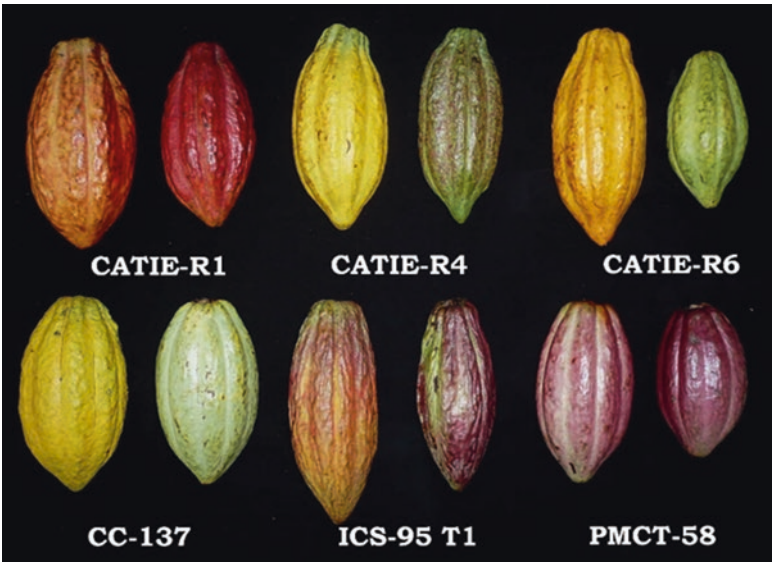

Fig. 12.3 CATIE *R* clones, resistant to frosty pod disease

transmitted by conventional cuttings and grafting. For example, Quainoo et al. (2008) observed that progressive elimination of the cocoa swollen shoot virus (CSSV) in infected trees occurred from primary embryogenesis to secondary embryogenesis.

The use of tissue culture via somatic embryogenesis (SE), using immature flowers, for multiplication of cacao planting material has only been done on a limited scale in cocoa producing countries such as Brazil, Ecuador, Ghana, Indonesia, Puerto Rico and Vietnam (Guillou et al. 2014; Maximova et al. 2008). Nestlé has developed a collection of SE trees in Ecuador, which is maintained in the field and the greenhouse (Guillou et al. 2014). Many of the 140 accessions conserved there are also cryopreserved (stored as living cells/tissues and/or organs in a frozen state) (Guillou et al. 2014). At CATIE, Costa Rica, the development of somatic embryos was investigated in elite cacao clones, CATIE R1, CATIE R4, CATIE R6, CC 137, ICS 95 Tree 1, PMCT 58 and UF 273 (Mata Quirós 2013; Solano 2008). Somatic embryos were obtained in both studies and some were established in the field (Fig. 12.4).

12.2.1.2 Cryopreservation

Cryopreservation can be used for long-term storage of cacao somatic embryos (SE) (Fang et al. 2004; Maximova et al. 2008). Cryopreservation techniques have been adopted at Reading University and by Nestlé (Fang and Wetten 2011; Guillou et al. 2014). The encapsulation-dehydration of cacao SEs allows for appropriate management of the water content of the propagules during cryopreservation. There was a

Fig. 12.4 Somatic emblings (clonal plants generated via somatic embryogenesis that are ready for establishment in the field) in Costa Rica. (Source: Solano 2008)

72% recovery rate in the study at Reading University (Adu-Gyamfi and Wetten 2012; Fang et al. 2004).

Despite the innovations in tissue culture and cryopreservation of cacao, cost-effective and efficient alternatives to conservation of cacao as living collections of trees are yet to be adopted. A large genotype effect has been observed on the success rate for primary somatic embryo regeneration (Maximova et al. 2005). Furthermore, measures must be adopted to monitor and preclude somaclonal variation that can occur with the use of propagules that develop from callus (Adu-Gyamfi and Wetten 2012; Adu-Gyamfi et al. 2016). However, the potential of somatic embryogenesis and cryopreservation for mass vegetative propagation of cacao is enormous (Maximova and Guiltinan in Sena Gomes et al. 2015) with opportunities for automated handling in bioreactors (Niemenak et al. 2008), and further advances in the application of in vitro techniques in cacao are anticipated.

12.2.2 Morphological Characterization of Conserved Cacao Genetic Resources

Morphological characterization data are useful for the identification of cacao accessions and to recognize important traits related to yield and potential constraints to it. The data generated also assist in the detection of duplicates or mislabelled accessions (Sounigo et al. 2003a). Strategic use of characterization data allows selection of genotypes for future breeding/germplasm enhancement programs.

Information on cacao germplasm of value to breeders is available online through the International Cocoa Germplasm Database (ICGD) (End et al. 2000; Turnbull and Hadley 2016) and *Tropgene*. CRC has also disseminated characterization information in a Cacao Clones Manual (Sankar et al. 2008), field guides (Johnson et al. 2004) and articles (Bekele et al. 1994, 2003, 2006, 2008a, b; Iwaro et al. 2003, 2010; Khan et al. 2008). CATIE has produced several catalogues and publications with information on cacao clones in its collection (Engels 1981; Enríquez and Soria 1967, 1981; Johnson et al. 2007; Phillips-Mora and Enríquez 1988; Phillips-Mora et al. 2013; Zhang et al. 2009a).

12.3 Cytogenetics and Self-incompatibility

Cacao is outbreeding (allogamous) and diploid (2n = 20). Bartley and Cope (1973) explained the importance of understanding the incompatibility of cacao to advancement in cacao breeding. Cacao possesses a unique gameto-sporophytic self-incompatibility system, which has been elucidated by Cope (1962) and Knight and Rogers (1955). It is a late-acting mechanism (Silva et al. 2011) since failure in gametic nuclei fusion occurs in the embryo sac. Genetic control of

self-incompatibility is apparently mediated by a nuclear multi-allelic S locus (Knight and Rogers 1955), which is affected by other independent loci, two complementary genes, A and B (Cope 1962). Self-compatible trees are most likely homozygous for the inactive S allele (Eskes and Lanaud 2001).

Cornejo (2013) found that different populations undergo different selfing rates, 0.25–0.9, and this coincides with the range of inbreeding coefficients estimated from individually sequenced genomes. Eskes and Lanaud (2001) stated that Upper Amazon Forasteros are generally self-incompatible. However, some amount of self-pollination occurs due to the mixing of compatible and incompatible pollen by insect pollinators (Lanaud 1987). Determination of the self-compatibility status of potential selections and hybrids for release to farmers is thus a priority for breeders. Furthermore, manual pollination is necessary to produce true hybrid seeds (Eskes and Lanaud 2001).

Posnette (1950) demonstrated that crosspollination in cacao occurs preferentially among neighboring trees. It is thus recommended that cross compatible trees be planted together in cocoa orchards to overcome the negative impact of self-incompatibility on the productivity of cacao. Where it is necessary to have homogeneous plots of trees, manual pollinations, as described by Monteiro et al. (2009), can be done to maintain productivity (Falque et al. 1996). The latter is best done before noon.

12.4 Current Status of Cocoa Breeding

12.4.1 Selection Criteria

There are several challenges to global cocoa production, which include low productivity, small farm acreages, increasing pest and disease pressure, climate change, difficulty to satisfy growing consumer markets, the relatively high costs of production and the need to maintain good quality. Cocoa breeding programs must thus be designed to address the various constraints that affect the crop as well as optimize its production and productivity, and the quality of cocoa beans produced.

Among traits which superior varieties should have are fermented and dried seeds with a weight >1 g (Soria 1977, 1978), a high number of beans per pod, high butterfat content and resistance to pests and diseases (Glendinning 1963; Toxopeus 1972), preferably horizontal/non-specific and durable disease resistance (Simmonds 1994; Tiburcio et al. 2010). Durable resistance is demonstrated when a cultivar is tolerant to infection over a wide geographic area and over a significant period of time while under strong disease pressure (Johnson 1993).

Other selection criteria include vigor, uniform plant type, compactness in tree size, precocity (early flowering and maturing) (Ahnert 2009), tolerance to drought stress and quality expressed in terms of bean flavor, purity and food safety.

Since bean weight and bean number per pod have a fair to high heritability (Cilas et al. 2010; Glendinning 1963; Jacob and Toxopeus 1971; Toxopeus 1972), infor-

mation on seed and pod indices is particularly useful to breeders. A low pod index, which is the number of pods required to produce 1 kg of dried cocoa, is desirable since it is normally associated with large bean size (preferred by chocolate manufacturers) and is a reliable indicator of good yield potential. A maximum pod index of 16.5 pods was a standard set for selection in an Imperial College of Tropical Agriculture survey (Pound 1932). However, in many cases breeders select genotypes with higher pod indexes to favor other relevant traits such as disease resistance. Yield efficiency (a function of dry bean yield divided by plant vigor measured as trunk girth to obtain less vigorous, more efficient and productive cacao trees) has gained precedence in Côte d'Ivoire (Eskes et al. 1993, 1996; Sounigo et al. 1994) and Papua New Guinea (Efron et al. 2005b, c). The number of healthy pods produced per tree is also a critical component of yield.

Cacao thrives under specific environmental conditions (soil and climate) and is susceptible to a range of fungal and other diseases as well as pests, including insects such as mirids (*Sahlbergella singularis*, *Distantiella theobroma* and *Helopeltis* sp.) and the cocoa pod borer (larvae of the moth, *Conopomorpha cramerella*), along with rodents and parrots. Some insects can cause younger trees to wither or *dieback*.

Among the diseases caused by fungi are witches' broom (WB) (in South America and the Caribbean) caused by the *Moniliophthora perniciosa*; Moniliasis or FP (Fig. 12.5), caused by *Moniliophthora roreri* (in Central America and South American producing countries such as Ecuador) (Aime and Phillips-Mora 2005;

Fig. 12.5 Cocoa fruit/pod with frosty pod infection caused by *Moniliophthora roreri*

Phillips-Mora et al. 2011), and recently in Jamaica; and black pod (BP) or Ppr (elsewhere but particularly in Africa) caused by *Phytophthora* spp. (Akrofi et al. 2015; Guest 2007; Iwaro et al. 1997a, b, c).

In Southeast Asia, the cocoa pod borer (CPB) and the disease, vascular streak dieback (VSD) caused by *Oncobasidium theobromae*, cause considerable damage. In West Africa (Benin, Côte d'Ivoire, Ghana, Liberia, Nigeria, Sierra Leone and Togo), the cocoa swollen shoot virus (CSSV), genus *Badnavirus*, is a serious constraint to production (Dzahini-Obiatey and Adu-Ampomah 2010; Padi et al. 2013c).

Diseases and pests can destroy 20–30% or more of total cocoa production, and high yield and disease resistance have received the most attention from breeders (Gutiérrez et al. 2016; Lopes et al. 2011). However, according to Akrofi et al. (2015), despite the progress made in international collaborative efforts such as the two international, multi-stakeholder projects spearheaded by the International Cocoa Organisation (ICCO), funded by the Common Fund for Commodities and administered by Bioversity International between 1998 and 2009 (hereinafter referred to as CFC/ICCO/BI Project) (Eskes 2011; Eskes and Efron 2006; Eskes et al. 2000), cacao varieties available to farmers are not yet fully tolerant to *Phytophthora* pod rot disease (BP) and particularly to *P. megakarya* or to WB and FP pod rot.

In West Africa, the emphasis, since the 1940s, has been on resistance to CSSV and establishment ability (Adu-Ampomah 1996; Posnette 1948). In Southeast Asia, resistance to VSD, CPB attack and BP have been primary objectives. In Brazil, resistance to BP and WB were of primary importance, but more recently in Central America, Mexico, Brazil and Ecuador, focus has shifted to FP tolerance.

In Trinidad and Tobago and Ecuador, sensory/flavor attributes are desirable traits to ensure that the coveted aromatic profiles for which these countries are well known (fruity and floral/*Arriba*, respectively) are maintained (Monteiro et al. 2009). In the breeding programs at CEPLAC, Brazil and CATIE, Costa Rica quality traits are important selecting criteria (Lopes et al. 2011; Monteiro et al. 2009; Phillips-Mora et al. 2013). Currently, in Ghana and elsewhere, flavor attributes are also gaining importance (Assemat et al. 2005; Sukha et al. 2008). In the near future, nutraceutical properties may also be assessed as it has been done in Trinidad and Tobago by Pilgrim et al. (2015) and by Onomo et al. (2015) in Cameroun.

The choice of suitable parents for cacao breeding traditionally depended on the availability of characterization/evaluation data, where attributes are easy to score by observation of morphology or simple screening procedures, along with information on general combining abilities of prospective candidates. However, polygenic traits, which are significantly affected by environment are more difficult to score without a tool for identifying and following the major genes influencing the phenotype. BP, FP and WB disease resistance fall into the latter category, and recent research has facilitated the tagging of these traits with molecular markers to identify quantitative trait loci (QTLs) (Araújo et al. 2009; Argout et al. 2008, 2011; Brown et al. 2007; Clément et al. 2003a, b; Collard et al. 2005; Crouzillat et al. 2000a, b; Epaina 2012; Feltus et al. 2011; Lanaud et al. 1999, 2003b, 2004a, b; Zaparoli et al. 2009). The

identification of QTLs and candidate genes (Pflieger et al. 2001) for the aforementioned characters can facilitate the early selection of superior genotypes.

12.4.2 Constraints to Breeding

Cacao breeding has been constrained to date by the long breeding cycle of the crop. The production of sufficient fruits in progeny to be evaluated may not be realized until a plant is about 2–5 years old. A single selection cycle frequently takes more than a decade (Monteiro et al. 2009), and two or more cycles of selection are often required before a new variety can be released to farmers with an expectation of good performance (Phillips-Mora et al. 2013). This is not uncommon even with annual crops where the release of new varieties can entail up to 20 years of trials (Briggs and Knowles 1967). It is important to consider that most economically-important traits in cacao are polygenic and governed by different modes of inheritance (dominant, codominant, incompletely dominant, additive, polygenic additive, complex) that complicate simultaneous selection for multiple traits. Therefore, it is essential that improvements be made in successive stages of selection.

In addition, the availability of land and resources to conduct large-scale pre-breeding and breeding trials has been a limiting factor. The CFC/ICCO/BI project entitled *Cocoa Germplasm Utilization and Conservation: A Global Approach* (1998–2004) (Eskes and Efron 2006) was the first major international, collaborative cocoa improvement program to date. This project did much to improve the status of global cacao pre-breeding and germplasm evaluation efforts. The main objective for this project was germplasm enhancement for BP and WB disease resistance along with yield potential.

The relationship between genetics and the environment to which cacao varieties must be adapted in the future must be understood when designing breeding programs. The projected impact of climate change must be taken into consideration as well as the management practices and specific growing environments in which new varieties will be distributed. These factors pose challenges to breeders since they must consider unknown variables when selecting and breeding new varieties and stand to lose favor with cacao farmers if commercial varieties are not adaptable and do not perform as desired in farmers' fields. A thorough understanding by breeders of the relationship between traits and environment and G×E interaction and a systematic approach to breeding is thus required (Comeau et al. 2007). Multi-locational trials for testing promising genotypes are highly recommended.

Another constraint was identified by Bartley (1967) as the inability to predict the performance of clones as parents in breeding programs. He stated that the yield of a clone was no guide to its value as a parent. This makes the process of selection cumbersome, time-consuming and expensive since it requires mass screening of progeny and recurrent selection to facilitate identification of superior genotypes.

12.4.3 Breeding Programs

Some of the breeding programs that have been conducted to date are highlighted in Table 12.2. Since the 1990s, recurrent selection programs were conducted in Brazil, Costa Rica, Côte d'Ivoire, Ghana, India, Malaysia, Papua New Guinea and Trinidad and Tobago.

12.5 Breeding Strategies

12.5.1 Breeding for Yield Improvement

Several cocoa breeders, including Bartley (1996) and Adomako and Adu-Ampomah (2005), have stated that breeding for yield improvement in cacao is challenging. Some improvement has been realized through breeding and selection in various national programs (Table 12.2). Such improvement has been attributed partly to additive genetic gain (Adomako and Adu-Ampomah 2005; Efron et al. 2005b; Lockwood and Pang 1994; Lockwood and Yin 1996; Tan 1990). Eskes and Lanaud (2001) cited evidence that general combining ability predominates for cocoa productivity.

In Ghana, Adomako and Adu-Ampomah (2005) observed that genetic gain in cacao yield may be lost when farmers use F_2 seed to generate new plantings, thereby increasing genetic variation and tree-to-tree variation. Based on actual yields attained currently and considering that potential yields of over 3000 kg per hectare have been realized in Malaysia (Lockwood and Pang 1994), there is still much improvement in yield to be achieved in most cocoa producing countries (Ahnert 2009; Bartley 2005; CacaoNet 2012; Efron et al. 2005b; Eskes et al. 1996; Lockwood 2003). Aikpokpodion et al. (2010) described the program for yield improvement in Africa at the 6th INGENIC Workshop, in 2009 (http://www.incocoa.org/data/ingenic_workshop_6_Rep_2009.pdf). Farms in West Africa were described as generally low yielding essentially due to the prevailing pests and diseases (mirids, BP, CSSV) and advanced age of the tree stock. The need for high yielding, precocious planting material was highlighted and would be an objective of the future breeding activities in the region.

Table 12.2 Some highlights of cacao improvement programs for yield, disease resistance and other agronomic traits

Country	Mating design	Breeding objectives	Main findings	Reference
Brazil	Recurrent breeding scheme	Multi-trait selection for resistance to witches´ broom (WB), black pod (BP), frosty pod (FP), high yield, high butterfat content and other traits.	500 progenies, accumulating 30 thousand trees, 39 clones and 3 hybrids released to farmers;	Lopes et al. (2011), Monteiro et al. (2009), Pires et al. (1998), and Silva et al. (2011)
	Bi-parental crosses; complete diallel design	Improvement in yield (number of pods per plant), resistance to WB and *Ceratocystis* wilt	The progenies CEPEC 94 × CCN 10, RB 39 × CCN 51, CCN 10 × VB 1151 and PA 169 × CCN 10 were the most productive	Yamada et al. (2013)
Cameroun	6 × 6 diallel crossing scheme	Multi-trait selection for improved yield, yield potential, resistance to black pod (BP) diseases	Heritability of *Phytophthora* resistance was 0.2; higher than for yield (0.13). Expected genetic gain was about 35% for resistance at 1% level of selection.	Ndoumbé et al. (2001)
Costa Rica	Six segregating populations (from bi-parental crosses) for molecular studies, 532 clones, and 292 hybrid families are now under evaluation	Multi-trait selection for disease resistance (frosty pod (mainly) and BP, high-yield and good quality (flavor). Pyramiding of FP resistant genes.	CATIE R-1, CATIE R-4, CATIE R-6, CC 137, ICS 95 (type 1), PMCT 58 selected as superior clones	Phillips-Mora et al. (2012)

(continued)

Table 12.2 (continued)

Country	Mating design	Breeding objectives	Main findings	Reference
Côte d'Ivoire	Reciprocal recurrent selection scheme	Selection for adaptation and for resistance to Ppr, good yield potential, self-compatibility, large seed size and good flavor attributes;		Paulin et al. (1994)
	5 × 5 diallel and in a 4 × 2NC II factorial design;	Resistance to BP (laboratory and field)	Superior parental types include: five Lower Amazon clones, eight Trinitarios, and 11 Upper Amazon Forasteros	Tahi et al. (2006a, b)
	Reciprocal recurrent selection scheme	Resistance to black pod disease (Ppr) and yield	Described in Sect. 5.2.3	Pokou et al. (2009)
Ghana	Bi-parental crosses	Improvement in yield, establishment capabilities, to cocoa swollen shoot (CSSV) resistance and *P. megakarya* resistance;	Success in yield improvement but not in CSSV resistance.	Adu-Ampomah (1996) and Legg and Lockwood (1977)
	Diallel or factorial	Field resistance to CSSV virus disease;		Adomako (2006, 2007)
		Combining ability analysis of BP disease incidence		
Malaysia	Reciprocal recurrent selection scheme	Resistance to cocoa diseases, particularly vascular streak dieback (VSD)	Crosses between superior clones were established in variety trials and over 97 promising progenies were generated, some with enhanced resistance to VSD and black pod disease as well as high butterfat content.	Chong and Shepherd 1986, Lockwood and Pang (1996), and Lamin et al. (1999)
Nigeria	Bi-parental crosses, progeny trials; diallel crossing scheme (started in 1960)	Establishment ability, vegetative vigor, precocity, yield, resistance to BP and CSSV	Release of superior varieties to farmers	Toxopeus (1972), Aikpokpodion and Adeogun (2011) and Aikpokpodion et al. (2005)

(continued)

Table 12.2 (continued)

Country	Mating design	Breeding objectives	Main findings	Reference
Papua New Guinea	Bi-parental and poly crosses, reciprocal recurrent selection scheme	Durability of field resistance to BP disease, yield, vigor, compact architecture	Release of promising hybrids for field trials	Saul-Maora et al. (2003), Efron et al. (1999, 2002a, 2005a, b, c)
		Resistance to vascular-streak dieback		Efron et al. (1999) and (2002b)
Trinidad and Tobago	Reciprocal recurrent selection scheme	Resistance to *Ceratocystis* wilt and WB, large bean size and weight, high yield	Release of 20 superior commercial clones to farmers	Freeman (1969, 1982), Shripat (1993), and Maharaj et al. (2011)

12.5.2 Conventional Hybridization

Hybridization (in bi-parental crosses and in diallel (complete and partial) and factorial designs (North Carolina II model)) has been the favored breeding method for improving yield in cacao (Dias et al. 2003; Eskes and Lanaud 2001; Gotsch 1997). Ahnert (2009) outlined a method for ideotype breeding, which seeks to increase production potential through selection of individual traits in accordance with a specified model. Traits identified for inclusion in such an approach included precocity (early bearing), plant vigor and growth rate, growth habit, durable disease resistance, seed size, shape and butterfat content, compatibility status and yield. A recurrent scheme with F_1 crosses and self-pollinated generations, in a circular partial diallel mating design, was recommended.

The consensus of opinion is that sustainable progress in cocoa breeding for improved production and yield requires systematically screening for genetically superior individuals in segregating populations (Eskes et al. 1996; Lockwood et al. 2007; Monteiro et al. 2009; Pires et al. 1999). Bartley (1996) concluded that an effective breeding strategy utilize the *products of recombination from controlled breeding programs* to generate new clonal cultivars on a continuing (recurrent) basis as improvement progresses. Hybrids between heterozygous parents, including double crosses and triple crosses, where the parents are derived from different source populations, are the best potential sources of superior selections (Adu-Ampomah et al. 2006). Noteworthy for elite selections derived from such a strategy are the TSH breeding program in Trinidad (Freeman 1969, 1982; Montserin et al. 1957), the CATIE-R series (Phillips-Mora et al. 2013), and the CCN program in Ecuador, conducted by the late Homero Castro, which eventually produced the now widely adopted CCN 51 variety. In Trinidad, Freeman performed backcrossing of hybrid progenies (F_1 and F_2) to the parents that transmitted favorable traits such as WB resistance (from the SCA 6 parent) and large bean size (from ICS 1) (Maharaj et al. 2011).

The innovative use of doubled haploids to generate desirable homozygous lines of cacao to be distributed to farmers or used as parents for the production of hybrid seed has been attempted in cacao (Lanaud 1987; Sounigo et al. 2003b). This is an area that requires further work (Guiltinan 2007).

Recent activities in West Africa involved the conduct of clonal and hybrid trials, and a regional progeny trial to assess new clones from populations that were not used before, along with controlled crosses and commonly used varieties. In Ghana and Nigeria, the marker-assisted selection program has identified putatively resistant material to WB and BP in five families. In the case of selecting for WB resistance, this is a proactive measure since this disease does not occur here. QTLS for BP and CSSV resistance are being sought in a segregating population of over 250 plants in Ghana and Côte d'Ivoire.

12.5.2.1 Intravarietal Breeding Strategies and Recombinant Inbreeding Lines (RILs)

Recombinant inbreeding lines (RILs) and intravarietal breeding strategies are used in breeding to accumulate genes of interest. Although RILs are not common in cacao, a well-known example of an inbred line reproduced through several generations of inbreeding (through open pollination) in cacao is the West African Amelonado (WAA). Nacional of Ecuador and Matina from Costa Rica may be considered other examples. Nacional was maintained in a fairly uniform state over time, although self-incompatibility would have allowed some sib-mating and probably resulted in it being described as Forastero despite the Criollo characteristic of large, pale beans in the pure form (Enríquez 1993).

Intravarietal crosses have been made in several cacao breeding programs. LA × LA; UA × UA; T × T as well as within population crosses such as PA × PA; NA × NA; IMC × IMC and ICS × ICS and other such crosses are common (Monteiro et al. 2009). In Ghana and Malaysia, intra Amazon Forastero crosses have resulted in productive hybrids (Lockwood and Pang 1996). Eskes et al. (1996) described such crosses made in Cameroun and Côte d'Ivoire, and Efron et al. (2005a) reported on such crossing schemes in Papua New Guinea. Pires (2003) concluded from their research in Brazil that accumulating favorable alleles for specific traits and pyramiding of genes for durable resistance to WB may be achieved through simultaneous inter-crossing within groups.

12.5.2.2 Intervarietal Breeding Strategies

Hybridization (intervarietal breeding) and selection has been widely applied in cacao with undesirable progeny being culled during the various stages (Bartley 1996), and select or superior trees cloned through vegetative propagation. The latter tend to display heterogeneity (Eskes and Lanaud 2001). The generation of a large number of progeny is recommended to obtain better results (Efron et al. 2005b;

Lockwood 2003). Progenies of outstanding trees are usually planted to conduct a second and third cycle of crosses and selection (Baudouin et al. 1997).

12.5.2.3 Reciprocal Mating Designs with Backcrossing – Reciprocal Recurrent Selection Scheme

The objective of achieving relatively rapid progress in cacao breeding combined with long-term improvement has largely been achieved through reciprocal recurrent selection schemes (RRSS) (Eskes et al. 1996; Lockwood and Pang 1996), as that adopted in Trinidad with the Trinidad Selected Hybrid Program (Freeman 1969; Maharaj et al. 2011). RRSS have also been adopted for perennial crop breeding by CIRAD, France (Baudouin et al. 1997; Eskes and Lanaud 2001), Costa Rica, Côte d'Ivoire, Ghana, and elsewhere in cocoa producing countries. The advantages of such schemes include increased frequency of genes and desirable associations, the enabling of effective recombination, and partial fixing of heterosis (Baudouin et al. 1997), and additive gene action observed in cacao (Eskes et al. 1996) that can allow for the exploitation of transgressive segregants (superior to parents in traits expressed). Most of the traits of interest have been found to be under polygenic control and general combining ability is common (Dias 2001; Eskes and Lanaud 2001).

The process involves mass screening and selection of suitable parents from populations such as Upper Amazon (UA_0) or Lower Amazon (LA_0) or Trinitario (T_0), production of hybrids from the promising parents (recombinations such as $LA_1 \times$ or $UA_1 \times UA_{1\ or}$ $UA_1 \times LA_1$ or $UA_1 \times T_1$ or $LA_1 \times$ Criollo and so on), selection among progeny, hybrid testing (as described below in Sect. 12.6), backcrossing to parental types or recurrent reciprocal crosses, and varietal development. This is followed by at least two other cycles of crosses and selection of promising crosses based on genetic tests, evaluation of progenies in the field, and subsequent varietal development and release to farmers (Monteiro et al. 2009; Pokou et al. 2009) (Fig. 12.6).

Control plants or testers, comprised of outstanding performers that are cloned, should be included in each cycle of selection for comparison with the performance among progenies (Ahnert 2009; Efron et al. 2005b). Hybridization between distant individuals or distinct populations has been found to lead to increased chances of achieving heterosis and genetic gain (Baudouin et al. 1997; Dias et al. 2003; Eskes and Lanaud 2001; Eskes et al. 1996; Lachenaud et al. 2007; Lanaud et al. 2003a; Pang and Lockwood 2008; Toxopeus 1972).

In Côte d'Ivoire, a RRSS was initiated in 1990 (Pokou et al. 2009). The aim was simultaneous improvement of the two main genetic groups for increased resistance to BP and yield. The varieties involved are from the UAF genetic group, which provides good combining ability for early yield (Paulin et al. 1994), adaptation and resistance to BP, and a mixture of Lower Amazon Forastero (LA) and Trinitario (T) (LA + T) that combine good yield potential, self-compatibility and large seed size with good flavor attributes. The scheme included two cycles of recurrent selection to improve traits with good heritability (such as bean size and resistance to BP),

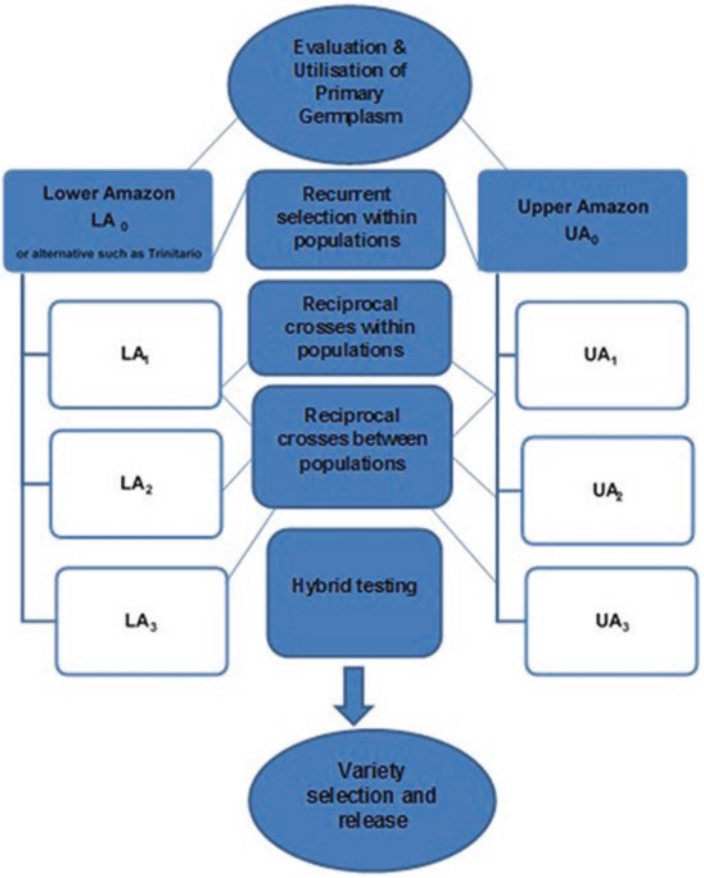

Fig. 12.6 Schematic for a reciprocal recurrent selection scheme (RRSS). (Source: Adapted from Baudouin et al. 1997)

followed by RRS to exploit additive as well as dominant gene effects. The first cycle included the two main groups and involved 250 intra-group progeny. Among the second cycle parental clones, single trees from the first cycle intra-group progeny as well as additional selected parental clones were selected. The control varieties used were the same for the inter-group hybrid varieties and intra-group crossing plots. The first cycle parents included SCA 6, IMC 57 and PA 150 (UA) and ICS 6, ICS 95, IFC 1, GS 29, SNK 12, UF 676 and VENC4-11 (LA + T). Pokou et al. (2009) concluded that it is feasible to exploit both the best inter-group as well as the best intra-group crosses to achieve success in cacao breeding.

In Ghana, a RSS was adopted using four bi-parental crosses including six Amazonian and one Trinitario clone (Pang and Lockwood 2008). Over time, the performance of clones in terms of growth, yield and yield efficiency was compared to that of their progeny grown as clones. There was no evidence of pronounced

hybrid vigor, but the authors concluded that simple recurrent selection is effective in cacao when optimum planting densities for the seedling versus clonal plantings are adopted.

12.6 Varietal Screening and Utilization

Van Hall (1932) adopted a method of selection and evaluation of individual trees among progenies as well as evaluation of the progenies of selected clones (resulting from natural pollination) in clonal trials to determine whether the clones were superior to the progeny generated through open pollination. The results were inconclusive and several breeding programs have undertaken one or both types of screening of hybrid progeny: single tree among progenies (Bartley 1957) and mass screening of progenies of selected clones (Ahnert 2009).

Individual tree or clonal selection, based on superior phenotypic traits such as yield potential, bean size and disease resistance, was employed by Pound when selecting the Imperial College Selections in Trinidad among 50,000 local landraces (Pound 1935), and elsewhere in the Caribbean by Cope with the Grenada Selections produced through bi-parental crosses (Cruickshank and Murray 1966). Clone selection was also done in Latin America as in Costa Rica by the United Fruit Company with the UF selections (Hunter 1990; Johnson et al. 2009) and further afield as in Nigeria and Ghana (Lockwood et al. 2007; Posnette 1943). These single tree screening exercises met with some success due to their large scales.

Elite cacao clones are usually chosen for vegetative mass propagation followed by distribution to farmers (Bartley 1996). This was the strategy adopted by the Trinidad Ministry of Agriculture for the Trinidad Selected Hybrids (Freeman 1969), but it was elaborated by first evaluating clones on twelve different estates to determine stability over environments.

However, predicting the yield of a tree's performance as a clone or seedling is difficult due to the large tree-to-tree variation in cacao and low broad sense heritability (Bartley 1957). Toxopeus (1969) also observed that the yield of mother trees does not correlate well with that of clonal progeny. Cilas (2005) described a statistical approach to improve the efficiency of individual cacao tree selection for quantitative traits in progeny trials to select trees in RSS. This entailed combined individual-family selection, smoothing methods derived from spatial statistics and longitudinal data analysis. Cilas (2005) also proposed methods to improve experimental designs by increasing the number of trees per genotype. Bertrand and Cilas (1990) described a method to produce true twins from one seed for such experiments.

The optimal strategy for clone selection from among progeny is generally agreed to be family level selection (Simmonds 1996), followed by evaluation of large numbers of clones from among superior families in properly designed and managed trials (Efron et al. 2006; Lockwood et al. 2007). In Cameroun, a combined individual-family multi-trait selection index was applied (Ndoumbé et al. 2001) as recommended by Cilas (2005).

At Prang Besar Research Station in Malaysia, Chong and Shepherd (1986) conducted one of the most successful programs of selection of elite clones by mass screening in a large-scale breeding program. Lockwood et al. (2007) observed that only 1 among 1000 trees was found worthy of selection based on ease of establishment, low vigor, and precocity, justifying the need for mass screening. The clone selection program in Ghana (Lockwood et al. 2007; Padi et al. 2015) was also effective since it involved 5000 ortets, many of them including the best selections for low incidence of BP from farmers' fields.

Mass screening of progenies is labor-intensive and time-consuming especially where reproductive traits such as bean weight are evaluated. There is a need to more effectively utilize genomic tools for early prediction of the potential performance of hybrids in the recommended mass screening of hybrids to generate superior clones (Dias et al. 2003, 2004).

12.6.1 Farmer Participatory Approach to Screening of Planting Material

The importance of adopting a farmer participatory approach to cocoa breeding must be emphasized. Farmers are the ones who utilize improved cacao planting material and can evaluate them in terms of all of the aforementioned traits. They also conserve *landraces* or *heirloom varieties*, as described by Guarino and Lobell (2011). Over the past decade, *Farmer Participatory approaches* have been adopted to select superior genotypes or landraces in farmers' fields, utilize these selections in germplasm enhancement and so eventually improve cocoa production. Farmer Field Schools have also been held to educate farmers on how to screen their planting material for superior traits such as good yield and disease resistance as well as to apply good agricultural practices to maximize productivity. Success was achieved during the international project entitled *Cocoa productivity and quality improvement: a participatory approach*, which was conducted 2004–2009 (Eskes 2011).

Farmer Field Schools (FFS) are now conducted routinely in West Africa, Southeast Asia, Latin America and the Caribbean. Between 2011 and 2013, the Cocoa Research Centre spearheaded a FFS initiative within the Caribbean that trained farmers in good agricultural practices (GAPs) and screening of planting material. Cocoa artisans and entrepreneurs were also trained, during this project, in good manufacturing practices (GMPs) for food safety compliance.

In Papua New Guinea, some of the advanced farmers' selections were found to yield up to 60% more than even the best improved varieties (Eskes 2011). The adoption and further evaluation of improved varieties (hybrids) by farmers are facilitated and accelerated when farmers are part of the process (Aikpokpodion et al. 2005; Neilson et al. 2014). Thus, future cocoa breeding strategies should ideally incorporate a farmer participatory approach to maximize output. However, the low frequency of genes in the farms related to traits of particular interest impose a limitation

to the participatory approach and make it mandatory to also search for them in cacao genebanks. That is the case for resistance/tolerance to frosty pod rot.

12.7 Genetic Analysis and Screening for Superior Hybrids

Superior hybrids must not be selected based solely on favorable phenotypes or genotypes. The narrow sense and broad sense heritability of the superior traits exhibited by hybrids must be determined to fully assess their breeding value. According to Bartley (1996), successful hybrids would result from the careful exploitation of heterosis for the components of yield and factors that contribute to greater adaptability to varying environments. According to Eskes and Lanaud (2001), Upper Amazon Forasteros (UAF) and their hybrids are more adaptable to growing environment than Amelonados (LAF) and Trinitarios. Hybrid trees exhibiting superior traits must also be subjected to determination of their self and cross compatibility status, which would allow selection of self and cross-compatible hybrids for further improvement.

Breeders also require information on the combining abilities of parental types (progenitors) in order to choose the best candidates for cocoa improvement (Adomako 2006). When a complete diallel mating design (random model) is used in breeding, it is possible to determine general and specific combining abilities (GCA and SCA) for quantitative traits of select hybrids (Acquaah 2012; Ahnert 2009; Efron et al. 2005a; Gutiérrez et al. 2016). The latter cater to the *randomness of the hybridization process* (Dias and Kageyama 1995; Dias et al. 2003). Such information is gleaned through genetic analysis to determine components of variance among progenies, which are estimated as outlined by Falconer (1989). The mean square values computed for GCA, SCA and the error terms in analysis of variance (ANOVA) can be used in determining the additive, non-additive and environmental variance. GCA is a measure of the average performance of a progenitor/parent in a group of crosses. It is an indicator of breeding value.

Griffing's (1956) method is useful to calculate combining ability in diallel crosses. Cervantes-Martinez et al. (2006) demonstrated a rigorous statistical method and the utility of deriving GCA and SCA for bi-parental crosses in Costa Rica, as did Nyadanu et al. (2012), in Ghana.

Tan (1990) described how information derived on GCA effects and the mean performance of hybrid progenies in Papua New Guinea was used to select 20 high-yielding crosses with good pod and bean characteristics for commercial use. Lockwood and Pang (1994) were able to deduce, in this way, that yield is inherited additively.

Furthermore, genetic distance estimates based on performance and molecular data (Dias et al. 2003, 2004; Lanaud et al. 2003b) may also be used to identify prospective parental types and hybrids with putative heterosis for yield and other quantitative traits of economic importance.

12.7.1 Hybrid Screening for Traits of Economic Interest

Screening for superior parental types among progeny may involve selection among heterozygous genotypes followed by inbreeding and subsequent identification, among the inbred progeny (S_0), individuals that are homozygous for the desired condition (Monteiro et al. 2009). The production of clones from superior progeny also ensures uniformity in the desired trait or traits. Selected clones should be assessed for their value as parents. However, it must be borne in mind that many farmers prefer seedling material because of its growth habit and ease of establishment due to the rooting system (Wood and Lass 1985).

Field assessments for yield components (number of pods, pod index, yield per hectare), stem diameter, vigor, precocity and yield efficiency, disease resistance and other traits of interest are routinely conducted during the process of conventional breeding. Tan and Tan (1990) noted that the resistance to black pod disease (Ppr) observed in the hybrid, K82, in Papua New Guinea is probably horizontal resistance. They concluded that breeding for high-yielding cultivars combined with Ppr resistance is the most effective way of controlling Ppr in small holdings in Papua New Guinea, and recommended K82 as a good candidate. Iwaro et al. (2009) described the genetic gain achieved in Trinidad through hybrid screening for favorable yield potential along with BP resistance. This is being leveraged to further improve the Trinidad and Tobago Ministry of Agriculture's TSH breeding program (Iwaro et al. 2010; Maharaj et al. 2011).

12.7.2 Assessing Yield Components and Productivity

Bean weight, bean number and pod index (PI) are moderately heritable (Toxopeus 1972) and are indicators of yield potential. In order to maximize productivity of cocoa estates, pod index as a measure of yield potential should be low (not exceeding 20–25), trees should produce many pods (more than 20 per tree each harvest) and the yield of cocoa per hectare should exceed 800 kg per hectare at a planting density of 1600 trees per hectare.

To measure yield in cacao, the method of Lachenaud (1991) can be used. Yield per tree is estimated from the average total number of harvested pods (PN) per tree and the average total wet bean weight per pod (TWBW). During each harvest, the total wet bean weight per tree (WBW) is estimated using a random sample of pods (PN × TWBW). Yield in mt of dry cocoa per annum and per unit area (hectare or acre) is then deduced from the equation:

$$\mathrm{WBW} \times d \times \alpha$$

where d is the planting density (e.g. 1666 trees/ha) and α is a coefficient of transformation of wet bean weight to dry bean weight, fixed at 0.35, on the recommendation of Lachenaud (1991).

Tahi et al. (2007) recommended simple methods for measuring pod production in field trials. In Ghana, Padi et al. (2012) found that combined selection for rapid increase in tree cross-sectional area and precocity is potentially useful as an early indicator for identifying high yielding families in breeding programs. Padi et al. (2013b) concluded that ortet selection for yield will be more effective in families where the progenitor clones combine good yield with favorable general combining ability for vigor and yield. The yield efficiency or relationship between yield and vigor measured by trunk girth at 30 cm height is used to identify high yielding trees (Toxopeus 1969) since it identifies whether there is a favorable distribution of assimilates directed towards fruit production compared to vegetative growth (Eskes and Lanaud 2001).

In selecting for superior yield, several studies have indicated that interaction between clone and planting density affects yield, and optimal planting density for specific varieties must also be determined (Lockwood and Yin 1996; Maharaj et al. 2005; Mooleedhar and Lauckner 1990; Shripat and Bekele 1999; Sitepu et al. 2005; Souza et al. 2009). In addition, physiological traits such as assimilate accumulation and partitioning should be considered since they affect yield (Alvim 1977; Daymond et al. 2011).

Adaptability of potentially high-yielding cultivars to varying environments and temporal stability of yield are important considerations for breeders. These factors were studied by Carvalho et al. (2003), who screened superior cacao varieties and hybrid trees for yield, bean quality and resistance to WB in Rondônia State, Brazil. The IMC 67 × BE 8 cross in their study was found to be adapted to unfavorable environments and was highly predictable in terms of the total number of healthy fruits produced. Furthermore, it was concluded that total number of healthy fruits, total weight of fresh seeds and mean weight of fresh seeds per fruit were stable over time and could be measured early in trials once fruiting is established, and prior to 8 years after planting as was previously advocated.

Some consideration must also be paid to effects of agronomic practices that affect the realization of the yield potential of promising hybrids or clones in a sustainable manner (Oberthür et al. 2018). Ahenkorah et al. (1987) demonstrated the importance of shade and fertilizer on the yields of Amazon cocoa in Ghana. High yields (of more than 1000 kg dry cocoa ha^{-1}) could not be maintained beyond 15 years of intensive cropping.

The components of yield and their interactions may be complex and require careful statistical analyses, as demonstrated by Paulin et al. (1994), Cilas (2005) and Efron et al. (2005a, c, 2006). Factorial mating designs (such as the North Carolina Design II) (Acquaah 2012) and those that examine spatial and temporal factors also demand rigorous analysis that include the use of Ripley's $K(r)$ functions (Oro et al. 2012). Path analysis is useful to study yield components of cacao hybrids (de Almeida et al. 1994).

12.7.3 Screening for Disease and Pest Resistance

Since breeding trials, by necessity, must be large to increase the chances of success, it is imperative that early and easy screening tests be available to rapidly screen the progenies for outstanding individuals in terms of disease and pest resistance (Nyassé et al. 1995; Surujdeo-Maharaj et al. 2003, 2004, 2009; Tahi et al. 2000, 2006a, b). Several advances have been made in screening for resistance to BP and WP as well as yield efficiency (Gutiérrez et al. 2016). Sackey (2003) described novel methods for screening for CSSV resistance.

Although some significant genotypic responses to pest attack have been observed in cacao (Eskes and Lanaud 2001), high levels of pest resistance have not been recorded to date. Resistance to mirid attack in Côte d'Ivoire (Sounigo et al. 1994), cocoa beetle (*Steirastoma breve*) attack in Venezuela (Morillo et al. 2008) and to cocoa pod borer attack by the larvae of *Conopomorpha cramerella* (Snellen) in Malaysia (Lamin and Sáedi 1996) was found to be significantly linked to genotypic effects. Valenzuela et al. (2014) developed yield loss models for infestation of cocoa with cocoa pod borer. Due to increased mortality of the larvae with harder pod walls observed by Azhar (1988) and Azhar and Long (1996), routine evaluation of pod wall hardness is done at the International Cocoa Genebank Trinidad (Bekele and Butler 2000; Bekele et al. 1996a, 2009). Pest resistance is not usually a selection criterion in cacao breeding programs. However, it was considered in selecting an international clone collection (Eskes and Sounigo 2000; Sounigo et al. 2006) for evaluation in the collaborative CFC/ICCO/BI project on Cocoa Productivity and Quality Improvement, a Participatory Approach (Eskes 2011).

12.7.3.1 Early Screening Tests

Significant achievements in developing efficacious early screening tests for disease resistance have been developed (Cilas and Despréaux 2004; Iwaro et al. 1997a, 2005; Surujdeo-Maharaj et al. 2003). These are being used currently to more quickly screen hybrids and clones for selection (Efombagn et al. 2011). Nyassé et al. (1995, 2002, 2003) developed and tested a leaf inoculation test to assess the resistance to *Phytophthora megakarya* in a 6 × 6 diallel mating design. Nyassé et al. (2003) found a significant genetic correlation between pod rot incidence in the field and early leaf test data.

Surujdeo-Maharaj et al. (2003, 2004, 2009) described an optimized agar-droplet method, which can effectively be used for screening for WB resistance in the greenhouse. Broom-based diameter of brooms in the greenhouse was found to be significantly correlated ($r = 0.71$) with field resistance to WB assessed as infection frequency of vegetative shoots observed over 2–3 years (Surujdeo-Maharaj et al. 2009).

At CATIE, effective artificial inoculation tests for resistance against FP (moniliasis) have been developed and implemented (Phillips-Mora and Galindo 1988;

Phillips-Mora et al. 2005, 2011). Phillips and Galindo (1989) also developed a pod inoculation method to test for resistance to BP (Fig. 12.7).

In addition, progress has been made to elucidate the modes of inheritance of certain traits such as resistance to BP caused by *Phytophthora palmivora* (Iwaro et al. 1997a, b, c), and the host-pathogen interaction (Surujdeo-Maharaj et al. 2001). Iwaro et al. (1997a) found congruence between the results of a post-penetration inoculation assay for BP resistance using leaves (early test) and pod resistance after artificial infection.

Despite the utility of early screening, the importance of evaluation of inherent resistance under field conditions remains important (Eskes and Lanaud 2001) since the likelihood of escapes exists due to conditions unfavorable to pathogen survival in the field and the time of pod ripening (late in the dry season). With the advent of

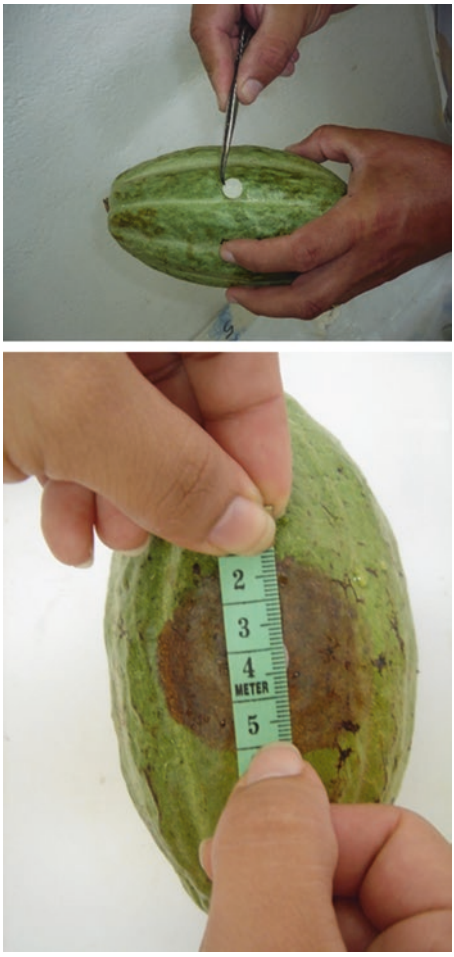

Fig. 12.7 Pod inoculation method to test for resistance to BP

genomics, it is now possible to apply marker-assisted selection to expedite the process of screening of progeny.

12.7.4 Multi-location Varietal Trials

Multi-locational trials (MLTs) are indispensable for determining the potential of elite parents across a range of environments. The existence of environmental effects and genotype/environment interactions may obscure useful genotypic variation (Simmonds 1981; Toxopeus and Wessel 1970; Van der Vossen 1999), and require that varieties be locally bred so that they are adapted to local conditions (Lopes et al. 2011; Susilo 2011). Quick progress can be obtained through fixation of interesting genotypes by clonal selection.

Results from MLTs with cacao in Brazil (Lopes et al. 2011; Pinto et al. 1993), Ghana (Lockwood 1980), Côte d'Ivoire (Paulin et al. 1994) and Papua New Guinea (Efron et al. 2005c) indicate that stability in agronomic traits among various hybrids is not uniform. Adaptability studies must thus be conducted routinely in testing of favorable hybrids prior to release in countries and regions outside of where they are bred.

An achievement of the CFC/ICCO/BI project on Cocoa Germplasm Utilization and Conservation: a Global Approach (1998–2004) (Eskes and Efron 2006) was the conduct of multi-locational clonal and hybrid trials, involving introduced and local genotypes. These were established at ten different sites for observation of their performance. The goal was to select superior genotypes and determine their breeding value and stability over environments in terms of agronomic and other traits of interest. However, this was not fully realized after the conclusion of the project due to the limitations in funding for the required long-term observations.

In Indonesia, Sitepu et al. (2005) also noted significant clonal × site effect for 22 clones grown at different planting densities in two environments. However, a Finlay-Wilkinson (1963) analysis revealed that some clones were adapted to the two environments studied. In Sulawesi, McMahon et al. (2015) concluded from their study that the performance of twelve clones, including PBC 123 from Malaysia, was affected by the locality in which they were grown as well as their genotype. They underscored the importance of testing clones under different environmental conditions. The benefits of such an approach was also demonstrated in Papua New Guinea (PNG) by Marfu et al. (2009), who described multi-locational testing as the last step in breeding of hybrids before distributing to farmers. Their study of adaptability of the hybrids under different soil types, rainfall, and disease and insect pest pressures facilitated the identification of possible genotype x environment (G x E) interaction.

Multi-locational testing was initiated in PNG in 1995 and, in 2003, the polyclonal varieties, HC1-Big and HC1-Small (with different plant stature or vigor), were released by the PNG Cocoa and Coconut Institute (CCI) to farmers. Thirty-eight promising hybrid clones were also included in MLT in 2000, and promising

high-yielding clones with resistance to Ppr and VSD were identified (Marfu et al. 2009). Noteworthy was the interaction noted by Marfu et al. (2009) between genotype, environment and management (agronomic practices) that has also been recorded by Struik and Yin (2009).

12.8 Utilization of Hybrids

Utilization of cacao hybrids is becoming more effective due to collaboration among regional breeders' groups (CacaoNet 2012). In 2009, the INGENIC Asia/Pacific Working Group reported at the 6th INGENIC Workshop on their initiative in which each collaborating institute produced seed from 3 hybrid crosses involving their best clones and established at least 200 trees of each hybrid at each participating institute. At that time, 900 hybrid seeds and budwood from 6 clones had been exchanged within the group. The hybrids are being observed in field trials. The next phase of this project is to introduce new material with FP and WB resistance, good productivity and quality attributes.

12.8.1 Trinitarios Utilized in South-East Asia – Papua New Guinea, Indonesia and Malaysia

Among the locally-adapted Trinitario hybrids, purportedly originating in Trinidad and later selected in Papua New Guinea as interesting for breeding due to favorable agronomic traits, were K 13, 20, 21, 82, K 6-101, KA2-101, KA 2-106, KA5-210 (Efron et al. 1999; Pang 2006). K 82 and KA2-106 were reported by Efron et al. (1999) to have higher levels of resistance to Ppr and VSD compared to other Trinitarios and were used successfully in later breeding programs along with KA2-105, KA2-102 and KT 140 (Efron et al. 2005a). The outstanding hybrids, SG1 and SG2, incorporate the hybrid vigor of UAF cacao and locally-adapted Trinitario hybrids and are widely utilized. Selection is based on tree size to suit specific planting densities (Efron et al. 2005a).

In Indonesia, McMahon et al. (2015) found KA2-106 to be one of 8 hybrid clones, among the 49 studied, to show a good level of resistance (designated R) to VSD. This clone and K 82, which was ranked moderately resistant, both from PNG, were used as resistant standards in their study. They also found that the Trinitario clone, DRC 16, which was developed in Java, was resistant to BP (ripe pods).

Lockwood and Pang (1993) found all the Trinitario (hybrid) clones, such as UIT 1 and 2, used in clone trials in Malaysia to be low-yielding compared to UAF. However, Lamin et al. (1999) found that ICS 95 and UIT 1 were good parents for breeding in terms of their VSD resistance.

12.8.2 Trinitarios Utilized in West Africa

Aikpokpodion et al. (2010) described the Trinitario accessions imported into Nigeria from Trinidad in 1944. A group of *red-fruited Trinitarios* were found to be phenotypically distinct from the West African Amelonado (WAA), and the Upper and Lower Amazon Forastero (UAF and LAF) germplasm (primary and improved germplasm/hybrids) in farmers' fields in Nigeria. The Trinitario hybrids had distinctively large beans and fruit size relative to the other germplasm. However, limited adoption by farmers of Trinitario hybrids and clones in the Nigerian cocoa breeding program of the Cocoa Research Institute was noted. This reflects the preference for CSSV and *Phytophthora*-resistant WAA and UAF. WAA is also noted for its distinctive cocoa flavor.

In Cameroun, Efombagn et al. (2009) found that the Trinitario selections/hybrids of the SNK and SNK 600 series from the genebank had larger pod/fruit weight and size, dry seed weight and pod index than the germplasm they collected in farmers' fields. Furthermore, the Trinitarios were phenotypically distinct from the UAF studied, and the ICS Trinitarios had higher seed and pod values than the locally selected Trinitarios. They have thus been fairly-widely adopted in Cameroun, and Trinitario beans in Cameroun occupy a market niche.

12.8.3 Hybrids Bred in Trinidad and Tobago

Trinidad selected hybrids (TSH) clones are outstanding in terms of yield, vigor, ease of establishment, precocity, resistance to *Ceratocystis* wilt and witches' broom disease and have displayed desirable fruity, spicy and other flavor attributes. Twenty TSH clones are widely adopted commercially in Trinidad and Tobago. These include TSH 919, 1188, 1076, 1095, 1102, 1104, 1220, 1347 and 1364 (Maharaj et al. 2011) (Fig. 12.8). TSH 565 and TSH 1188 are also widely cultivated in countries such as Brazil, Peru, Colombia and Costa Rica. Some TSH cultivars (seedlings) are also grown in the Caribbean cocoa- producing countries and a few have been utilized in breeding in Brazil and Costa Rica.

12.9 Breeding for Tolerance to Diseases and Pests

Diseases are currently considered the most serious biological (biotic) constraint to cocoa production (Gutiérrez et al. 2016; Ploetz 2007). Five major diseases of cacao, viz., *Phytophthora* pod rot (black pod, BP), witches broom (WB) (caused by *Moniliophthora perniciosa*), cocoa swollen shoot virus (CSSV), vascular streak dieback (caused by *Ceratobasidium (Oncobasidium) theobromae*, and moniliasis (frosty pod (FP), caused by *Moniliophthora roreri*) have been reported to account

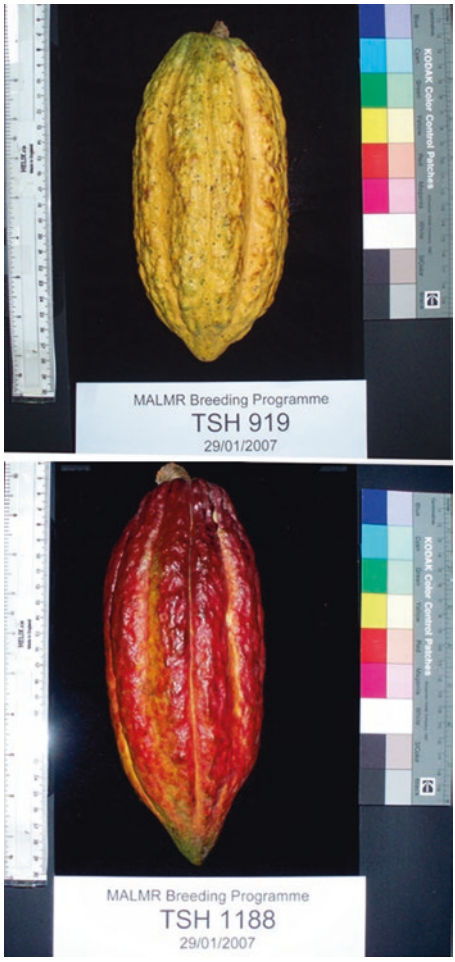

Fig. 12.8 Two commercial Trinidad Selected Hybrid (TSH) clones with superior traits

for over 30% of the losses in cocoa production annually (Flood and Murphy 2004; Ten Hoopen et al. 2012). FP is emerging as a very serious constraint in Central and South America (particularly in Ecuador, Colombia, Mexico, Costa Rica, etc.) (Aime and Phillips-Mora, 2005), and will devastate world cocoa production if it were to spread from the centers of origin and domestication of cacao to West Africa and Southeast Asia.

In West and Central Africa, *Phytophthora megakarya* is currently the main factor limiting yield in affected areas (Adomako 2007; Akrofi 2015; Akrofi et al. 2015; Opoku et al. 2000). Breeding for resistance to diseases such as BP is thus critical. It has been cited as the preferred method of disease control because it is relatively cost-effective and environmentally acceptable (Iwaro et al. 2010; Luz et al. 1999; Simmonds 1986; Toxopeus 1969).

Screening of germplasm collections for sources of resistance to major diseases of cacao has been undertaken in some cocoa-producing countries (Bartley 1981, 1986; Iwaro et al. 2000, 2003, 2005; Nyadanu et al. 2012; Phillips et al. 2013). Several clones have been identified as demonstrating resistance to specific diseases in different countries. Some of these were included in the CFC Project Collection (Sounigo et al. 2006). Of note is SCA 6 for witches' broom disease (Soria 1974), with less significance in Ecuador (possibly due to the virulence of the pathotype existing there), IMC 47, POUND 7 and PA 150 for BP resistance (Eskes and Lanaud 2001) and UF 273, UF 712, ICS 95 and PA 169 for resistance to FP (CacaoNet 2012; Phillip-Mora et al. 2012). Derived clones from the cross UF-273 × PA 169 (CATIE-R4 and CATIE-R6) have demonstrated high levels of resistance to FP (Phillips-Mora et al. 2013).

Breeding for disease resistance has been hampered by the factors that have been described under *Constraints* (Sect. 12.4.2) as well as the fact that improvement is often sought for multiple traits simultaneously. Variation in pathogens and pathogenicity and host-pathogen interaction further complicate breeding for disease resistance. The complexity of dealing with cacao diseases was discussed by Gutiérrez et al. (2016) and Ploetz (2016).

In West Africa, the germplasm used in breeding for disease resistance comprises mainly traditional populations of Trinitario, Amelonado and F_3 Amazon cacao, and of open-pollinated populations of selected hybrids (N'Goran et al. 2006). Black pod resistant trees have been identified in farmers' fields in West Africa; in Cameroon, Ghana and Côte d'Ivoire (Efombagn et al. 2004, 2011; Kébé et al. 1999; Opoku et al. 2007; Pokou et al. 2008).

In Brazil, genetic *resistance* to WB in cacao with Scavina ancestry was observed to break down over time (de Castro Virgens Filho 2009). This was attributed to the hypothesis that with the evolution of the disease, resistance can only be durable if it is encoded by multiple genes (under polygenic control). It was recommended that a combination of disease control methods be utilized such as the use of genetic resistance, application of chemicals and biocontrol agents as well as phytosanitation. Collaborative research is ongoing to develop and distribute resistant varieties that combine both FP and WB resistance using traditional and molecular breeding. Other traits such as desirable flavor will also be incorporated.

At the Indonesian Cocoa and Coconut Institute, research is in progress to assess factors that could contribute to a lower incidence of cocoa pod borer (CPB) attack. Clones with high density of trichomes and higher levels of tannin granules and lignin early in pod development were observed to have lower CPB attack, according to Susilo (2009). There is also a regional trial to assess the putative relationship between pod wall hardness and resistance to CPB.

In Mexico, 11 hybrid families that have shown resistance to FP were evaluated for agronomic performance including pod index, as an indicator of yield. The families of promising FP resistant crosses, UF 273 × PA 169 and PA 169 × UF 273, were found to be most promising with yields greater than 500 kg of dry cocoa per hectare (Solís Bonilla et al. 2015). A similar result was observed in the CATIE's breeding program in Costa Rica (Phillips-Mora et al. 2012).

The inheritance of resistance to disease in cacao has been found to be quantitative for BP and FP but not for WB (Eskes and Lanaud 2001; Iwaro et al. 1998) and general combining ability pervades (Simmonds 1994). A fairly high degree of heritability for resistance to diseases such as BP has allowed genetic gain in breeding (Eskes et al. 1996) and pre-breeding (Iwaro et al. 2005, 2010) in cacao. Iwaro et al. (1997b, c) also elucidated the mechanisms of resistance to BP in leaves and pods.

Guiltinan et al. (2008) and Schnell et al. (2005a,b, 2007) have described recent applications of genomic tools in cacao breeding for disease resistance. Refer to Sect. 12.17 for details. A major and minor QTL for WB and FP diseases were located in an F_2 mapping population derived from Trinidad Selected Hybrid (TSH) 516, a cross between Imperial College Selection (ICS) 1 and Scavina (SCA) 6. This QTL was used in the marker-assisted selection (MAS) program of Brown et al. (2005).

12.10 Breeding for Drought Tolerance in the Face of Climate Change

The effects of climate change on cacao have been receiving increasing attention within the research community. Abiotic stress factors, such as drought, flooding, high temperature and irradiance, pose a threat to sustainable cocoa production (Oyekale et al. 2009). There is a need to optimize water use, soil and microclimatic conditions through cultivation of improved, stress-tolerant planting material (Padi et al. 2013a). This is particularly urgent since Baligar et al. (2008) concluded that cocoa is ineffective in limiting transpiration in dry air compared with other rainforest trees. Fortunately, the application of research findings and the adoption of technology to address climate change in cacao is becoming more evident (Stigter 2008).

Access to diverse crop genetic resources should benefit selection and breeding of varieties with adaptive traits for abiotic along with biotic stress tolerance (Beebe et al. 2011; McCouch et al. 2013). Since plants under abiotic stress are affected physiologically and biochemically, adaptation to stress can be facilitated by morphological, physiological and biochemical responses in crops (Saibo et al. 2009). The latter include deeper root systems, stomatal control/conductance and efficient water use and translocation of nutrients within the plant.

In Maracaibo, Venezuela, Ávila-Lovera et al. (2016) found that maximum photosynthetic rate, stomatal conductance and water use efficiency varied among genotypes studied and with season. In Brazil, dos Santos et al. (2014, 2018) observed that useful traits to screen for drought tolerance were leaf and total dry biomass, relative growth rate and magnesium content of leaves. Drought tolerance candidate genes, involved in ABA biosynthesis during photosynthesis, were identified, and tolerant genotypes were found to have more efficient antioxidant metabolism due to significant increases in guaiacol peroxidase (enzymatic) activity in response to oxidative stress.

In West Africa, selection of well-adapted varieties with abiotic stress tolerance is a major goal of cocoa breeding (Läderach et al. 2013). In Ghana, where drought is a serious constraint, genetic variation and traits related to drought tolerance in cacao grown under shade versus without shade were studied (Anim-Kwapong and Frimpong 2005; Ofori et al. 2015). Stem growth, survival, leaf chlorophyll content and plant vigor were deemed useful traits for drought tolerance screening. Daymond et al. (2011) suggested that specific leaf area be used as a criterion for breeding for drought tolerance due to its correlation with water use efficiency within the plant.

In Trinidad, Antwi (1994) investigated the effects of soil water deficit in cacao and Galyuon et al. (1996a, b) examined the effects of irradiance. More recently, studies on climate change adaptation to drought and heat stress, in different varieties of cacao, were initiated.

New technologies such as genomics and the application of GIS may be applied in the future to predict the best growing environments for cacao. Ramirez-Villegas and Thornton (2015) described a methodology for classifying crop growing environments, determining stress profiles of crops such as maize (*Zea mays*) and beans (*Faba* sp.) and assessing the potential benefit of improved breeding practice. This approach addresses the constraint due to genotype-by-environment interactions, which often restrict progress in plant breeding to achieve broad adaptation and/or for adaptation to specific conditions within a region. Such an approach in cacao would be useful and requires increased attention to conducting multi-locational trails and assembling detailed GIS data and molecular tools to identify favorable candidate genes. In addition, Carr and Lockwood (2011) underscored the need to dedicate resources to studying water relations in mature cacao in the field to prepare for impending climate change impacts on moisture availability.

12.11 Breeding for Other Traits

The completed mapping of the cacao genome will facilitate the identification of marker-trait associations in cacao that can be used in breeding for abiotic stress tolerance, along with biotic stress tolerance, and for desirable flavor attributes and high nutraceutical value, among other favorable traits. Technological approaches such as genome-wide association studies and breeding with genomics as well as genomic prediction will play an increasingly important role in cacao breeding in the future.

12.12 Determination of Genetic Diversity in Cacao Using Molecular Tools

Molecular tools have been widely used to fingerprint the genetic diversity of cacao in most cocoa-producing countries: in Brazil (Carvalho et al. 2003; Marita et al. 2001; Pires et al. 2003; Santos et al. 2005; Sereno et al. 2006); Ecuador

(Loor-Solorzano et al. 2013; Loor et al. 2009); Honduras and Nicaragua (Ji et al. 2013; Trognitz et al. 2013); Bolivia (Zhang et al. 2012); French Guiana (Lachenaud and Zhang 2008); Peru (Zhang et al. 2006); Mexico (Whitkus et al. 1998); the Caribbean (Boza et al. 2013; Irish et al. 2010; Johnson et al. 2009; Martinez et al. 2015; Motilal et al. 2010); West Africa (Aikpokpodion 2012; Efombagn et al. 2006, 2008; Opoku et al. 2007; N'Goran et al. 1994, 2000; Pokou et al. 2014), and Southeast Asia (Indonesia) (Dinarti et al. 2015; Susilo et al. 2011). The research findings of Motamayor et al. (2008) were among the most significant since they revealed 10 genetic groups among 1241 wild type cacao accessions from a broad geographic area.

Ronnig and Schnell (1994) found that Trinitarios differed genetically from Forasteros based on allozyme data for cacao germplasm conserved at the USDA/American Cocoa Research Institute collection in Florida. They also found that Caribbean and Central American groups were distinct from South American types. Laurent et al. (1994) distinguished amongst Criollo, Trinitario and Amazon Forastero groups of cacao using ribosomal probes to analyze DNA samples from Brazil, Costa Rica, Côte d'Ivoire, France, Trinidad and Venezuela. N'Goran et al. (1994) studied diversity, using RAPD markers, among samples from diverse geographical origins. Lower Amazon Forasteros (LAF) were clearly differentiated from Upper Amazon Forasteros (UAF) and Criollos. The Trinitarios grouped between the LAF and Criollos. N'Goran et al. (2000) were also able to distinguish Criollo from Amazon Forasteros using RFLP analysis. Lerceteau et al. (1997) analyzed samples form varied locations including from Costa Rica, Ecuador, France and Mexico. They discriminated between Criollo and Forastero clones using RAPD and RFLP markers. Motamayor et al. (2002) further differentiated between *modern* and *ancient* Criollo from South and Central America.

Zhang et al. (2008) deduced from their research that the Refractario is a distinct genetic group. Motilal et al. (2010) observed that Refractarios were clustered between the UAF and Trinitario samples studied. They also examined the genetic diversity within Belizean Criollos.

A comprehensive description of the genetic diversity of cacao in West Africa was presented by Aikpokpodion (2012). The genetic diversity and population structure of cacao in Nigeria was elucidated by Aikpokpodion et al. (2010). In Ghana, Opoku et al. (2007) assessed the genetic diversity in cacao using 17 mapped microsatellite markers. The germplasm studied included 104 trees at the CRIG seed gardens and 235 trees in farmers' fields, representing all the cocoa-growing regions of Ghana. Thirty-eight parental clones from the CRIG's collection were also assessed. The highest genetic diversity was observed among breeders' and parental collections compared to within farmers' fields. However, diversity in the farmers' collection was comparable. It was possible to differentiate [Fst = 0.076] between the breeders' collection and farmers' collection.

Susilo et al. (2011) used simple sequence repeat (SSR) markers to study the genetic diversity of Java, Indonesia fine/flavor cocoa. A heterogeneous genetic background was discerned, which excluded direct parentage of pure Criollo cacao or a single cross, as was previously believed. The clones, DR 1 and 38, which pro-

duce Java cocoa, were observed to have Trinitario, Upper Amazon and Lower Amazon Forastero ancestry.

Genetic diversity studies in cacao have shown some congruence between conventional classification of cacao based on phenotype and that based on genetics. Significant genetic diversity was observed among the UAF group by several scientists, including Laurent et al. (1994), N'Goran et al. (1994, 2000); Motamayor et al. (2003), Sounigo et al. (2005) and Zhang et al. (2006, 2009b), among others. Future studies, involving both morphological and molecular analysis of cacao, should elucidate patterns of diversity within cacao genebanks, and reveal whether ex situ conservation has effectively captured a reasonable amount of the genetic diversity that is estimated to exist in the wild (in situ) (Bekele et al. 2006). If not, further collection in the wild should be undertaken since much of the diversity of cacao is purportedly untapped and is under threat due to deforestation (Anon. 1981).

Progress in genotyping cacao in the field should be accelerated due to innovations such as the optimization of a SNP assay suitable for field studies, which was achieved by Livingstone III et al. (2011). The latter developed a fluorescence microplate reader and standard thermocycler to replace real-time PCR analysis. This will also reduce the cost of SNP genotyping of cacao.

12.12.1 Verification of Genotype Identities Using Molecular Tools

Livingstone et al. (2011) stated that genetic gain obtained through selection in breeding programs may be impeded due to mislabeling of clones (off-types). Schnell et al. (2007) also commented on the how misidentified parents can hinder progress in breeding. The latter may possess traits that differ from those that were selected in synonymous candidates (bearing the same clone name, but genetically different). Livingstone et al. (2011) demonstrated the use of SNP markers in detecting mislabeling among 186 trees, representing 19 clones in the Institute of Agricultural Research for Development (IRAD) breeding program in Cameroun. SNPs have the advantage of being easy to use, cost-effective, reliable and unambiguous across different platforms compared to microsatellites (SSR) markers (Livingstone et al. 2011). The usefulness of the application of SSR and SNP fingerprinting for verification of genetic identities of varieties used in breeding and parentage analysis was demonstrated by Takrama et al. (2005) and Padi et al. (2015) in Ghana, and by Motilal et al. (2011, 2012) and Sounigo et al. (2003a, 2006) in Trinidad.

12.13 Cacao Genomics

The application of molecular methods has been actively pursued in cacao and research on the cacao genome has been in progress for more than two decades. There have been significant achievements that can accelerate progress in breeding

for improved cacao planting material. These include linkage mapping of the cacao genome to identify quantitative trait loci (QTLs), genomic prediction, estimation of the breeding value of genotypes and marker-assisted selection (MAS) (Allegre et al. 2012; Argout et al. 2011; Brown et al. 2008; Crouzillat et al. 2000a,b; Guiltinan 2007; Kuhn et al. 2012; Lanaud et al. 1999, 2004a, b, 2017; Lerceteau et al. 1997; Pugh et al. 2004; Saski et al. 2011; Schnell et al. 2007; Wilkinson 2003) (Tables 12.3 and 12.4).

Guiltinan (2007), Guiltinan et al. (2008) and Clément et al. (2004) described the value of the cacao bacterial artificial chromosome (BAC) libraries for gene discovery in cacao. Lanaud et al. (2009) performed meta-QTL analysis of resistance traits to *Phytophthora* spp. and other diseases in cacao. Kuhn et al. (2012) identified 19 COSII SNP markers that co-locate with existing QTLs in cacao, which are used for genotyping and detecting off-types or mislabeled material in cacao breeding programs. They have also been utilized in genetic mapping and for identifying co-location of genes that regulate traits of interest in cacao. Feltus et al. (2011) employed a BAC-based physical map to identify candidate genes for seed shape, pod weight and black Pod resistance contained within QTL intervals.

Prospects for improved cacao planting material appear encouraging based on recent developments that capitalize on successes in conventional breeding and involve the application of genomics (Figueira et al. 2005; Guiltinan 2007; Guiltinan et al. 2008; Kuhn et al. 2012; Wilkinson 2003). In 2010, years of application of molecular analyses, gene discovery and genomics resulted in the release of a cacao genome map by two independent research teams. One team involving Mars, IBM and USDA produced a sequence that covers 92% of the genome with approximately 35,000 genes. The sequencing was done using the highly homozygous cultivar, Matina 1-6 (Motamayor et al. 2013; Saski et al. 2011). The other research team sequenced an ancient Mayan cacao variety, a homozygous Criollo, B97-61/B2, which was domesticated in Belize (Argout et al. 2011, 2017). They identified the genes that influence the production of flavonoids in cacao, which have cardiovascular benefits, and isolated the gene encoding the melting point of chocolate as well as of hundreds of genes potentially involved in pathogen resistance. They assigned 99% of these protein-coding genes to one of the 10 chromosomes in the Criollo cacao tree studied (Argout et al. 2017).

The size of the cacao genome has been estimated as 430 Mbp for the cultivar B97–61/B2 (Argout et al. 2011). Motamayor et al. (2013) observed it to be 445 Mbp in cv. Matina 1–6.

12.14 Molecular Breeding

With the advent of the mapping of the cacao genome, advances in genomics such as QTL mapping, genome wide association mapping (Da Silva et al. 2016; Marcano et al. 2007, 2009; Motilal et al. 2016), identification of candidate genes and haplotype inferencing (Cervantes and Brown 2004; Fouet et al. 2011; Gutiérrez-López

Table 12.3 Some QTL studies conducted in cacao pre 2011

Quantitative Trait Loci (QTL) studies based on controlled crosses	References
Quantitative trait loci (QTLs) associated with resistance to witches' broom disease (caused by *Moniliophora perniciosa*). Three QTLS detected (1 on linkage group (LG) 4 and another on LG 9 for ICS 39×CAB 0214; one on LG 8 for ICS 39×CAB 0208	de Albuquerque (2006)
Mapping of QTL for butter content and hardness. One QTL associated with butterfat content was mapped at LG 9 and two QTLs for butter hardness were identified at LG9 and LG7.	Araújo et al. (2009)
QTLs located for resistance and agronomic traits. Five QTLs for frosty pod resistance were found on three LGs 2, 7, and 8. Three QTLs for black pod resistance were found on LGs 4, 8, and 10. QTLs for average trunk diameter growth and pod color were found on LG 4.	Brown et al. 2007
Two QTLs for resistance to witches' broom disease were found, one producing a major effect and one a minor effect (on LG 5 and 6). Both showed dominance effects.	Brown et al. (2005)
QTL mapping for bean traits and the number of ovules per ovary. One to six QTLs were detected for bean traits (length, weight, and shape index) and one and four QTLs for the number of ovules per ovary particularly on LG 4.	Clément et al. (2003a, b, 2004)
One major QTL detected for agronomic performance, which was correlated to a QTL for early flowering and trunk diameter on LG 10. A QTL for number of beans per 100 g was located on LG3.	Crouzillat et al. (2000a, b)
Inheritance of polygenic resistance to *Phytophthora palmivora* in two related cacao populations.	Crouzillat et al. (2000b)
Mapping QTLs for witches' broom resistance in ICS 1×SCA 6 population in Brazil. A putative QTL was detected associated with the microsatellite mTcCIR35.	Faleiro et al. (2006)
Mapping of QTLS FOR bean shape, pod weight and black pod resistance.	Feltus et al. (2011)
Mapping five QTLs of BP resistance (detected for hybrid, T60/887). Five QTLs of resistance were detected for T60/887. However, none were common between the three traits measured; number of rotten pods in the field, and disease symptoms expressed due to inoculation of leaves and of pods.	Flament et al. (2001)
QTLs for BP resistance were sought using SNPS homologous to COSII sequences. LG 5 was associated with an important QTL for BP resistance. Nineteen COSII SNP markers that co-locate with existing QTLs in cacao were identified.	Kuhn et al. (2012)
Four QTLs for resistance to BP were detected in several progenies, particularly on chromosome 4.	Lanaud et al. (1999, 2004a, b)
QTLs linked to fat content, seed size and sensorial traits.	Lanaud et al. (2003b)
76 QTLs observed in 16 experiments were subjected to meta-analysis using BioMercator. Several *hotspots* of QTLs associated with disease resistance were found.	Lanaud et al. (2009)
QTLs for BP resistance found in a segregating population in Trinidad on LG 1, 2, 3, 4, 6 and 8.	Motilal et al. (2003)
QTLs detected in UPA × UF 676 progeny at two sites in Côte d'Ivoire for pod and bean traits on LG 2, 5 and 8. QTLs for percentage of rotten pods on LG 1 and 8, QTL for number of ovules on LG 4.	N'Goran et al. (1996)

(continued)

Table 12.3 (continued)

Quantitative Trait Loci (QTL) studies based on controlled crosses	References
QTL mapping performed to detect QTLs for three species of *Phytophthora*. Multiple QTLs involved in resistance to *Phytophthora* were detected. this should facilitate MAS and breeding for durable resistance to BP through pyramiding of different resistance genes located in different chromosomal regions.	Risterucci et al. (2003)
QTL mapping for *Ceratocystis* wilt (CW) resistance. QTLs detected on LGs 3 and 9 associated with CW resistance.	Santos et al. (2012)
A major QTL flanked by the marker, AV14.940, was identified in the linkage group (LG) 11. It explained roughly 35% of the resistance to WB observed.	Queiroz et al. (2003)

Table 12.4 Some cacao mapping populations pre 2011

Progeny	Country	Number of trees	Reference
SCA 6 × ICS 1(F_2 population)	Brazil	146	Brown et al. (2005) and Faleiro et al. (2006)
UF 273 × POUND 7	Costa Rica	256	Brown et al. (2007)
Combined populations 1 and 2			Schnell et al. (2007)
UPA 402 × UF 676	Côte d'Ivoire	181	Lanaud et al. (1995), N'Goran et al. (1996) and Risterucci et al. (2003)
Catongo × Pound 12	Costa Rica	55	Crouzillat et al. (1996)
T60/887 × (IFC 5 × IFC 1)	Côte d'Ivoire	112	Flament et al. (2001)
DR 1 × Catongo	Côte d'Ivoire	107	Clément et al. (2001)
S 52 × Catongo	Côte d'Ivoire	101	Clément et al. (2001)
IMC 78 × Catongo	Côte d'Ivoire	128	Clément et al. (2001)
Nacional × Venezolano Amarillo (selfed F_2 progeny)	Ecuador	171	Crouzillat et al. (2003)

et al. 2016; Utro et al. 2013), genomic selection and marker-assisted selection have become fairly new tools at the disposal of the cacao breeder (Guiltinan et al. 2008). These tools can be adopted in a re-invigorated approach to breeding. They will facilitate the translation of climate-smart, heritable variations in cacao into varieties to achieve increased yields and enhanced adaptation to biotic stresses (pests and diseases) as well as to abiotic stresses due to climate change, as recommended by Mba et al. (2012).

12.14.1 Marker-Assisted Selection Aided by Bioinformatics

Reliable molecular markers are useful for locating QTLs and candidate genes and facilitate marker-assisted selection (MAS) of genotypes with desirable traits. This application of molecular markers is the basis of *genomic breeding* (Edwards and Batley 2010; Stuber et al. 1992, 1999; Varshney et al. 2005, 2014; Wilkinson 2003). Noteworthy is that fact that molecular markers are heritable and thus invaluable for selection of phenotypic traits with low heritability. Single nucleotide polymorphisms (SNPs) are markers proven indispensable for mapping candidate genes, marker-assisted breeding and MAS, and cloning of genes underlying traits based on genomic maps (Batley and Edwards 2007).

MAS could be a useful tool to identify useful traits in cacao such as (abiotic) stress tolerant traits, once quantitative trait loci (QTL) for this quantitative trait have been located (Collard et al. 2005). It leverages the burgeoning available molecular data to select for candidate genes and favorable genotypes, allowing for genotype by environment interaction, and targeting adaptive phenotypic traits (Jha et al. 2014). Skillful use of bioinformatics and computational biology is then required to allow the often voluminous and complex data generated to be summarized and interpreted accurately.

Schnell et al. (2005a, b, 2007) stated that traditional cacao breeding programs have had *marginal* success in producing disease resistant material with other favorable economic traits. A project was thus launched to apply modern molecular genetic techniques to cacao breeding. The objective was to develop a MAS breeding program for developing disease resistance in cacao (Brown et al. 2005, 2007, 2008). However, as noted by Struik and Yin (2009), polygenic control, epistasis and QTL × E × M interactions (where QTL represents the genetic component, E the environment and M the management system) can complicate the application of MAS approaches.

Nevertheless, MAS is expected to allow relatively quicker progress in pyramiding or accumulating several genes of interest in segregating breeding populations relative to what is possible with traditional breeding. Cacao, as a perennial crop, has a long breeding cycle. Desirable traits are conventionally screened based on the phenotypes of mature plants when reproductive traits are involved. MAS can reduce the time required to breed a new crop variety by half (Mba et al. 2012). However, the cost of MAS is prohibitive (Ribaut et al. 2010). Fortunately, high-throughput molecular screening methods are becoming increasingly available at reduced costs.

Genetic distances data for select cacao genotypes, derived using molecular markers such as SSRs and SNPs, can expedite advances in cacao breeding (Batley and Edward 2007; Dias et al. 2003; Lanaud et al. 2004a, b; Livingstone et al. 2012; Olasupo et al. 2018). Genetic similarity datasets can be used for constructing additive genetic and dominance matrices to obtain the best linear unbiased prediction (BLUP) of specific combining ability and general combining ability in cacao. Genetic distances can be used to predict the accuracy of genotypic values through the application of the Monte Carlo method (Balestre et al. 2009).

QTLs for resistance to diseases such as BP and FP have been identified in cacao using SSR markers (Brown et al. 2007), and WB (Queiroz et al. 2003) in family mapping populations. This technology has also been utilized in Côte-d'Ivoire to develop lines with resistance to current and potential disease threats such as Ppr caused by *Phytophora megakarya*. Mapping populations are thus being used to identify additional QTLs (Pokou et al. 2008) (Table 12.3).

In order for breeders to identify markers that are linked to the specific genes and use these to facilitate selection of potentially useful cacao genotypes, the method of *bulked segregant analysis* is useful (Wilkinson 2003). This entails separating members of a segregating mapping population into two halves based on phenotype (positive or negative for the trait being considered). DNA is then extracted from plants representing each phenotype and pooled together (one pool for each allelic state (Wilkinson 2003)). The DNA samples can then be analyzed using appropriate multilocus markers to identify the presence or absence of bands linked to the gene expressed. Confirmatory fine mapping and RNA-Seq can follow to unravel the candidate genes in the segregating population, as was done in upland cotton for candidate genes controlling cotton fiber quality (Liu et al. 2016). Identification of candidate genes using markers closely linked to them thus facilitates MAS (Collard and Mackill 2008) by allowing the detection by molecular breeders of haplotypes that express desirable conditions. This is *breeding with genomics*, which was exemplified when Rehem et al. (2010) undertook genetic mapping of cacao seedlings of the Parinari series in Brazil to identify carriers of the lethal gene, *Luteus-Pa*.

12.14.2 Mapping Populations

Past QTL studies in cacao were generally developed from controlled crosses. Brown et al. (2005) used a genetically segregating population produced by selfing of the Trinidad Selected Hybrid, TSH 516, a progeny from the cross of parental clones, SCA 6 × ICS 1. The F_2 population consisted of about 140 progeny. Some highlights of this research are provided in Table 12.3. Several other mapping populations used for QTL mapping and improvement in cacao are presented in Table 12.4.

12.15 Association Mapping

Association (*population*) mapping is currently being applied in cacao. It involves searching for genotype-phenotype correlations in unrelated individuals (Bradbury et al. 2007; Breseghello and Sorrells 2006; Li et al. 2011), and is based on nonrandom association of alleles in a population or linkage disequilibrium. It is an alternative to classical QTL (family) mapping studies, which have demonstrated that some molecular markers explain a considerable amount of phenotypic variance in quantitative traits (Paterson et al. 1988; Stuber et al. 1992), but are constrained by

the paucity of large productive progenies from known parental origin for perennial crops such as cacao.

Linkage maps with genetic markers covering the whole genome facilitate the elucidation of genes or chromosomal regions influencing traits of interest (Allegre et al. 2012; Botstein et al. 1980). Since the cacao genome has been fully mapped and there is tight coverage with molecular markers (Argout et al. 2011, 2017; Saski et al. 2011), association mapping and admixture mapping studies (Stack et al. 2015) are now facilitated. Marcano et al. (2009) conducted a genome-wide mapping study for yield factors and morphological traits in cacao.

Royaert et al. (2011, 2016) identified marker-trait associations for self-compatibility and resistance to witches' broom, respectively, in a segregating mapping population of cacao. Sounigo et al. (2012) found several associations related to SSRs and yield in Cameroun. Motamayor et al. (2013) identified candidate genes regulating pod color in cacao. Da Silva et al. (2016) performed genome wide association mapping studies (GWAS) to locate markers for self-incompatibility in a mapping population involving 295 trees. Putative markers were located on chromosome 4 within 196 kb suggesting the location of a major gene in this region. Motilal et al. (2016) employed GWAS to identify genes linked to seed and disease traits.

12.16 Functional Genomics for Cacao Improvement

The utility of functional genomics for cacao improvement has been demonstrated by several research teams in the past 10 years. For example, Leal et al. (2007) observed differential expression (between genotypes) of genes associated with disease response (23 transcripts), which were up-regulated at the asymptomatic stage of WB expression. Gene induction was observed at 48 and 120 h after inoculation with the pathogen for the resistant (CAB 214) and susceptible (ICS 39) genotypes, respectively. The induction peaks corresponded to morphological and biochemical changes previously described during colonization. These results can be applied in developing tools for WB resistance screening of cacao genotypes.

12.16.1 Candidate Gene Identification

12.16.1.1 Disease Resistance

To date, several molecular markers including SNPs have been identified from candidate genes putatively linked to WB (Borrone et al. 2004; Brown et al. 2005; Lanaud et al. 2004a, b; Lima et al. 2009; Royaert et al. 2016; Teixeira et al. 2012, 2015). Fister et al. (2016) have published a transformation protocol using cacao leaves to express transgenes of interest. This protocol should have value in functional genetic assays such as pathogen bioassay, metabolic analysis and gene

expression analysis. It can reduce the time required to test candidate genes involved in defense responses in cacao.

Shi et al. (2013) undertook the characterization of a putative NPR3 gene from cacao, viz., (TcNPR3). They found that this gene functions in a manner comparable to the *Arabidopsis* NPR3 gene in regulating the defense response. However, TcNPR3 did not perfectly match the *Arabidopsis* NPR3 mutation when eliciting its pathogen defense response. It is possible that some other useful defense function of TcNPR3 may be found in cacao. This discovery can contribute to the breeding of resistant cacao varieties against pathogens using molecular approaches or biotechnological strategies.

Bailey et al. (2014) pursued research to determine whether *Moniliophthora roreri*, which causes frosty pod disease (FP), shows a differential molecular response during the process of successfully infecting *tolerant* clones. Infected pods at all stages of symptom expression were collected in the field from trees of two highly susceptible clones, viz., POUND 7 and CATIE 1000, and three tolerant clones, UF 273, CATIE R7 and CATIE R4. Metabolite analysis was carried out on susceptible clones, POUND 7 and CATIE 1000, as well as tolerant clones, CATIE R7 and CATIE R4. A total of 873 *M. roreri* genes, which were differentially expressed among the clones, were identified with RNA-Seq analysis. Among these were genes encoding transcription factors, heat shock proteins, transporters, enzymes modifying membranes or cell walls and metabolic enzymes, such as malate synthase and alternative oxidase. Validation of the observed differential expression between clones was done for 43 *M. roreri* genes using the real-time quantitative reverse transcription polymerase chain reaction. This study demonstrated that *M. roreri* genes associated with stress metabolism and responses to heat shock and anoxia were induced early in tolerant clones. This has significant implications for marker assisted selection for FP tolerance through the selection of haplotypes expressing these early stress responses and allied defense responses to the disease pathogen (Cervantes-Martinez and Brown 2004).

Legavre et al. (2015) performed transcriptional screening and gene expression analysis to unravel putative defense genes in cacao associated with resistance to *Phytophthora megakarya* infection (BP). Genes involved in stress signal transduction, general and specific defense genes and genes that mediate hormone signaling pathways were identified. Noteworthy was the discovery of three genes from a family encoding a protease inhibitor that displayed varying levels of expression in resistant versus susceptible *cacao* clones. Furthermore, a motif was identified among promotor sequences, which was present only in genes that were expressed at a higher level in the resistant plants.

12.16.1.2 Drought Tolerance

Bae et al. (2008) identified genes involved in polyamine biosynthesis. Levels of polyamine are associated with drought response. This finding should facilitate the process of enhancing drought tolerance in cacao by altering polyamine levels

through genomic selection. Bae et al. (2009) also identified another putative means of improving drought tolerance in cacao. The method involves the endophytic fungus, *Trichoderma hamatum* (isolate DIS 219b), which was found to promote growth and delayed the onset of drought symptoms, accompanied by changes in gene expression. Colonization by *T. hamatum* (isolate DIS 219b) was observed to promote root growth and consequently to prevent changes in stomatal conductance, net photosynthesis and wilting under drought conditions.

12.16.1.3 Cocoa Butter Biosynthesis

Zhang et al. (2015) investigated the stearoyl-acyl carrier protein (ACP) desaturase (SAD) gene responsible for cocoa butter biosynthesis. Their findings will facilitate screening with biomarkers for genotypes with desirable fatty acid compositions to accumulate genes for desirable cocoa butter quality and disease resistance such as to oomycete pathogens such as *Phytophthora capsici*.

12.17 Proteomics in Cacao

The application of proteomics is already underway in cacao. Using bioinformatic tools, Freire et al. (2017) analyzed the open read frame (ORF) and the promoter of *Theobroma cacao* L. phylloplanin (TcPHYLL) in order to determine the presence of this compound, which could be useful for the control of WB. Phylloplanins are potential antimicrobial compounds (proteins), found on the surface of some plants, which play an important role in controlling infection. A protein similar to phylloplanins was identified in the *T. cacao* L. genome database. Freire et al. (2017) observed that the accumulation of transcripts of TcPHYLL in meristems increased on the first day after inoculation with *Moniliophthora perniciosa*, the causative pathogen of WB, and it was reduced after the second day. TcPHYLL is the first characterized phylloplanin in a perennial tree.

Mares et al. (2017) determined a comparative proteomic profile of *Moniliophthora perniciosa* basidiospores during germination and identified 316 proteins, including some associated with fungal filamentation such as septin and kinesin. Such an approach is useful for elucidating the mechanisms underlying host invasion and to develop strategies for control of WB and other diseases.

12.18 Genetic Transformation and Mutagenesis

Advances in biotechnology can also accelerate genetic improvement in cacao by enabling genetic transformation/gene manipulation. Transgenic cacao embryos using non-tumorigenic strains of *Agrobacterium tumefaciens* have been produced,

but due to industry restrictions this procedure will not be implemented commercially. The field performance of somatic embryo derived plants still needs to be assessed in detail, and trials are in progress (Goenaga et al. 2015; Sena Gomes et al. 2015).

Mutation breeding and mutagenesis have not been used in cacao due to the aforementioned industry perspective. Fortunately, cacao is out-breeding and is rich in inherent variability that can be exploited in breeding. Nevertheless, the acceleration of breeding progress may be facilitated by mutation breeding. Adu-Ampomah et al. (1996) successfully used gamma radiation to induce genetic variability for resistance to cocoa swollen shoot virus (CSSV) in cacao in Ghana. Somatic embryogenesis was employed for the propagation of desirable genotypes and in vitro mutagenesis and pollen mutagenesis were being applied for induction of virus-resistant cacao trees (Quainoo et al. 2008).

12.19 Conclusions and Prospects

Cocoa breeding is a laborious and lengthy process requiring a combination of proper planning, expertise and continuity as well as biological, human and financial resources. It is imperative and urgent to have further advancement in cocoa breeding to meet emerging threats and challenges that hinder sustainable cocoa production globally. Indeed, the current situation in West Africa, where more than 70% of the world's cocoa is produced, highlights the inadequacy of cocoa breeding efforts to date. There has been a significant increase in acreage of cultivated cacao in West Africa, a 100% increase by 2012 from roughly 5 million ha in 1980. However, this has only generated an increase in yield of 11–12% (Hawkins and Chen 2016). Despite the fact that the hectarage of harvested cocoa in Africa has increased, the yields per hectare have plateaued. Based on the agricultural efficiency indicators, it is very plausible to forecast that the West African cocoa producers such as Côte d'Ivoire and Ghana may not be able to supply cocoa sustainably to meet the growing global demand for bulk cocoa**.** There is still an urgent need to increase productivity and production through breeding for increased yield, yield efficiency and disease resistance and to satisfy the new market demands.

Collaborative efforts including pre-breeding and farmer participatory breeding, as those being pursued by the regional breeding groups under the umbrella of the International Group for the Genetic Improvement of Cocoa (INGENIC) and CacaoNet, must be doubled to fulfil some of the requirements to achieve a sustainable cocoa industry. Breeders can contribute towards environmental sustainability by developing superior varieties that do not require large quantities of fertilizers, fungicides, pesticides and energy. The utilization of such genotypes in energy efficient farming systems, which incorporate social and economic strategies for sustainability, as described by Aikpokpodion and Adeogun (2011), will assure sustainable cocoa production.

A structured, multi-sector, international approach is required to conserve, characterize, evaluate and utilize cacao germplasm as a safeguard against the impact of climate change and other emerging abiotic as well as biotic threats. MAS and breeding with genomics have a role in this process (Bekele and Bekele 2017; Brady and Provart 2007). Such a collaborative approach has already been adopted, albeit not on a continuous basis, during the CFC/ICCO/BI project entitled *Cocoa Germplasm Utilization and Conservation: A Global Approach* (1998–2004) (Eskes 2011; Eskes and Efron 2006; Eskes et al. 2000). Several primary germplasm varieties were identified during this project, and enhanced genotypes with combined resistance to both WB and BP diseases were developed through pre-breeding at the Cocoa Research Centre (CRC), Trinidad (Iwaro et al. 2010). The yield potential, bean traits, precocity, vigor and flavor profiles of these individuals were assessed (Iwaro et al. 2010) and are still being assessed at CRC. The most promising improved varieties were sent to and quarantined at the International Cocoa Quarantine Centre, Reading University, U.K. They are now available for distribution to the project partners in cocoa producing countries. Enhanced genotypes, with Trinitario ancestry from the project in Trinidad, have been transferred to the Ministry of Agriculture, Trinidad and Tobago. They have been crossed with Trinidad Selected Hybrid (TSH) cacao to introgress genes encoding enhanced resistance to Black Pod disease into this elite TSH population. This will advance this successful breeding program (Maharaj et al. 2011) through the development of further improved cacao planting material. Such approaches should be expanded and ameliorated globally.

Further work on improving propagation methods such as somatic embryogenesis, and advancing the prospects of successful cryopreservation and safe germplasm transfer will facilitate the release, transfer and multiplication of improved cultivars. Ongoing research on overcoming the limitation of self-incompatibility in cacao will hopefully lead to significant results.

Biotechnological innovations should facilitate accelerated progress in cacao breeding in the future. However, as stated by Warren (1993) and Lopes et al. (2011), in order to gain maximum benefits from new technology, the established ones are required for evaluation of new planting material and elucidating the genetic control of economically important traits.

For optimal results, it is essential that large breeding programs and multilocational trials, including those which involve breeding with genomics and MAS, must be adequately funded and properly planned. Secure funding for the maintenance of cocoa genebanks and for assuring the continuation of breeding programs is a major issue. Furthermore, there must be continuity in this research effort (Baudouin et al. 1997) so that cocoa breeding can finally fulfil the needs of the producers and consumers in a sustainable manner.

Appendices

Appendix I: Some of the Most Widely Used Clones in Cacao Breeding and Others with Multiple Superior Traits

Cacao accession	Traditional classification	Pod Index[1] (assessed in Trinidad)	Disease resistance and other favorable trait	Comment
IMC 67	UAF (Iquitos)	20.0	Resistance to witches' broom disease (WB), cocoa swollen shoot virus (CSSV) and *Ceratocystis* wilt, good yield potential[BE]	Utilized at 34 locations (ICGD online)[T]
SCA 6	UAF (Contamana)	43.5	Tolerance to WB (widely); produces many fruits per tree	Utilized at 30 locations (ICGD online)
ICS 1	Trinitario	19.9	Resistance to black pod disease (BP) and WB [E P]	Utilized in 29 locations (ICGD online)
			Pod resistance to WB[D L]	
			Hard pod wall, large bean weight, favorable yield potential[Be]	
ICS 6	Trinitario	17.5	Large bean weight, favorable yield potential, potentially tolerant to *Ceratocystis* wilt	Utilized at 26 locations (ICGD online)
SCA 12	UAF (Contamana)	31.0	Resistance to WB	Utilized at 26 locations (ICGD online)
ICS 95	Trinitario	22.0	Fair yield potential, adaptable, tolerance to frosty Pod (FP), tolerance to vascular streak dieback (VSD)	Utilized at 25 locations (ICGD online)
UF 667	Trinitario	28.0	Resistance to VSD	Utilized at 24 locations (ICGD online)
UF 676	Trinitario	17.0	Large bean weight, favorable yield potential (Trinidad and Tobago, Malaysia)	Utilized at 24 locations (ICGD online)
ICS 100	Trinitario	19.0	Large bean weight, favorable yield potential, Nicaraguan Criollo pedigree	Utilized at 24 locations (ICGD online)

(continued)

Cacao accession	Traditional classification	Pod Index[1] (assessed in Trinidad)	Disease resistance and other favorable trait	Comment
SPA 9	Unclassified	29.3	BP resistance	Utilized at 23 locations (ICGD online)
UF 221	Trinitario	17.0	Favorable yield potential, bean size and weight	Utilized at 23 locations (ICGD online)
ICS 39	Trinitario	22.1	Favorable yield potential, Nicaraguan Criollo pedigree	Utilized at 22 locations (ICGD online)
POUND 7	UAF (Nanay)	22.0	Good general and specific combining ability for yield^{MM} (favored parent in Costa Rica, Ghana). Resistance to black pod	Utilized at 22 locations (ICGD online); Adomako and Adu-Ampomah (2005)
ICS 16	Trinitario	17.3	Favorable yield potential, bean size and weight	Utilized at 21 locations (ICGD online)
PA 121	UAF (Marañon)	30.6	Potential resistance to BP and WB, Favorable yield per hectare, used in breeding in Brazil	Utilized at 21 locations (ICGD online); Bekele et al. (2008b)
CC 10	Unclassified	14.7	Highly favorable yield potential, bean weight and size	Utilized at 20 locations (ICGD online)
ICS 60	Trinitario	16.5	Pod resistance to WB^{D}, favorable yield potential and bean size and weight	Utilized at 20 locations (ICGD online)
GS 36	Trinitario	23.2	Fair yield potential, bean size and weight	Utilized at 20 locations (ICGD online)
ICS 40	Trinitario	20.8	Fair yield potential, Nicaraguan Criollo pedigree	Utilized at 19 locations (ICGD online)^{T}
UF 613	Trinitario	26.9	Moderately fair yield potential, potential resistance to BP, Criollo pedigree	Utilized at 18 locations (ICGD online)
SIAL 93	LAF	24.8	Fair yield potential	Utilized at 18 locations (ICGD online)
ICS 8	Trinitario	19.8	Favorable yield potential, good bean weight and size	Utilized at 18 locations (ICGD online)

(continued)

Cacao accession	Traditional classification	Pod Index[1] (assessed in Trinidad)	Disease resistance and other favorable trait	Comment
ICS 89	Trinitario	28.3	Good combining ability	Utilized at 17 locations (ICGD online)
UF 705	Trinitario	–	Fair yield potential (Costa Rica)	Utilized at 16 locations (ICGD online)
CC 11	Unclassified	–	Favorable yield potential (Malaysia)	Utilized at 16 locations (ICGD online)
SCA 9	UAF (Contamana)	19.7	Resistance to VSD^{B}, favorable yield potential	Utilized at 16 locations (ICGD online)
PA 35	UAF (Marañon)	–	Fair yield potential, bean weight (Malaysia)	Utilized at 16 locations (ICGD online)
NA 33	UAF (Nanay)	39.8	Moderate tolerance to VSD (Malaysia), BP (Brazil) and CSSV	Utilized at 16 locations (ICGD online)
UF 11	Trinitario	13.9	Excellent yield potential, bean size and weight, tolerant to VSD (Malaysia)	Utilized at 15 locations (ICGD online)
NA 32	UAF (Nanay)	30.6	Tolerant to CSSV, limited tolerance to BP (Togo)	Utilized at 15 locations (ICGD online)
GS 29	Trinitario	17.6	Favorable yield potential, bean weight and size	Utilized at 15 locations (ICGD online)
UF 29	Trinitario	19.3	Favorable yield potential	Utilized at 15 locations (ICGD online)
CATONGO	Amelonado	35.6	Homozygous mutant	Utilized at 15 locations (ICGD online); Clément et al. (2003a, b)
PA 150	UAF (Marañon)	27.4	Resistance to BP^{IL}, good combining ability	Utilized at 15 locations (ICGD online); Adomako and Adu-Ampomah (2005), Freeman (1982), and Paulin et al. (1994)
IMC 47	UAF (Iquitos)	27.4	Potential resistance to BP and WB and tolerance to CSSV	Utilized at 15 locations (ICGD online)

(continued)

Cacao accession	Traditional classification	Pod Index[1] (assessed in Trinidad)	Disease resistance and other favorable trait	Comment
PA 107	UAF (Marañon)	30.3	Favorable butterfat content (more than 55%)	Utilized at 15 locations (ICGD online)
RIM 2[MEX]	Criollo/Trinitario	39.7	Good bean size, potential moderate and resistance to BP	Utilized at 14 locations (ICGD online)
RIM 10[MEX]	Criollo/Trinitario	–	Good bean size, yield potential (Mexico)	Utilized at 14 locations (ICGD online)
POUND 10	UAF (Nanay)	42	Good butterfat content (Malaysia), potential resistance to CSSV (Ghana)	Utilized at 14 locations (ICGD online)
CAS 1	LAF	44.1	Fair bean size, potential tolerance to BP (Costa Rica)	Utilized at 14 locations (ICGD online)[T]
IMC 14	UAF (Iquitos)	25.2	Fair yield potential, butterfat content	Utilized at 14 locations (ICGD online)
ICS 98	Trinitario	30.5	Potential resistance to WB	Utilized at 14 locations (ICGD online)
EET 400	UAF (Curaray)	19.8	Potential WB and *Ceratocystis* wilt resistance, favorable yield potential	Utilized at 14 locations (ICGD online)
NA 34	UAF (Nanay)	26	Potential resistance to CSSV	Utilized at 14 locations (ICGD online)
UF 168	Trinitario	23.8	Fair yield potential, good bean size (Costa Rica, Malaysia)	Utilized at 14 locations (ICGD online)
PA 13	UAF (Marañon)	31.3	Potential BP resistance (Côte d'Ivoire, Mexico, Costa Rica)	Utilized at 14 locations (ICGD online)
BE 10	LAF (Amelonado)	26.8	Fair yield potential, butterfat content	Utilized at 14 locations (ICGD online)
RIM 117[MEX]	Criollo/Trinitario	18.7	Favorable yield potential (Mexico, Trinidad and Tobago), good bean size	Utilized at 13 locations (ICGD online)
ICS 45	Trinitario	21.2	Potential WB and BP resistance, fair yield potential, bean size	Utilized at 13 locations (ICGD online)

(continued)

Cacao accession	Traditional classification	Pod Index[1] (assessed in Trinidad)	Disease resistance and other favorable trait	Comment
CC 41	Unclassified (UF 276 × unclassified)	28.0	Tolerant to WB (Brazil)	Utilized at 13 locations (ICGD online)
CC 38	Unclassified (UF 276 × MATINA?)	26.1	Fair yield potential (Costa Rica, Malaysia)	Utilized at 13 locations (ICGD online)
PA 7	UAF (Marañon)		Moderate resistance to BP, good combining ability, tolerance to CSSV (Ghana), Good female parent in crosses designed for *Ceratocystis* control in Bahia, Brazil	Utilized at 13 locations (ICGD online); Bekele et al. (2008b); Adomako and Adu-Ampomah (2005).
PA 56	UAF (Marañon)	32	Good combining ability for wet seed weight (Trinidad and Tobago)	Utilized at 13 locations (ICGD online)
ICS 84	Trinitario	28.3	Fair yield potential, bean weight (Malaysia)	Utilized at 13 locations (ICGD online)
ICS 68	Trinitario	15.9	Favorable yield potential, bean size and weight	Bekele et al. (2009)
UF 12	Trinitario	14.8	Favorable yield potential, bean size and weight, pod resistance to BP^{I}	Utilized at 8 locations (ICGD online)
JA 5/31	Refractario	16.4	Favorable yield potential	Iwaro et al. (2003)
ICS 5	Trinitario	16.9	Favorable yield potential, bean weight and size	Bekele et al. (2014)
IMC 97	UAF (Iquitos)	17.0	Favorable yield potential, fair bean size	Iwaro et al. (2003)
Silecia 8 (EET 395)	Trinitario	17.4	Favorable yield potential, bean size	Bekele et al. (2006)
EET 59[ECU]	Unclassified	17.61	Favorable yield potential, resistance to $BP^{D\,E\,I\,P}$	Iwaro et al. (2003); Utilized at 7 locations (ICGD online)
POUND 18	UAF	17.7	Resistant to *Ceratocystis fimbriata*, potential resistance to BP, WP, favorable yield potential (many beans)	Maharaj et al. (2011); Shripat (1993)

(continued)

Cacao accession	Traditional classification	Pod Index[1] (assessed in Trinidad)	Disease resistance and other favorable trait	Comment
ICS 85	Trinitario	18.1	Favorable yield potential, pod resistance to WB[D]	Iwaro et al. (2003)
ICS 43	Trinitario	18.9	Fair yield potential, good bean weight, Nicaraguan Criollo pedigree	Utilized at 8 locations (ICGD online)[T]
ICS 75	Trinitario	19.07	Fair yield potential, bean weight and size	Utilized at 12 locations (ICGD online)[T]
IMC 10	UAF (Iquitos)	19.0	Favorable yield potential	Utilized at 8 locations (ICGD online)
EET 272	Unclassified: Forastero 'Amarillo' type × Unclassified	25.0	Potential resistance to BP, WB, *Ceratocystis* wilt, fair yield potential	Utilized at 11 locations (ICGD online)
IMC 78	Iquitos	23.0	Fair yield potential, potential resistance to WB and CSSV	Utilized at 7 locations (ICGD online); Clément et al. (2003a, b)
ICS 48	Trinitario	20.0	Fair yield potential, bean weight and size	Utilized at 10 locations (ICGD online)
EET 399	Unclassified: Silecia 1 × unclassified	24.4	Tolerance to VSD, moderate resistance to BP[M]	Utilized at 12 locations (ICGD online)
ICS 10	Trinitario	21.0	Moderate resistance to FP, potential resistance to BP, flower and cushion resistance to WB[D], good yield potential	Ducamp (1994)

[Ba]Bartley and Chalmers (1970)
[Be]Bekele et al. (1996b)
[B]Bong and Lee (1999)
[D]Ducamp (1994)
[E]Enríquez and Soria (1999)
[I]Iwaro et al. (1999)
[L]Luz et al. (1999)
[MM]Morera and Mora (1991)
[M]Morera (1996)
[P]Phillips-Mora (1999)
[T]Turnbull and Hadley (online)
Pod index is derived by dividing 1000 by the product of bean number and individual bean weight in grammes

Appendix II: Listing of Major Cacao Research Institutes

Institute	Acronym	Location	Specialization	Contact
Centro de Pesquisas do Cacau	CEPEC	Brazil	Conservation, genetic improvement, screening for yield, disease resistance, quality, gene discovery.	Dr. Jose Luis Pires joseluis@cepec.gov.br http://www.ceplac.gov.br/pesquisa.htm
The Executive Commission for the Cocoa Farming Plan	CEPLAC	Brazil	Genetic diversity enrichment, conservation, improvement for yield, disease and pest resistance etc. Physiology, nutrient studies, heavy metal research, application of biotechnology for gene discovery, breeding with genomics.	Dr. Uilson López uvlopesbr@gmail.com http://www.ceplac.gov.br/index.asp
Institut of Agricultural Research for Development	IRAD	Cameroun	Partners with CIRAD, France on genetic improvement for disease and pest resistance, yield and agroforestry	Dr. Mousseni Efombagn, efombagn@yahoo.fr http://iradcameroun.cm/fr
Corporación Colombiana de Investigación Agropecuaria	CCIA	Colombia	Conservation, genetic improvement, genomics	atencionalcliente@corpoica.org.co http://www.corpoica.org.co/menu/ps/prod/cacao/
Tropical Agricultural Research and Higher Education Center	CATIE	Costa Rica	Conservation, genetic improvement for yield, disease resistance (especially for Frosty Pod), quality etc., with the application of biotechnology	Dr. Rolando Cerda. rcerda@catie.ac.cr; https://www.catie.ac.cr/en/
Centre national de recherche agronomique	CNRA	Côte d'Ivoire	Genetic improvement for yield, productivity disease control through conventional methods and the application of molecular tools	Dr. Desire Pokou pokoudesire@yahoo.fr; Tahi Gnion Mathias tahi_mathias(at)yahoo.fr http://www.cnra.ci/

(continued)

Instituto Dominicano de Investigaciones Agropecuarias y Forestales	INDIAF	Dominican Republic	Conservation, genetic improvement, quality improvement	Ms. Marisol Ventura López mventura(at)idiaf.gov.do http://www.idiaf.gov.do/
Instituto Nacional Autónomo de Investigaciones Agropecuarias	INIAP	Ecuador	Conservation, genetic improvement, diversity studies, disease control, flavor and quality, cadmium remediation, agroforestry	Dr. Rey Gastón Loor rey.loor@iniap.gob.ec; Freddy Amores freddy.amores(at)iniap.gob.ec http://www.iniap.gob.ec/web/cacao/
Centre de Coopération Internationale en Recherche Agronomique pour le Développement	CIRAD	France	Genomics, genetic improvement and breeding, control of pests and diseases, agronomy, physiology, cropping systems, agroforestry, postharvest processing	Dr. Christian Cilas christian.cilas@cirad.fr; Dr. Claire Lanaud claire.lanaud@cirad.fr http://www.cirad.fr/
Cocoa research Institute of Ghana	CRIG	Ghana	Genetic improvement, breeding, agronomy, physiology, biochemistry, entomology, pathology, soil science, value-added product development	Dr. Francis Kwame Padi padifrancis@yahoo.co.uk http://crig.org.gh/
Central Plantation Crops Research Institute	CPCRI	India	Conservation, genetic improvement, mixed cropping	Dr. Elain Apshara elain_apshara(at)yahoo.co.in
Indonesian Coffee and Cocoa Research Institute	ICCI	Indonesia	Conservation, genetic improvement,	Dr. Eben Haeser eben.haeser@londonsumatra.com
Malaysian Cocoa Board	MCB	Malaysia	Conservation, genetic improvement, molecular studies	Dr. Haya Ramba hayaramba(at)koko.gov.my
Cocoa Research Institute of Nigeria	CRIN	Nigeria	Genetic improvement, disease and control, Value-added product development	Dr. Daniel Adewale d.adewale(at)gmail.com or dadewale(at)cgiar.org http://www.crin-ng.org/index.php

Cocoa and Coconut Institute	CCI	Papua New Guinea	Conservation, genetic improvement	Dr. James Butubu butubu(at)yahoo.com.au
Instituto de Cultivos Tropicales	ICT	Perú	Germplasm collection, conservation, genetic improvement for pest and disease resistance and quality, cadmium remediation and heavy metal studies	Dr. Enrique Arevalo-Gardini e.arevalo@ict-peru.org; e.arevalo.ict@terra.com.pe; enriquearevaloga@gmail.com http://www.ict-peru.org/
Cocoa Research Centre	CRC	Trinidad and Tobago	Conservation, germplasm enhancement, characterization, evaluation, utilization of cocoa genetic resources, research on diversity, genomics, disease control, flavor and quality studies, cadmium uptake and remediation	Prof. Pathmanathan Umaharan pathmanathan.umaharan@sta.uwi.edu http://sta.uwi.edu/cru/index.asp
International Cocoa Quarantine Centre, University of Reading	ICQC, R	United Kingdom	Provision of intermediate quarantine in a temperate country for the safe movement of cacao germplasm between producing countries	Dr. Andrew Daymond a.j.daymond(at)reading.ac.uk
Penn State University	PSU	USA	Plant development, genetic transformation, propagation, engineering, functional genomics, proteomics, metabolomics, regulation of gene expression, plant defense responses, starch biosynthesis	Prof. Mark Guiltinan mjg9@psu.edu Dr. Siela Maximova snm104@psu.edu http://plantscience.psu.edu/research/labs/guiltinan
United States Department of Agriculture, Agricultural Research Service, Tropical Agriculture Research Station	USDA/ ARS	USA and Mayaguez, Puerto Rico (PR)	Conservation, genetic improvement, genomics, marker-assisted selection, diversity studies, genomics-assisted breeding	Dr. Ricardo Goyenaga Ricardo.Goenaga@ARS.USDA.GOV Dr. Dapeng Zhang dapeng.zhang@ars.usda.gov; Dr. Lyndel Meinhart Lyndel.Meinhardt@ars.usda.gov; Dr. Osman Guitterez osman.gutierrez@ars.usda.gov
Instituto Nacional de Investigaciones Agrícolas	INIA	Venezuela	Rescue and conservation of genetic resources, particularly Criollos, characterisation, genetic improvement including for disease resistance, flavor and quality	Dr. Alvaro Gómez Morales Email: Agomez153(at)gmail.com http://www.inia.gov.ve/

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Chapter 13
Oil Palm (*Elaeis* spp.) Breeding in Malaysia

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Abstract The planted area of oil palm has grown to over 5.81 million ha by 2017, uplifting the economy and livelihood of Malaysians over the last 100 years since its beginning as a plantation crop in 1917. The narrowness of the active gene pool was recognized as one of the major drawbacks to a successful and rapid selection progress in oil palm breeding. The industry has made significant strides by incorporating germplasm material sourced from its center of origin in Africa and South America into on-going breeding programs. This resulted in the development of 13 new types of genetic material (PS series) by the Malaysian Palm Oil Board that have been distributed to the industry. An active tissue culture program has also facilitated cloning of high yielding palms, where clonal uniformity offers the opportunity to increase yields of up to 30%. Research and development efforts to further improve the productivity of oil palm received a boost when its genome was sequenced and released in 2013, which laid the foundation for applying genome-based technologies in oil palm research. With the discovery of a few genes at the molecular level together with oil palm genome, sequence data, we expect further integration of conventional and molecular breeding. Biotechnology research is the core area to opening up new avenues for oil palm improvement toward an era of precision agriculture and sustainability.

Keywords Biotechnology · Breeding · Genetic resources · Molecular breeding · Oil palm

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13.1 Introduction

Among cultivated oil crops, oil palm has the highest productivity and hence sufficient to fulfill the increasing demand for vegetable oil which may reach 240 million mt by the year 2050 (Barcelos et al. 2015). Currently, the top five palm oil producing countries are Indonesia (41.5 million mt), Malaysia (20.5 million mt), Thailand (2.9 million mt), Colombia (1.6 million mt) and Nigeria (one million mt) (Index Mundi 2018). Meanwhile, palm oil area harvested reached 11.3 million ha in Indonesia, followed by Malaysia, Nigeria, Thailand and Colombia with 5.3, 2.5, 0.78 and 0.47 million ha, respectively (Index Mundi 2018). This chapter highlights progress made in the genetic improvement of oil palm based on conventional and modern breeding technologies with emphasis on the Malaysian experience.

13.1.1 History and Origin

The oil palm of commerce (*Elaeis guineensis* Jacq.) is now generally regarded to originate from West Africa or the third land bridge between South America and Africa (Chevalier 1934; Hartley 1988; Rosenquist 1986). However, Cook (1942) and Corner (1966) favor a South American origin. Fossil, linguistic, archeological and historical evidence supports that hypothesis of African origin. Crone (1937) reported that many early European explorers of Africa have described the palm based on its occurrence and products. However, no similar description was found in the records of South American explorations. The two main arguments for the South American origin are that the oil palm is found in the wild in Brazil and all its relatives are natives of that continent.

In studies of the African flora, Chevalier (1934) recorded the distribution of oil palms from Senegal and (now) South Sudan in the north through tropical West Africa to Mozambique in the southeast. Zeven (1967) then recorded the most northerly point of spontaneous occurrence as near Saint Louis (16°N) in Senegal. He considered that the main oil palm belt begins in Guinea-Conakry and spreads south via Cameroon, Gabon, Dahomey, Nigeria, Togo, Ivory Coast, Ghana, Angola, Equatorial Guinea, Liberia, Congo-Kinshasa and Sierra Leon. Namibe (16°S) in Angola is considered as the southernmost point of the natural distribution of oil palm in West Africa. From Central West Africa, the oil palm belt stretches eastwards through Congo-Kinshasa, northern Angola, Rwanda and Burundi and across the Congo-Kinshasa/South Sudan border to the eastern shores of Lake Tanganyika. In East Africa, it spreads south to Mozambique and across to the island of Madagascar. It is believed that much of the spread, especially eastwards, was due to human dispersal (Zeven 1967). It is generally agreed that soil and water availability as well as temperature, especially at high elevations, have restricted the spread of oil palm.

13.1.2 Distribution in Southeast Asia

The earliest record of oil palm introduction in Southeast Asia was in 1848 and involved four palms in the Bogor Botanic Gardens, Indonesia (Hartley 1988). These palms were from the Amsterdam Botanic Gardens (of unknown source) and Mauritius or Reunion Island. Hartley (1988), Gerritsma and Wesel (1997) as well as Pamin (1998), suggested that these palms possibly originated from one single parent palm in Africa as they were all quite similar. Progenies of these palms became the foundation stock for the Southeast Asian industry after having been transferred to Sumatra in 1875.

Since 1860, several experimental plots were established in different parts of Indonesia using seeds from the four Bogor palms. The Deli palm was derived from one plot located in Deli, Sumatra (Pamin 1998). In Malaysia, the Deli palms were planted in 1911 and 1912 at Rantau Panjang, Selangor. In 1917, seedlings from the Rantau Panjang palms were used for the establishment of Malaysia's first oil palm plantation located at Tenammaran Estate and later planted in Elmina Estate in 1920 (Kushairi 1992) where the Deli *dura* palms became the commercial planting material for decades (Kushairi and Rajanaidu 2000).

13.1.3 Taxonomy and Classification

Uhl and Dransfield (1988) subdivided the family Palmae (monocotyledons) also known as Arecaceae into six subfamilies, namely Coryphoideae, Calamoideae, Nypoideae, Ceroxyloideae, Arecoideae and Phytelephantoideae. The subfamily Arecoideae is divided into tribes and subtribes. The genera *Elaeis* and *Barcella* fall into the tribe *Cocoaeae* and subtribe *Eleaidinae*. *Elaeis guineensis* and *E. oleifera* are two species from the genus *Elaeis* (Fig. 13.1) while *Barcella odora* (Trail) Drude is monotypic (Henderson 1986). The taxonomic classification of oil palm is shown in Table 13.1.

The African oil palm, *Elaeis guineensis* Jacq. received its botanical name from Jacquin (1763) in his account of American plants. The name *Elaeis* originated from *elaeion*, meaning oil in Greek. Meanwhile, the distribution of this species, *guineensis*, to the coast of Guinea-Conakry in Africa demonstrated attributes described by Jacquin.

13.1.4 Biology of the Oil Palm

The oil palm is an erect, single-stemmed palm tree which can grow to 20 m or more in height. It has an adventitious root system where the tertiary and quaternary roots are mostly found in the upper 35 cm of the soil, while some of the root biomass can

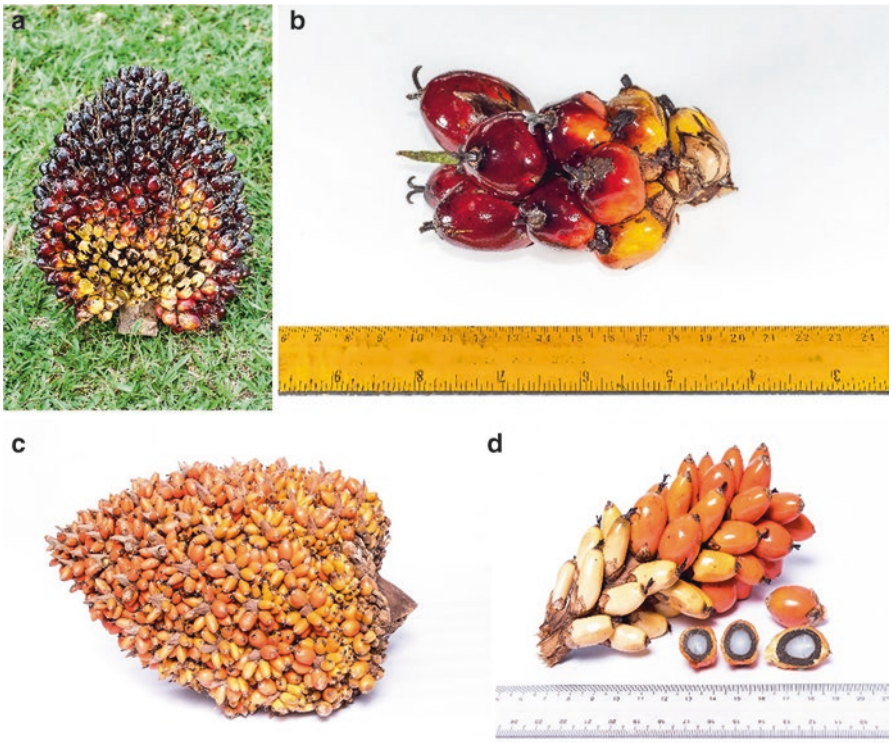


Fig. 13.1 Oil palm bunches and fruits. (**a**) *Elaeis guineensis* bunch, (**b**) *E. guineensis* fruits, (**c**) *E. oleifera* bunch, (**d**) *E. oleifera* fruits

Table 13.1 The taxonomic classification of *Elaeis guineensis* and *E. oleifera*

Classification	*Elaeis guineensis* Jacq.	*Elaeis oleifera* (Kunth) Cortés
Kingdom	Plantae (plants)	Plantae (plants)
Subkingdom	Tracheobionta (vascular plants)	Tracheobionta (vascular plants)
Superdivision	Spermatophyta (seed plants)	Spermatophyta (seed plants)
Divison	Magnoliophyta (flowering plants)	Magnoliophyta (flowering plants)
Class	Liliopsida (monocotyledons)	Liliopsida (monocotyledons)
Subclass	Arecidae	Arecidae
Order	Arecales	Arecales
Family	Arecaceae (palm)	Arecaceae (palm)

grow deeper than 1 m. The cylindrical stem diameter varies between 20 and 75 cm, topped by 35–60 spirally arranged pinnate leaves. Each leaf consists of a petiole rachis of about 150 cm long with 300–400 leaflets. According to Corley and Gray (1976a), leaves are actively produced (30–40 per year) at the immature plant stage which is from second to fourth years after field planting. Leaf production will slowly decrease (20–25 per year) at mature plant stage of 8 years and onwards. The

economic life span of commercial oil palms is about 25–35 years, while wild palms may live up to 200 years.

Inflorescences Oil palm produces distinct male and female flowers (inflorescences) on the same palm, termed *monoecious* (Fig. 13.2). However, based on detailed investigation, each flower primordium is capable of becoming a male or female inflorescence in alternating cycles of varying duration (Beirnaert 1935). Hermaphrodite flowers are produced when both male and female organs are fully developed. Each leaf axil contains an inflorescence and while twin inflorescences are rare, several inflorescences can abort before emergence (Corley and Tinker 2016). The young inflorescence takes about 2 years from initiation to reach the central spear stage. It then takes another 9–10 months for flowering and anthesis. After the inflorescence has fully emerged, an inner and outer spathe covers it tightly for almost a month. The outer spathe opens 6 weeks before anthesis, follow by the inner spathe about 2–3 weeks later. As the inflorescence advances in development, both spathes disintegrate (Corley et al. 1976). The number of fully developed inflorescences and total leaf production determines the number of inflorescences per palm.

Sex Ratio Palm sex ratio is the quotient between the number of female inflorescences and the total number of inflorescences produced (Corley and Tinker 2016). Generally, there is an interval of about 20 months between sex differentiation and anthesis. Sex ratio is key to yield achievement. It is influenced by both genetics and the environment including fertilizer application, planting densities and the

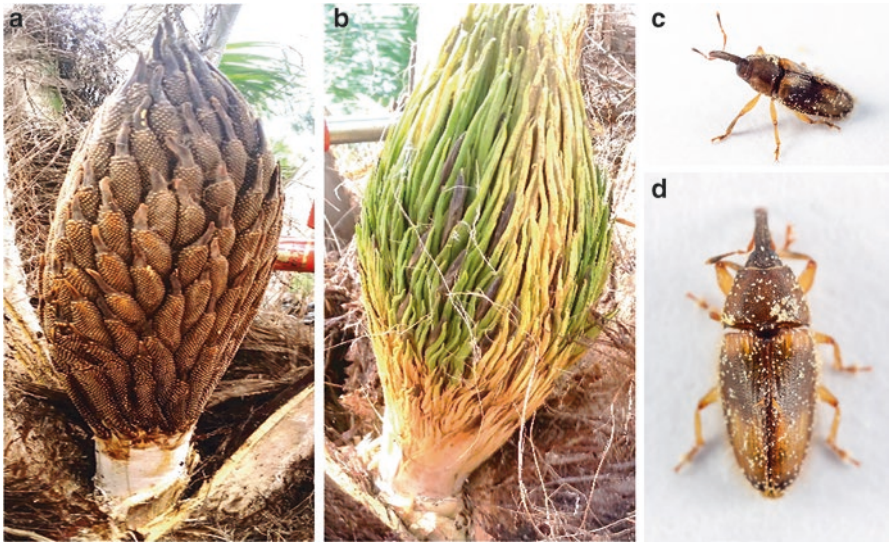


Fig. 13.2 Oil palm inflorescences and insect pollinators. (**a**) Male inflorescence, (**b**) Female inflorescence (**c–d**) weevil *Elaeidobius kamerunicus*

availability of sufficient water. Latiff (2000) reported that sex ratio declines from 98% in young palms to 35% in older palms.

Pollination Oil palm pollen is wind dispersed and often required artificial pollination in Southeast Asia before the successful introduction of the insect pollinator *Elaeidobius kamerunicus* Faust, in Malaysia in 1979, followed by Indonesia (Fig. 13.2). The introduction of this weevil increased pollination and fruit production from 20% to 30% in 1981 (Basri et al. 1983; Syed et al. 1982).

Fruit Bunch Fruit bunches (infructescences) are produced 3–4 years after field planting and fruit ripeness takes 5–6 months after pollination. The ripe fruits become detached after concurrent events of fruit ripeness from top to bottom of the bunch and from outer to inner fruits. Between 3 and 15 years of age, bunch weight would increase from below 5 kg to 25 kg (Corley and Gray 1976b). Each bunch consists of about 1500 fruits of 5–20 g in weight (Hartley 1988). The oil palm fruit consists of an epicarp, mesocarp (which produces palm oil), endocarp (shell) and endosperm or kernel (which produces palm kernel oil). Janssens (1927) and Smith (1935) recognized three fruit forms based on shell thickness, namely *dura* (thick-shell), *tenera* (thin-shell with fiber-ring around the endocarp) and *pisifera* (shell-less). In addition, oil palm fruits also vary in the epicarp skin color and these are known as *nigrescens*, *virescens* and *albescens*, with *nigrescens* the most common (Fig. 13.3).

13.2 Sustainable Cultivation

13.2.1 Optimum Requirement for Oil Palm Cultivation

The remarkable growth of the palm oil industry in Malaysia began with the planting of 54,000 ha in 1960, and reached an astonishing increase to 5.81 million ha by 2017 (MPOB 2018). However, due to land scarcity, the growth rate of oil palm planted areas in Malaysia has declined gradually from 3.14% (1975–1985) to 2.62% (2006–2016) (Kushairi 2017; Parveez 2017).

The lowland humid tropics are the natural environment of the oil palm. Its native areas receive a rainfall range of 1780–2280 mm year^{-1}, with 2–4 months of dry season. However, in some areas, as low as 640 mm annual rainfall has been recorded while the highest recorded was 4200 mm. Generally, oil palm is a lowland crop and can be grown on a wide range of tropical soils with suitable pH (4–8) and sufficient moisture (Duke 1983).

In Malaysia, both inland and coastal soils support oil palm growth (Corley and Tinker 2016). Ndon (2006) recommended temperatures of 22–24 °C (minimum) and 29–33 °C (maximum) to achieve the best yields from oil palm. Meanwhile, Goh

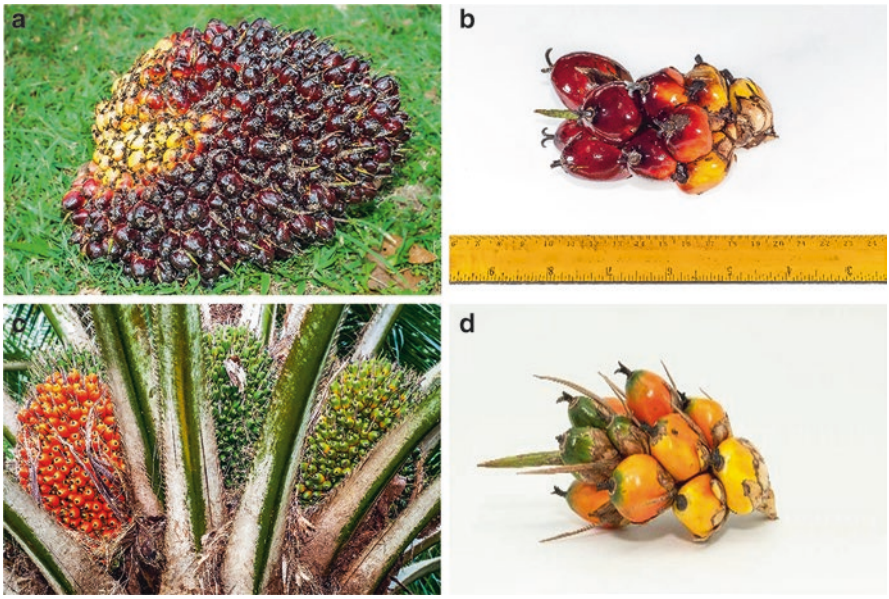


Fig. 13.3 Oil palm epicarp skin color. (**a–b**) Nigrescens, (**c–d**) Virescens

(2000) reported that 2000–2500 mm year^{-1} of rainfall is highly suitable for oil palm while less than 2000 mm year^{-1}, or several dry months, can lead to poor fresh fruit bunch (FFB) yields.

13.2.2 Cultivation Practices

Oil palm cultivation practices by the oil palm industry have progressed over the years based on the latest technologies and approaches introduced by the industry. For oil palm cultivation, the clean-clearing method involving multiple processes of biomass burning was formerly the practice for land clearing. However, due to a ban imposed by the government of Malaysia on open burning in 1998, various zero burning techniques of land clearing have been applied, which has brought about higher returns compared to the clean-burn method (Mohd Nor 2003).

Generally, 12–15 month-old seedlings with a minimum height of 1 m and 12–13 functional leaves are recommended for field planting. An equilateral triangular pattern is applied for oil palm planting with different densities based on soil fertility, topography and other management factors (Table 13.2 and Fig. 13.4). In terms of irrigation, the oil palm crop requires sufficient irrigation for growth as well as for yield and biomass production. Meanwhile, soil mulching with oil palm empty fruit bunch (EFB) is widely practiced to conserve moisture content as well as improving oil palm nutrition and yield when supplemented with N and K fertilizers (Lim and Zaharah 2002).

Table 13.2 Planting density and distance between palms

Soil type	Planting density (palms ha^{-1})	Distance between palms (m)	Distance between lines (m)
Fertile mineral soil	136	9.2 × 9.2 × 9.2	8.0
Less fertile mineral soil, shallow peat	148	8.8 × 8.8 × 8.8	7.6
Medium deep peat, deep peat and most marginal soil	160	8.5 × 8.5 × 8.5	7.4

Source: Esnan and Idris (2009)

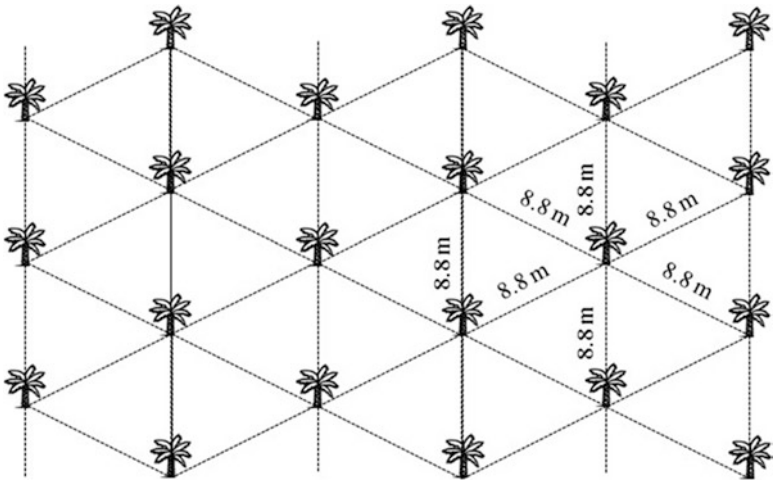


Fig. 13.4 An equilateral triangular planting at 148 palms ha^{-1} density

In controlling pest sustainably, integrated pest management (IPM) is key to minimize the use of pesticides by adopting biological, cultural, mechanical, physical or other less-hazardous strategies. Application of specially-formulated biopesticides such as the bacteria *Bacillus thuringiensis* to control bagworm and the fungus *Metarhizium anisopliae* to control rhinoceros beetle are some of the examples of IPM (Siti Ramlah et al. 2011). With reduced risk of pollution and release of toxic chemicals, IPM restores the natural equilibrium among the palm, its pests and their natural enemies.

Meanwhile, there are several elements in management practices which guarantee stability of the business, and one of the elements is profitability. Each organization must develop their own best-management practices which include site operations, accountancy, labor and materials to obtain optimum yield, production and profit (Ainie et al. 2015). Due to the need of sustainable implementation in oil palm premises, best practices are carried out as a strategy to strengthen the palm oil position in international markets. MPOB Codes of Good Agricultural Practices for Oil Palm Estates and Smallholdings were launched with the objectives to ensure that sustainably produced palm oil meets the requirements of food safety, quality,

environmental protection, biodiversity enhancement and greenhouse gas (GHG) reduction (MPOB 2009).

13.2.3 Issues and Challenges in Oil Palm

There are several issues which significantly impact the oil palm industry. Labor shortage, environment, palm age and replanting, plant infection and climate change are some of the current issues discussed below.

Labor Shortage In Malaysia, the oil palm plantation sector is highly dependent on manual labor from foreign countries, especially Indonesia. The land-labor ratio is commonly used to indicate the labor used in managing estates. Current land-labor ratio in the plantation sector is 11:1, where a worker can cover 11 ha of oil palm plantation. In 2012, Azman (2013) estimated that the sector was facing a labor shortage of 26,374 workers, with the highest shortage in Peninsular Malaysia (47.4%). Due to labor issues, the supply of palm oil in domestic and world markets has been affected.

Environment Deforestation, loss of biodiversity, sustainability, traceability, indiscriminate burning and net emitter of carbon dioxide are some of the environmental issues linked to oil palm cultivation. However, despite being the most productive of oil crops, oil palm has the lowest land use of only 5% as compared with other oil crops (Parveez 2017). Besides, the Malaysian oil palm industry is a highly regulated industry following over 15 laws and regulations such as the Land Acquisition Act 1960, Environmental Quality Act 1974, Pesticides Act 1974 (Pesticides Registration Rules), Occupational Safety and Health Act (1977), Environmental Quality (Clean Air Regulations) 1978 and Protection of Wildlife Act 1972 (MPOC 2018). In addition, the Environmental Impact Assessment (EIA) as well as Hazard & Critical Control Points (HACCP) requirements are also complied with by the industry. Over its 25 years of productive life cycle, the oil palm stores carbon dioxide and provides cover for a wide range of terrestrial flora and fauna species.

Palm Age and Replanting Generally, the peak yield for oil palm FFB is reached between the sixth and twelfth year after field planting. The cost of harvesting a metric ton of FFB increases with palm age while oil extraction rate (OER) and yield decrease (Corley and Tinker 2016; Ismail and Mamat 2002). Due to economic rationale, palms of at least 25 years old are usually considered necessary to replant.

Plant Infection One of the serious threats to the oil palm industry is basal stem rot (BSR) disease caused by *Ganoderma* fungal species. Three species have been reported to be pathogenic to oil palm, with *Ganoderma boninense* being the most destructive (Idris et al. 2000; Nurniwalis et al. 2009). BSR symptoms include external symptoms (e.g. foliar symptoms and appearance of *Ganoderma* fructifi-

Fig. 13.5 *Ganoderma* fruiting bodies. (Source: MPOB)

cation as shown in Fig. 13.5) as well as internal symptoms (e.g. root and stem decay) (Idris et al. 2009).

Climate Change The Malaysian oil palm industry has been significantly affected by El Niño and La Niña climatic conditions whereby these two events have caused prolonged hot and dry weather as well as cooler temperatures usually related to heavy rainfall season, respectively (Kamil and Omar 2016). Verheye (2010) reported that the occurrence of adverse weather conditions affect floral abortion, frond production, low survival of flowers after anthesis, sex ratio and reduced bunch weight at an earlier growth stage. These will consequently influence oil palm yields.

13.2.4 Improvement Strategies

For all the issues and challenges listed above, improvement strategies have been taken for future growth and expansion of the oil palm industry. Sustainable oil palm development is one aspect in the development of plantation and the national agriculture processing industry.

Advances in Field Mechanization In order to overcome labor shortage, mechanization approaches have been introduced to the oil palm plantation industry. A motorized cutter (Cantas), mechanical harvester, mechanical grabber, compact transporter, battery-powered wheelbarrow and power cart (three-, four-and eight-wheelers) are some of the machines invented for in-field FFB removal and transportation of the harvested bunches to the mill (Abd Rahim et al. 2010).

Sustainability Due to the sensitivity of environmental and social issues, the Malaysian oil palm industry actively participates in the certification standards set by

the Roundtable on Sustainable Palm Oil (RSPO). The Malaysian Sustainable Palm Oil (MSPO) Certification Scheme was then launched as a national scheme covering both upstream plantation and downstream applications of the oil palm industry in the country. This guide was developed by the Malaysian Palm Oil Certification Council (MPOCC), an independent organization set up in October 2015 to support sustainable management practices for palm oil production.

Accelerated Replanting The Malaysian government has introduced the National Key Economic Area (NKEA) where part of the Economic Transformation Program (TEP) involves a requirement to replant old and unproductive oil palms with new higher-yielding cultivars with the aim of increasing palm oil production without expanding the area under cultivation. In their study, Basri and Arif (2010) estimated that Malaysia requires continued accelerated replanting at a minimum rate of 155,000 ha per year.

Integrated *Ganoderma* Management (IGM) MPOB introduced IGM to combat the *Ganoderma* disease (Idris 2011). Several preventive technologies such as the application of biofertilizer Hendersonia GanoEF, hexaconazole treatment and chemical fertilizer GanoCare™ have been introduced to the Malaysian oil palm industry with the aim to reduce potential yield losses in oil palm cultivation (Idris 2012; Idris et al. 2014, 2015).

Mitigation of Greenhouse Gas (GHG) In tackling climate change, the global aim is to reduce GHG emissions in the use of resources and energy as well as in processing and transport. In processing, for example, a Green Palm Oil Mill and a biorefinery plant was implemented led by MPOB (Kushairi et al. 2017). Under the National Energy Policy, biomass is one of the renewable alternatives for electricity, and the oil palm creates an abundant amount of biomass which can be burned for power generation (Ooi 2012). Besides, application of trapped biogas from the anaerobic effluent ponds will also help to mitigate GHG emissions (Langeveld et al. 2016).

13.3 Breeding Program and Seed Production

13.3.1 Foundation Stocks of Current Breeding

As mention in Sect. 13.1.2, the four Bogor palms were the genetic basis of the oil palm industry in Malaysia. The Deli *dura* which is the basic breeding stock, is derived from selection among descendants of these four Bogor palms in Deli, Sumatra (Rajanaidu et al. 1979). Hartley (1988) reported that the Deli type is still considered as the best *dura* for breeding. Compared with those of *tenera*s and the African *dura,* Deli *dura* produce fewer but heavier bunches. Their fruits are darker

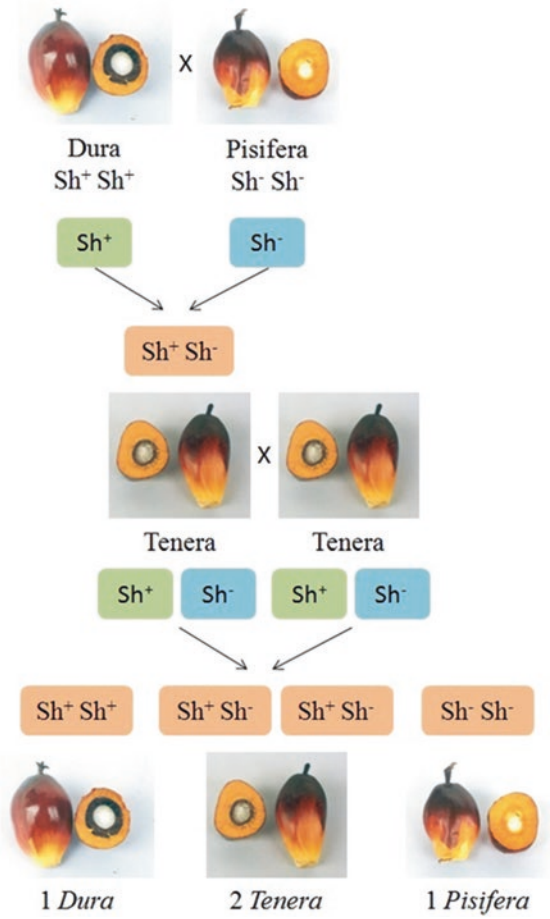
colored, larger and have greater mesocarp content with higher percentage of oil, as compared to African *dura*s (Hardon and Thomas 1968; Hartley 1988).

The Department of Agriculture (DOA) pioneered the oil palm breeding program in Malaysia in the 1920s followed by two other companies namely, the Oil Palm of Malaya (OPM) of Kumpulan Guthrie Berhad and Societe Financiere de Caoutchouces (Socfin) by 1933. Most efforts during that time focused on improving the *dura* palm with a thick shell to increase bunch weight. Through their breeding programs, four breeding populations considered as independent breeding populations of restricted origin (BPRO), were created. They were Serdang Avenue and Elmina (DOA), Ulu Remis (Guthrie) and Johore Labis (Socfin) (Rosenquist 1985). The history of these BPROs has been well documented by Hardon and Thomas (1968). The Malaysian Agricultural Research and Development Institute (MARDI) later managed DOA oil palm breeding materials and transferred them to the Malaysian Palm Oil Board (MPOB) (formerly the Palm Oil Research Institute of Malaysia, PORIM) when it was formed in 1979.

Beirnaert and Vanderweyen (1941) discovered the monogenic shell thickness which influenced oil yield in oil palm. The thick shell *dura* is controlled by dominant homozygote gene (Sh^+ Sh^+) whereas recessive homozygote gene (Sh^- Sh^-) controlled the shell-less *pisifera*. The cross between the *dura* and *pisifera* would produce 100% of heterozygote *tenera* hybrid (Sh^+ Sh^-) with thin shell (Fig. 13.6). Despite the fact that *pisifera* has a high mesocarp content of 95%, it is usually female sterile or semi-fertile and generally does not produce bunches. Therefore, *pisifera* is only used as the male parent in producing the *tenera* hybrid.

This single gene inheritance has been fully exploited in oil palm breeding to produce planting materials with higher oil yield. The first *dura* × *pisifera* (D × P) planting in Malaysia was initiated by DOA in 1953 (Kushairi et al. 1999). DOA collaborated with institutes in West Africa and private plantations in Malaysia to test and exchange *pisifera* and *tenera* materials which led to the import of the AVROS (*Algemene Vereniging van Rubber Planters ter Oostkust van Sumatra*) *tenera* × *pisifera* (T × P) seeds from Sumatra, Indonesia in 1957 and planted at Klanang Bahru Estate. The Djongo palm at the Eala Botanical Garden, Congo-Kinshasa, was the ancestral palm of the AVROS *pisifera* which was found to be a good general combiner with Deli *dura.* The Deli *dura* × AVROS *pisifera* (D × P) has been the common commercial planting material in Malaysia and worldwide since late 1959 (Kushairi et al. 1999).

The switch-over from *dura* to *tenera* (D × P) planting materials would increase yields by at least 30% (Kushairi 2009). Corley et al. (1976) and Rajanaidu et al. (1990) reported that the best experimental plot and selected progeny produced oil yields of 8.6 and 12.2 mt ha^{-1} $year^{-1}$, respectively. Meanwhile, Sharma and Tan (1999) reported that individual palms recorded 13.6 mt ha^{-1} $year^{-1}$ of oil yield. An oil yield of 18.2 mt ha^{-1} $year^{-1}$ is the maximum theoretical oil yield proposed by Corley (1998).



Dura
Shell thickness (2–8 mm)
Mesocarp to fruit ratio (35–55 %)
Kernel to fruit ratio (7–20 %)
Oil to bunch ratio (16 %)
No fiber ring

Pisifera
Shell-less
No fiber ring
Mesocarp/fruit ratio (95 %)

Tenera
Shell thickness (0.5–4 mm)
Mesocarp to fruit ratio (60–96 %)
Kernel to fruit ratio (3–15 %)
Oil to bunch ratio (26 %)
Presence of fiber ring

Fig. 13.6 Production of oil palm *tenera* hybrid and monogenic inheritance of shell thickness. Characteristics of *dura*, *tenera* and *pisifera* fruit forms, described by Hardon et al. (1985) and Hartley (1988)

13.3.2 Breeding Schemes

Production of commercial D × P seeds depends largely on both populations of *dura* (D) and *pisifera* (P). Therefore, oil palm breeding schemes are generally designed to develop both populations, and the most commonly-adopted schemes are the reciprocal recurrent selection (RRS) and modified recurrent selection (MRS).

The RRS scheme is the basis of programs in both Nigeria (by the Nigerian Institute for Oil Palm Research, NIFOR) and the Ivory Coast (by IRHO) (Gascon and de Berchoux 1964; Sparnaaij et al. 1963). In the RRS scheme, there are two base populations which are kept separately, the *dura* and *tenera/pisifera* populations. Test crosses are made between the two populations (D × T and D × P) where superior *dura* and

tenera palms are identified based on their performance. From the test-cross program, both parents of the superior individual palms are selfed where *dura* and *pisifera* palms from the self crosses are then used for seed production. General combining ability (GCA) as well as specific combining ability (SCA) effects are expected to be reproduced from this scheme. The best individuals from the two base populations are selfed and crossed among them to form the next generation. Meunier (1989) and Rajanaidu et al. (2000) reported that oil yield has increased by almost 18% per cycle by adopting the RRS scheme compared to the base population. The RRS schemes can save time if both selfings of all parents and test-crosses are made simultaneously. However, it requires a large experimental area to test all crosses and thus, the scheme is more appropriate for large national or international organizations (Soh 1987).

On the other hand, the MRS scheme mainly used in Malaysia involves intercrossing between selected parents. Based on family and individual palm performances (also known as the FIPS method), Deli *dura* and *tenera* parents are selected for further breeding and seed production. As *pisiferas* are usually female sterile, selection based on its phenotypic performance is unattainable. Therefore, *pisiferas* are selected as the male parents for progeny testing based on its *tenera* sib performance. The North Carolina Model I (NCMI) is used in progeny testing where a selected male parent (*pisifera*) is crossed with several individual *duras*. The *pisifera* is considered to have a good GCA if its mean performance with a number of *duras* is high. Meanwhile, the *pisifera* is said to have a SCA if only selected crosses showed high yields. Improved *dura* mother palms can be generated through selfs of the outstanding *dura* palms (Rajanaidu et al. 2000), while the next generation of *pisifera* is formed through *tenera* selfs and crosses (T × T) or *tenera* × *pisifera* (T × P) crosses. To improve the genetic variability, other populations can be introduced in the MRS scheme. Jalani and Ariffin (1994) reported that oil yield among Deli *dura* selections has increased (~3–5 mt ha^{-1} $year^{-1}$) compared to the Nigerian groves (~0.18 mt ha^{-1} $year^{-1}$) which proves the MRS to be largely successful. One of the advantages of MRS is that more recombinant crosses can be generated. However, the scheme has a long generation time as progeny testing of *dura* × *pisifera* (D × P) must be done before selection of parents for the next cycle.

13.3.3 Breeding Objectives

Six objectives in oil palm breeding were recommended by Rajanaidu et al. (2000). The targeted traits are increased oil yield, dwarf palms, improved oil quality, resistance to disease, physiological traits (such as bunch index i.e. the ratio of bunch dry matter to total dry matter production) as well as genetic × environment (G × E) interaction. Based on two brainstorming sessions within the oil palm industry held in 2002 and 2003, 10 priority traits were listed as breeding objectives as shown in Table 13.3 (Basri 2003; Mohd Din et al. 2005a, b).

Table 13.3 List of priority traits for oil palm breeding

Traits	Current	Benchmark
High oil yield	3.70 mt ha^{-1} $year^{-1}$	9.00 mt ha^{-1} $year^{-1}$
Ganoderma resistance	70%	90%
High bunch index	0.40	>0.60
Low height increment	45–75 cm $year^{-1}$	<30 cm $year^{-1}$
Long stalk	10–15 cm	>25 cm
Low lipase	22–73%	11–36%
High oleic acid	22–40%	>65%
Large kernel	5% kernel to bunch	20% kernel to bunch
High vitamin E	600 ppm	1000–1500 ppm
High carotene	500 ppm (*Elaeis guineensis*)	>2000 ppm (*E. guineensis*)
	1500 ppm (*E. oleifera*)	>3000 ppm (*E. oleifera*)

13.4 Genetic Resources

13.4.1 Introduction

As the Deli *dura* originates from the four Bogor palms, while the pollen source is descended from a limited number of *pisifera* origins, the genetic base of the oil palm planting materials in Malaysia is extremely narrow. The role of genetic resources for future developments is crucial since successful breeding and selection depends a great deal on genetic variability. Concerns generated by this awareness provided the initial thrust for the collection of new oil palm genetic materials (Hardon 1984). The genetic resource program at MPOB involves four major steps: (i) collection, (ii) evaluation, (iii) utilization and (iv) conservation.

13.4.2 Collection

The oil palm germplasm collection established represents an excellent source of new genetic diversity. These materials could successfully be used for the introgression of new genetic diversity into existing breeding populations as well as for the development of new breeding populations. The purpose of germplasm collection is to assemble material with a large indefinite amount of useful genetic variability within a restricted number of samples (Marshall and Brown 1975).

The first effort was accomplished through collections from Nigeria in 1973 which were then planted at the MPOB Research Station at Kluang, Johore, Malaysia for evaluation (Rajanaidu 1986). Other than Nigeria, exploration was widened to include other countries in Africa such as Cameroon, Congo-Kinshasa, Tanzania, Madagascar, Angola, Senegal, Gambia, Sierra Leone, Guinea-Conakry and Ghana (Table 13.4) (Rajanaidu and Rao 1987; Rajanaidu et al. 2013). These MPOB germplasm collections were also planted at the MPOB Research Station, Kluang, Johore, Malaysia. At

Table 13.4 List of germplasm collections

Year	Country	No. of accessions	Collectors
Elaeis guineensis			
1973	Nigeria	919	Rajanaidu, Arasu and Obasola
1984	Cameroon	95	Rajanaidu and Unilever
1984	Congo-Kinshasa	369	Rajanaidu and Unilever
1986	Tanzania	59	Rajanaidu and Ministry of Agriculture Officials, Tanzania
1986	Madagascar	17	Rajanaidu and Ministry of Agriculture Officials, Madagascar
1991, 2010	Angola	54, 127	Rajanaidu, Jalani Sukaimi, Mohd Din Amiruddin, Marhalil Marjuni and Ministry of Agriculture Officials, Angola
1993	Senegal	104	Rajanaidu, Jalani Sukaimi and Ministry of Agriculture Officials, Senegal
1993	Gambia	45	Rajanaidu, Jalaini Sukaimi and Ministry of Agriculture Officials, Gambia
1994	Sierra Leone	56	Rajanaidu, Ahmad Kushairi Din and Ministry of Agriculture Officials, Sierra Leone
1994	Guinea-Conakry	61	Rajanaidu, Ahmad Kushairi Din and Ministry of Agriculture Officials, Guinea-Conakry
1996	Ghana	58	Rajanaidu, Mohd Rafii Yusop and Ministry of Agriculture Officials, Ghana
E. oleifera			
1982	Honduras	14	Rajanaidu
1982	Nicaragua	18	Rajanaidu
1982	Costa Rica	61	Rajanaidu
1982	Panama	27	Rajanaidu
1982	Colombia	42	Rajanaidu
1982	Suriname	7	Rajanaidu
2004/2006	Ecuador	15	Rajanaidu, Ahmad Kushairi Din and Noh Ahmad

present, the MPOB field genebanks consist of 19 germplasm accessions collected from West Africa and South America.

13.4.3 Evaluation

Extensive screenings are continuously accomplished on these materials in order to utilize their genetic potential in oil palm breeding programs. Through breeding programs, it requires over 20 years from the collection of germplasm in the wild to the release of oil palm planting materials. During this time, specific data are

collected on an individual palm basis. Below are the methods used to evaluate oil palm genetic materials.

Yield Recording Bunch weight (BWT) and bunch number (BNO) data are collected on individual palm basis at each harvesting round beginning 36 months after field planting. Fresh fruit bunch (FFB) yield is the sum of BWT while average bunch weight (ABW) is the quotient between FFB and BNO. The yield record is summarized for 12-month periods, January through December each year.

Bunch Analysis The bunch analysis method was formulated by Blaak et al. (1963) and improved by Rao et al. (1983). It is used to determine the oil extraction and fruit quality components. For this purpose, 3–5 bunches are sampled from each palm. To minimize seasonal variation, ripe bunches with 1–10 loose fruits are randomly sampled between intervals of at least 3 months from the previous sampled palm (Rao 1987).

Vegetative Measurements The vegetative characters measure the physiological and vegetative growth of a palm. The non-destructive method of vegetative measurements (VM) as proposed by Corley and Breure (1981) is carried out 8 years after field planting.

13.4.4 Utilization

An important strategy in oil palm breeding program is to introduce exotic germplasm to increase genetic variability. The oil palm germplasm collections conducted by MPOB showed significant phenotypic diversity for valuable agronomy performance such as breeding for shorter palms, fruit shell thickness, high bunch number, high iodine value, high kernel, as well as high carotene and vitamin E contents. Selected accessions have been utilized to create superior *dura* and *pisifera* fruit type parental palm varieties based on phenotypic data (Corley and Tinker 2003; Rajanaidu et al. 2000). Palms that showed interesting traits were chosen from the germplasm collections and disseminated to members of the industry (Table 13.5).

13.4.5 Conservation

The management of human use of the biosphere is referred as conservation which may produce the greatest sustainable benefit to current generations while retaining its potential to meet the needs and aspirations of future generations. Ex situ and in situ conservation are the two basic approaches to conserve plant genetic resources. In situ conservation is protecting germplasm in its natural habitats. In contrast, ex situ conservation refers to preservation outside its natural habitats (Engelmann

Table 13.5 Planting materials and breeding populations transferred by MPOB to the industry

MPOB series	Characteristics	Year of transfer
PS1	Elite oil palm planting materials (dwarf)	1992
PS2	Planting materials with high iodine values	1992
PS3	Breeding for high kernel planting material	1996
PS4	High carotene *Elaeis oleifera* planting material	2002
PS5	Breeding populations selected for thin shell *teneras*	2003
PS6	Breeding populations selected for large fruit *duras*	2003
PS7	High bunch index breeding population	2004
PS8	High vitamin E breeding population	2004
PS10	Breeding populations selected for long stalk	2006
PS11	High carotene *E. guineensis* breeding population	2006
PS12	Breeding population for high oleic acid palm oil	2006
PS13	Breeding populations selected for low lipase	2008
PS14	Oil palm breeding population selected for high protein kernel	2015

1991). Maxted et al. (1997) reported that ex situ conservation includes methods like field gene banks, seed storage, pollen storage, in vitro storage and botanical gardens, while the in situ approach includes genetic reserves, on-farm and home garden preservation.

As MPOB harbors the largest oil palm germplasm collection in the world (Rajanaidu 1994; Rajanaidu and Jalani 1994), every effort must be made to preserve these resources for posterity. The main role of the MPOB gene bank is to guarantee the preservation as well as consistent availability of genetic resources for oil palm improvement. With high variability in the available material in the gene bank, MPOB strives to improve on high-quality breeding material for the oil palm industry. Ex situ living collection (field gene bank), cryopreservation and DNA gene bank methods are used to conserve the oil palm germplasm.

Ex Situ Living Collection The bulk of the oil palm genetic resources in MPOB are conserved in the form of an ex situ living collection (field gene bank). In a field gene bank, oil palm genetic resources are kept as live plants. The material in field gene banks is always readily available for characterization, evaluation, utilization and research, whereas germplasm preserved in the form of seeds or in vitro cultures must be germinated or regenerated and grown before it can be utilized. However, the preservation of germplasm in the form of field gene bank makes maintenance efforts very expensive. Apart from being exposed to diseases and extreme climate conditions, the materials need to be regenerated every 25 years (Williams and Hsu 1970). For these reasons, germplasm collections in the field are unsuitable for long-term conservation. Nowadays, in vitro culture techniques (cryopreservation) for long-term conservation has been developed as an alternative and complementary method for more efficient long term conservation.

Cryopreservation Cryopreservation is a long-term storage technique to preserve biological materials without deterioration at an extremely low temperature (−196 °C) for an unlimited period of time; this is a widely used method to conserve plant genetic resources (Engelmann 2000). Plant cryopreservation began with research on cryopreservation of mulberry twigs (Sakai 1965). Numerous studies on other plants successfully cryostored for conservation have been reported (Bajaj 1984; Makeen et al. 2005; Normah et al. 1994; Radhamani and Chandel 1992). This method has been applied for the preservation of many different types of plant material such as cells, shoot apices, protoplasts, seed, somatic embryos or excised zygotic embryos (Engelmann 2004). Cryopreservation which is low maintenance and requires limited space has turned out to be an essential tool for long-term storage of germplasm materials. It seems to be the most feasible method for storing recalcitrant seeds (such as those of the oil palm) and species that are vegetatively propagated.

Presently, the in vitro technology for the conservation of oil palm germplasm is concentrated almost exclusively on the cryopreservation techniques of somatic embryos (Engelmann et al. 1985), zygotic embryos (Grout et al. 1983) and pollen (Tandon et al. 2007). An initial report on oil palm cryopreservation was published by Grout et al. (1983). Zygotic embryos extracted from seeds were partially desiccated under laminar flow and successfully preserved in nitrogen. Dumet et al. (1993) reported that dehydration could be applied either by placing embryos for 0–10 h under laminar flow or in air-tight boxes containing silica gel for 0–18 h. Tandon et al. (2007) reported the practicality of cryogenically storing oil palm pollen for long periods without any significant loss in viability and germination rate. Cryopreservation of pollen grains for up to 8 years maintained as high as 54 ± 1.72% viability (compared with 62 ± 4.33% before storage) and 49 ± 1.2% in vitro germination rate (compared with 52 ± 2.08% before storage).

Many elements such as status of the source plant, personnel, materials, culture conditions, cryopreservation techniques, pre-treatment conditions, cryogenic facilities, organizations and post-thawing are involved for successful cryopreservation (Reed et al. 2004; Reinhoud et al. 2000). Cryopreservation of oil palm zygotic embryos is currently stored in the cryotank in MPOB. More than 68,000 zygotic embryos of accessions from Angola, Cameroon, Congo-Kinshasa, Gambia, Ghana, Guinea-Conakry, Madagascar, Nigeria, Senegal, Sierra Leone and Tanzania and are conserved. Zygotic embryo is preferred for oil palm cryopreservation because of its comparatively smaller size as compared to seeds and kernels. Larger tissue are more constrained by desiccation and freezing sensitivity. Similar successes in cryopreservation using zygotic embryos were reported in coffee (Abdelnour-Esquivel et al. 1992), rubber (Sam 1999) and coconut (Sisunandar et al. 2010).

DNA Gene Bank DNA banking is a simple, efficient and long-term method in oil palm conservation which provides a new resource for the readily available DNA. Reference samples for analyses of parentage or particular genotypes/crosses verification are some of the great potentials of these DNAs. Leaf samples are processed, packed and put into two plastic bags (20 grams each) and kept in −80 °C

freezer until needed. Meanwhile, DNA is extracted from oil palm spear leaves and stored at −4 °C in 1.5 ml Eppendorf tubes and labeled properly. Leaf and DNA samples are stored in two locations to minimize the risk of damage due to mechanical disturbances and other failures. The CTAB DNA extraction method is used and prior to storage, the quality of each DNA sample would be evaluated by restriction enzyme digestibility test (*EcoR1* and *HaeIII*). The quantity of DNA is also measured through absorbance readings by spectrophotometer (at A260/A280 ratio) to be within the range of 1.6–2.0 (Rahimah et al. 2006; Suzana et al. 2015).

13.5 Molecular Breeding and Genomics

13.5.1 Molecular Markers

Conventional plant breeding involves a series of stages from designing an appropriate breeding method, generation of genetic variability, parent selection and hybridization, selection of the best progenies, fixing and stabilization of preferred genotypes to the final stage of commercialization (Soh 2011). Approximately 15 years are required to evaluate one cycle of oil palm performance due to the long generation time. To speed up the process, selection of desired traits at nursery stage has often been explored through oil palm genomics and molecular breeding. DNA-based selection will be more reliable than phenotype-based conventional selection because variation in field traits is affected by environment and genotype as well as their interaction (G × E) (Corley and Tinker 2016). The association between molecular markers and specific genes controlling various traits can now be identified to speed up breeding programs via marker-assisted selection (MAS) which is undeniably more efficient than field phenotyping in terms of effort, time and space. Genomic tools have been developing very rapidly, from the origination of classical markers used in oil palm studies in the 1980s to more advanced markers being developed at present and regularly utilized in molecular studies.

13.5.2 Types of Molecular Markers

Molecular studies on the oil palm began with heredity studies using approaches based on the separation of protein markers (isozymes) (Ghesquiere 1984, 1985; Hayati et al. 2004). This type of marker, however, has certain limitations, such as the different enzymes present within and between tissues results in difficulties to obtain comparable samples from different plants. Due to this drawback, most researchers at that time progressed to DNA markers which are more reliable. Early work used several markers such as restriction fragment length polymorphisms (RFLPs) (Maizura et al. 2006; Mayes et al. 1997), rapid amplification of polymorphic DNA

(RAPD) (Rival et al. 1998; Sathish and Mohankumar 2007) and amplified fragment length polymorphisms (AFLPs) (Ying et al. 2007) are all based on polymerase chain reaction (PCR). A few years ago, microsatellite markers or simple sequence repeats (SSRs) took their place and are used widely in oil palm research (Hama-Ali and Tan 2014). SSRs have been exploited for genetic diversity analysis by Zaki et al. (2012), Zhou et al. (2015) as well as by Okoye et al. (2016). SSRs are also very useful for DNA fingerprinting in which the legitimacy of the crosses are verified to confirm their identity in reducing contamination during field planting.

Currently, an advanced marker called single nucleotide polymorphisms (SNPs) is commonly used in oil palm genetics and molecular breeding. SNPs are variations in a single nucleotide that occur at a specific position in the genome and are very powerful in the study of genetic diversity and evolutionary relationship, population structure, genome-wide association studies (GWAS), association mapping (AM) and genomic selection (GS) (Parida et al. 2012). The application of SNP genotyping has been favorable compared to other markers due to its abundance, robustness, high genetic stability and amenability to high-throughput genotyping analysis (Vignal et al. 2002).

13.5.3 Oil Palm Genome Sequence

A major breakthrough in oil palm genomic studies was the establishment of the complete 1.8-gigabase (Gb) African oil palm *Elaeis guineensis* genome sequence as shown in Fig. 13.7 (Singh et al. 2013a) which paves the way for mining the desired genes associated with valuable traits. Evidently, the SHELL gene was successfully identified, mapped and found to control a monogenic trait for the determination of *E. guineensis* fruit form, namely *dura*, *pisifera* and *tenera* (Singh et al. 2013b). This finding led to development of the SureSawit™ *SHELL*, and commercialized to reduce *dura* contamination in the field by screening fruit forms at the nursery stage. This kit can help smallholders and commercial plantations plant only D × P types and reduce cost from growth and maintenance of illegitimate palms.

Following that, SureSawit™ *VIR* Kit was also developed for the identification of *virescens* (Vir) palms instead of planting *nigrescens* (Nig) (Singh et al. 2015). Mature Vir bunches are more easily identified by the harvester due to fruit color changes from green when unripe, to orange when ripe. This can help to overcome the problem of harvesting under- or over-ripe bunches which contributes to poor oil productivity. Precision in harvesting time thus helps to avoid obtaining lower quality oil of unripe bunches and reduces the collecting of loose fruits from over-ripe bunches (Jack et al. 1998). With the integration of genomic tools, superior breeding materials could help enhance oil palm production by close to 33% without an expansion of area under cultivation. It is foreseen that it will help to increase sustainability of oil palm plantations to reduce the adverse effects in palm oil plantation and palm oil-producing regions.

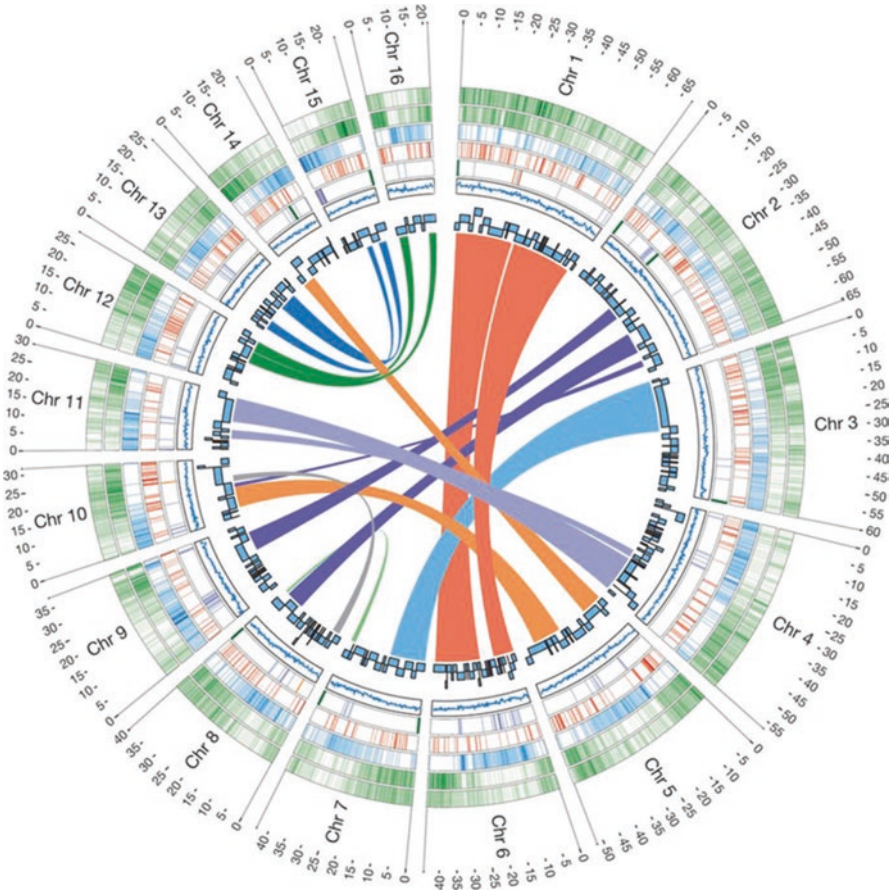


Fig. 13.7 The chromosomes of oil palm *Elaeis guineensis* which correspond to 16 linkage groups identified by genetic mapping. (Source: Singh et al. 2013a)

13.5.4 Linkage Mapping

The foundation in genetic analyses of quantitative traits loci (QTL) is the construction of linkage maps. A large number of markers are used to screen a large population of sibs (ideally 100 samples) to identify markers mapped on the same chromosome and tending to be inherited as a group.

The first QTL map in oil palm was constructed using RFLP (Mayes et al. 1997), AFLP (Chua et al. 2001), cDNA-RFLP (Singh et al. 2009) and SSR (Billotte et al. 2010). Some genetic maps were also created by a combination of AFLP, RFLP and SSR markers (Billotte et al. 2005; Seng et al. 2011; Singh et al. 2009). QTLs were successfully identified for FFB yield, oil to bunch (O/B) and vegetative traits (Jack et al. 1998; Jeennor and Volkaert 2014; Rance et al. 2001). MPOB found significant markers linked to carotene and oleic acid content (Rajinder et al. 2001) and also

fatty acid composition (FAC) (Singh et al. 2009). In tissue culture studies, Low et al. (2008) found markers associated with oil palm callogenesis and embryogenesis. Utilization of existing oil palm maps, however, is still restricted in breeding due to low marker density with big gaps between markers and QTLs (Ting et al. 2014). Thus, improvement was made by developing high density SNP and SSR-based genetic maps which were demonstrated later in the identification of significant linkages to IV and FAC (Ting et al. 2016). Due to yield losses encountered due to infection by *Ganoderma* spp., research agencies have increased their efforts to detect markers for *Ganoderma* resistance. Four loci were discovered by Tisné et al. (2017), whereby two loci were responsible in controlling the emergence of the early symptoms of *Ganoderma* attack, and the other two control the death of the palm. These loci provided significant information for use in MAS application for *Ganoderma* resistance.

Despite all the advantages, QTL mapping has certain limitations. Its resolution is restricted to suitable crosses with low polymorphism, limited number of alleles and small population sizes. Thus, its utilization is inappropriate for the search for genotypic-phenotypic correlations among unrelated populations. Linkages among independent populations were explored by linkage disequilibrium (LD) mapping or alternatively known as association mapping (AM) (Soto-Cerda and Cloutier 2012). AM offers higher mapping resolution with more extensive numbers of alleles and improving association between markers and traits (Flint-Garcia et al. 2005). Recently, Chuenporm and Volkaert (2017) identified two SNP markers with significant linkage to oil palm yield. Babu et al. (2017) also found markers associated with phenotypic data such as oil yield (OY), oil to dry mesocarp (O/DM) and fruit to bunch (F/B).

13.5.5 GWAS, GS and GBS

SNP markers were further utilized in a relatively new technique known as genomic wide association studies (GWAS) and genomic selection (GS). The few limitations of QTL mapping as previously described were resolved via GWAS as it provided higher resolution and used fully-studied and well-known materials for genotypic-phenotypic variation. In a comparison study on phenotypic selection using marker-assisted recurrent selection (MARS) and GWAS on small population size, the latter was proven to be more efficient and showed greater response in terms of gain per unit cost and time. GWAS has also been implemented to predict GS accuracy (Cros et al. 2015) and to select higher oil content (Bai et al. 2017) in oil palm. Figure 13.8 shows a comparison between year taken to make selection using GWAS, MARS and conventional phenotypic selection in oil palm breeding.

GS is a method expanded from MAS which utilizes the abundance of markers in the genome to precisely define breeding values of complex traits without accurate knowledge of their specific location (Goddard and Hayes 2007). GS enables early selection of elite materials and increases genetic gain per unit of time (Kwong et al.

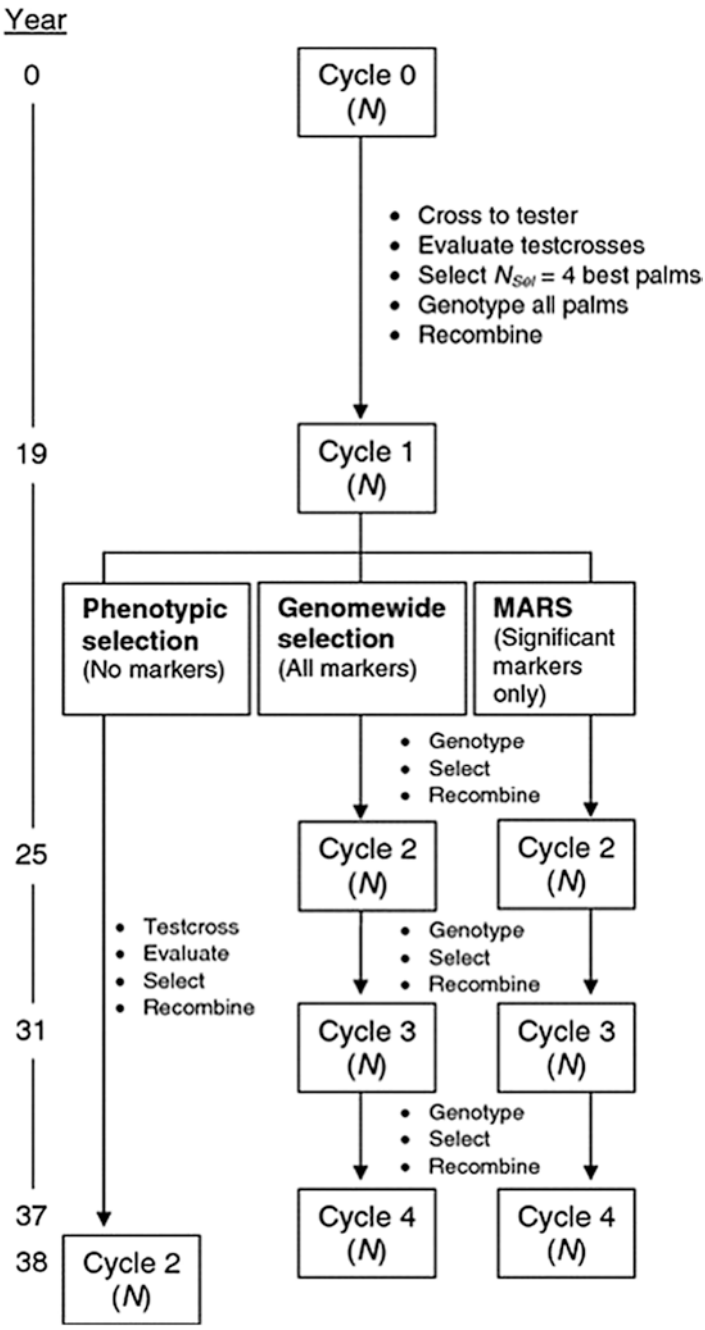


Fig. 13.8 Comparison between year taken to make selection using GWAS, MARS, and conventional phenotypic selection in oil palm breeding. (Source: Wong and Bernardo 2008)

2017) with an adequate GS accuracy in selecting candidates. In perennial crops like oil palm, it is useful to shorten breeding cycles and increase selection intensity (Cros et al. 2015) for phenotypic selection. However, information on its reliability is still limited as GS has not yet been extensively implemented in oil palm genome research.

Recently, linkage mapping, AM, GWAS and GS were performed using a next-generation sequencing method called genotyping by sequencing (GBS). GBS was first explained by Elshire et al. (2011) and is practical to study populations with complex genomes such as oil palm. Some of the advantages include; (i) reduced sample handling, (ii) cost effective, (iii) lower ascertainment bias than arrays, (iv) able to detect other variants such as small insertions, deletions and SSRs and (v) allows comparable analyses among samples even without a reference genome. GBS is also suitable for various analyses such as screening backcross lines, purity testing and AM. Three QTLs associated with palm height and one QTL for bunch weight were successfully discovered by Pootakham et al. (2015) using GBS approach.

13.5.6 Genome Editing

Natural breeding has long been practiced for the production of genetically modified (GM) plants to improve oil yield and quality, but requires large planting areas, and incurs high cost over a prolonged period of time. As the technology evolved, a new approach known as genome editing (GE) was introduced. GE is an improvement program that involves precise alteration of targeted genes in various cell types and organisms (Sattar et al. 2017). DNA insertion, deletion and replacement in the genome were modified using molecular scissors that are actually engineered nucleases. These nucleases are available in various forms with respective pros and cons. Initially, zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) were regularly used in GE technology. However, the latest contemporary clustered regularly interspaced short palindromic repeats (CRISPR) along with CRISPR-associated protein 9 (Cas9) are currently favored as they provide several benefits over the former.

GE technology allows for a larger scope of traits that could be modified and the determination of key genes that control complex traits by genomics tool will be more straightforward. Soh et al. (2017) optimistically predicted that GM crops will be accepted by societies and might be commercialized before early 2021. GE studies in oil palm are still considered in their infant stage but progressing forward towards the prospects of providing transgenic oil palm in local and international markets.

13.6 Tissue Culture

13.6.1 Introduction

Continuous yield improvement was attained through conventional breeding supported by agronomic principles (Kushairi et al. 2010). However, challenges in availability of arable land and shortage of labor faced by the industry have put limitations on conventional breeding in improving yield. Corley (2006) predicted that the potential palm oil yield could reach up to 18 mt ha^{-1} with an estimation of 50% oil to bunch (O/B), 80% fruit to bunch (F/B), 95% mesocarp to fruit (M/F) and 65% oil to wet mesocarp (O/WM). The switch in using oil palm *tenera* (D × P) planting materials from *dura* in the 1960s has boosted yields up to 30%. Unfortunately, for more than two decades, oil yield performance has stagnated at 3.5–3.9 mt ha^{-1} $year^{-1}$ (Kushairi et al. 2010). Due to limited land resources, the ideal approach to increasing yields is through increasing productivity per unit land area.

Through the latest advances in biotechnology, oil palm tissue culture techniques, combined with the ability to transfer genes from one parent to another using genetic engineering, can be one of the efforts to facilitate plant breeding as well as overcoming the above limitations. In tissue culture, large production scales of superior palms (ramets) carrying the same genetic material as their ortets are produced with theoretical yield advantages. Thus, clonal palms with high oil yield potential would increase oil yields without increasing arable land area and meet the Malaysian national aspiration of 35 mt ha^{-1} $year^{-1}$ of FFB (Tarmizi et al. 2011). Oil palm propagation via tissue culture was recommended by Jones (1974) in the 1970s and the tool is now actively implemented to speed up breeding programs.

13.6.2 Production and Performance of Clones

Annually, the world production of oil palm tissue culture plantlets is approaching three million. In 2010, oil palm ramet production in Malaysia was 2.5 million annually (Kushairi et al. 2010). Recently, almost five million ramets were produced annually from 14 licensed laboratories for local supply. Besides its uniformity, clonal yields are at least 20% more than the seedling plants (Khaw and Ng 1998; Kushairi et al. 2010). According to Soh (1986), clonal oil palm materials tend to increase yields by at least 10–15% under good agricultural practices (GAP).

Previously, the main limitation in cloning oil palm via in vitro production was a floral abnormality which affects palm oil production. There are several possible causes of abnormal development including somaclonal variation, explant source, age of culture, hormonal factors, habituation, genotypic factors and DNA methylation. In 2015, prediction and culling of possible mantling at the plantlet stage has been possible through exploiting the epigenetic information of *mantled* fruit, which is a floral abnormality condition of oil palms produced by in vitro cloning

(Ong-Abdullah et al. 2015). This breakthrough facilitates the establishment of superior clones and optimize land resources. In 2017, clonal planting materials, namely CPS1 (Clonal Palm Series 1), were developed featuring potential FFB yields of more than 30 mt ha^{-1} $year^{-1}$, O/B exceeding than 30% and oil yield ranges between 8–10 mt ha^{-1} $year^{-1}$ (Tarmizi et al. 2017). The outstanding palm from CPS1 was selected and used as an ortet for cloning. At a planting density of 198 palm ha^{-1}, CPS2 (Clonal Palm Series 2) displayed the potential of achieving 35.7 mt ha^{-1} $year^{-1}$ FFB with an oil yield of 10.8 mt ha^{-1} $year^{-1}$. This clone can improve productivity via a higher planting density. Besides, CPS2 has exhibited almost zero mantling (Samsul et al. 2018).

13.6.3 Tissue Culture Protocol

The earliest successful record of oil palm tissue culture was in the 1970s (Jones 1974; Rabechault and Martin 1976) leading to exploration of in vitro propagation from other organizations and was subsequently widely implemented to hasten their breeding programs. Efforts to improve the oil palm in vitro propagation protocols have been reviewed by Rohani et al. (2000, 2003). Tissue culture is not only limited to producing high-yielding oil palm materials, but it also used to produce superior lines with outstanding characteristics such as compactness, high bunch index, dwarf, tolerance to biotic and abiotic stresses, *virescens* fruit, etc. (Zulkifli et al. 2017). Thus, the goals to producing oil palm clones would rely on good breeding programs.

Due to low heritability, selection of individual palms will not contribute towards oil yield improvement (Soh et al. 2006). In fact, efficiency of cloning comes from the selection of high oil-yielding ortets from superior progenies. However, palms with higher bunch numbers and oil to bunch are more favorable as these parameters are more heritable (Syed Alwee et al. 2010). To ensure the quality of ramets, criteria for ortets selection were encoded by Department of Standards Malaysia as a standard MS 2099:2008 (SIRIM 2008). Data collection for FFB and O/B on individual palm basis was recorded from a minimum of 30 palms per trait for a period of four consecutive years. By following the proper Standard Operating Procedure (SOP), cloning planting materials can contribute 25% more yield compared to commercial D × P (Rohani et al. 2000).

Sampling of explants from desired palms with actively growing tissue or ortets is a crucial criteria (Elkington 1984). Normally, the explants used are young leaves, inflorescences or the tips of tertiary roots. Compared to roots, using leaves as explants requires no severe disinfection and reduces the risk of error. In addition, all palms produce callus from leaf explants, with rates ranging from 7–60% (Rival 2000; Wong et al. 1999).

The selected tissue sampled from mature oil palm must be immature, enclosed within a cylinder or cabbage of the leaf petiole. They are cultured on medium and subcultured every 3 months. After about a year, formation of an embryoid is initiated

by selecting the primary callus and transfer to another medium. For about a year, the transfer process would be repeated every 3 months to give cells time for embryogenesis. The embryoids expand and produce more compact clumpy, nodular structures called polyembryogenic (PE) cultures. However, the main inefficiency in tissue culture is that the embryogenesis rate in callus cultures was around 5% (Syed Alwee et al. 2010). The embryoid clumps are then subdivided into 1.5–2 cm diameter and subcultured many times for a long period. PE cultures are maintained on nutrient medium and subcultured bimonthly. Parveez et al. (2012) reported that PE subculture cycles are limited by up to 15 times (about 30 months).

13.6.4 Innovations in Oil Palm Tissue Culture

Although tissue culture is advantageous compared to the conventional genetic improvement approach, this method is also highly demanding in terms of cost and labor. The entire operation requires dedicated laboratory facilities with a sterile and controlled environment to accommodate and protect the cultures. Considering its sensitivity, a primary requirement is highly skilled workers. Hence, MPOB has taken a keen interest in improving the efficiency of the tissue culture process. Some of the improvements listed below (Fig. 13.9).

Double-Layer Rooting Technique The liquid medium is poured directly onto the established cultures, instead of transplanting it individually into a new medium (Zamzuri 2001). By implementing this method, the number of workers to handle individual shoots can be reduced and this simultaneously increases the working capacity by as much as 17.5 fold at the rooting stage. If test tubes are replaced with larger culture vessels, the required culture room area can be reduced by 2.6 fold. In addition, an 80% decrease on annual variable cost on wages and culture medium can be obtained.

Flameless Sterilizer Conventionally, tissue culture glass containers and surgical apparatus are sterilized by flaming. An electrical device, a flameless sterilizer was invented which contains a very hot coil that allows heat dispersion at maximum temperature of 300 °C (Zamzuri 2002). The use of the generated heat can be optimized by dual surface sterilization on the mouth parts of the vessel and surgical apparatus.

Liquid Culture The liquid culture method for oil palm also has been developed by MPOB as an alternative to wholly using solid culture. It involves a series of steps from selecting a good callus (friable type), formulation of the media, sieve analysis of aggregates, maturation initiation, embryoid regeneration and production of rooted plantlets in either solid or liquid medium (Tarmizi et al. 2011). The system is highly reproducible, versatile, efficient and offers prospects for automation as an

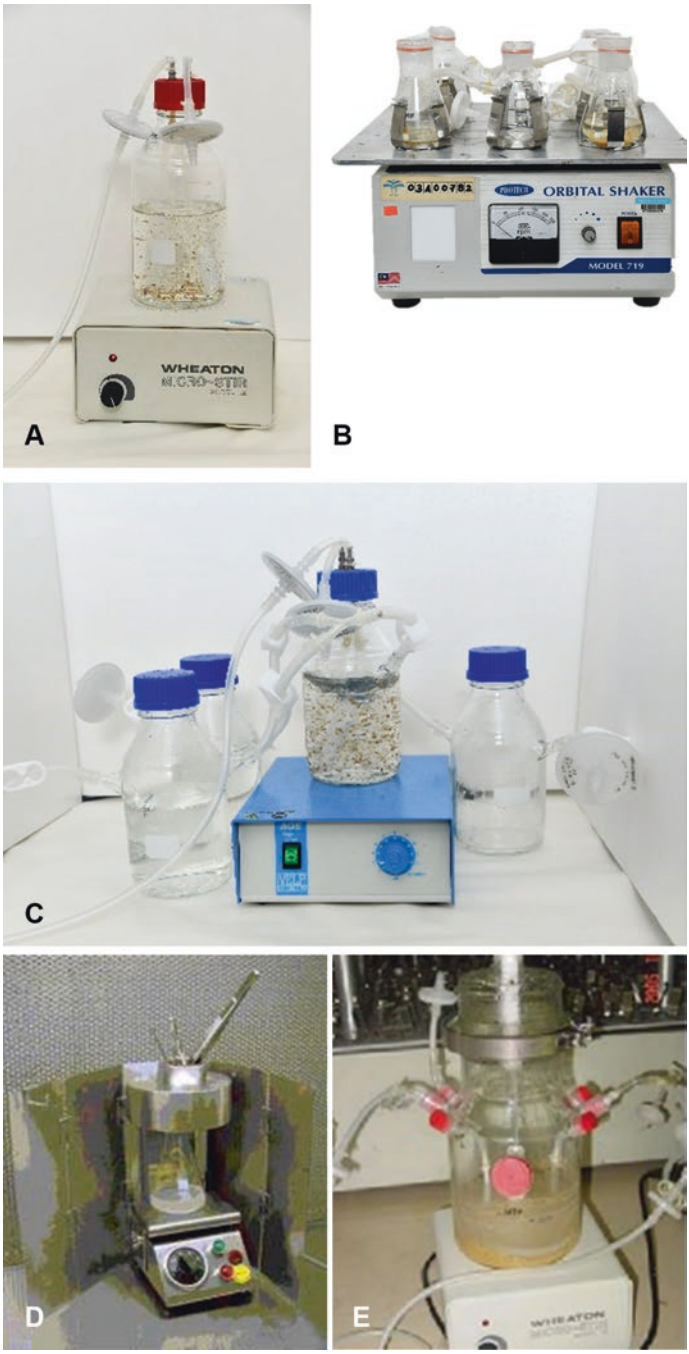


Fig. 13.9 MPOB innovations in oil palm tissue culture. (**a**) The 2-in-1 MoSlim, (**b**) MPOB Fast Transfer Technique (MoFaTT) in liquid culture system, (**c**) Simple Impeller with Fast Transfer Technique (SLIM-FaTT) in liquid culture system, (**d**) Flameless sterilizer, (**e**) MPOB Modified Vessel (MoVess) for liquid culture system

added value (De Touchet et al. 1991; Tarmizi 2002; Teixeira et al. 1995; Wong et al. 1999).

MPOB Fast Transfer Technique (MoFaTT) in Liquid Culture This technology allows for a convenient and rapid replenishment of liquid medium throughout the maintenance and maturation of oil palm suspension cultures. Moreover, it reduces the risk of contamination. This technique also simplifies medium replenishment and has the added potential for automation (Tarmizi and Zaiton 2005).

Bioreactors Bioreactors have been successfully used to multiply suspension cultures of different plant species (Okamoto et al. 1996; Yu et al. 2000). They can be monitored via online and growth conditions in six independent reactor vessels i.e. temperature, agitation and dissolved oxygen can be controlled (Tarmizi et al. 2011). Therefore, the bioreactor is the preferred alternative compared to the flask system when rapid large-scale proliferation is required (Tarmizi and Zaiton 2005). However, several cost effective apparatuses have been developed by MPOB as alternatives to bioreactors.

2-in-1 MoSlim MPOB simple impeller for liquid culture was developed to further enhance the liquid culture process. This led to the establishment of the two system combo, the Simple Impeller with Fast Transfer Technique (SLIM-FaTT) (Tarmizi and Zaiton 2006a)**.** Further improvements include a modified impeller for agitation of the liquid culture system (Tarmizi and Zaiton 2006b).

Oil Palm Tissue Culture Tracking System (OPTRACKS) Detailed information on the tissue culture process from ortet selection until the hardening and conditioning in the nursery can be tracked and safely stored through OPTRACKS. Culture vessels and nursery plants are tagged with bar codes containing the production batch and sequence number (Sambanthamurthi et al. 2009). Such information can be easily retrieved by scanning the code for fast tracking. This system has now been developed for DNA testing using RFLP (Cheah et al. 2002) and SSR probes (Rajinder et al. 2006).

13.6.5 Accelerating Breeding Programs via Tissue Culture

In oil palm plantations, cloning of selected *tenera* palms is considered the most promising approach in the direction of producing more uniform and high-yielding materials. Undoubtedly, tissue culture is a strategy adopted to accelerate improvement of planting materials in breeding programs. In MPOB, the large germplasm collection consisting of *Elaeis guineensis*, along with *E. oleifera* collected from Central and South America, is continuously evaluated for outstanding economical traits (Mohd Din et al. 2005a, b).

Ortets are regularly selected from D × P or D × T progeny trials where performance data are statistically complete and accessible (Yong and Mustafa Kamal 2005). For *pisifera* improvement programs, ortets are usually sought from T × T or T × P crosses (Soh 2010). Another way of cloning is through recombination program from the best progenies and parents. Meanwhile, cloning from seedlings of elite D × P may add to the pool for selection of ortet palms recommended by Soh et al. (2001) due to the low yield heritability between palms. In certain cases, the highest yielding commercial materials are also selected as ortets.

The technology for semiclonal and biclonal seed production was developed to overcome the insufficient supply of parental materials (Sharma 2006; Yong and Mustafa Kamal 2005). *Dura* and/or *pisifera* palms are cloned as parents and crossed to produce seeds through conventional D × P seed production. Semiclonal seeds are produced when one of the parents is a clone and biclonal seeds are produced when both parents are clonal palms. Sharma (2006) featured several positive sides of this type of seed production. Besides having a greater degree of uniformity, producing clonal seeds is more cost effective and with a lower risk of abnormal *mantled* fruit compared to tissue cultured plants.

13.7 Genetic Engineering

13.7.1 Introduction

Direct introduction of specific genes of interest into a genome can be made through genetic engineering, with no time required to grow and cross the plant (Parveez et al. 2011). The genes of interest may involve any genes from any species and are not limited only to compatible species of choice. Genetic engineering also allows a modification to be carried out with higher precision than with breeding. Thus, selection of desired palms with a reduction of time and cost is possible. Improvement of the precision and restriction in the amount of gene transfer may also broaden the genetic base of the oil palm (Sambanthamurthi et al. 2009).

Research associated with oil palm genetic engineering was initiated in 1987 in MPOB. The early aim of the research was to modify oil composition to increase oleic acid through palmitic acid (Cheah et al. 1995). The other targets are to produce novel and high-value products as well as oil quality modification which includes the oil palm producing high stearic acid, palmitoleic acid, ricinoleic acid and biodegradable plastics. Transgenic oil palms for the oleochemical industry, lubrication and nutraceuticals are also being produced for niche markets (Parveez et al. 2012).

Collaboration and coordination involving various research disciplines is needed in the genetic engineering program. Genes and promoters as well as the oil palm transformation methodology is carried out by the molecular biologists. Regeneration of the transformed tissues is done by the tissue culturists while field evaluation will be supported by the breeders. Parveez (2000) reported that particle bombardment is

the current method and routinely used for transforming oil palm. In oil palm engineering, Parveez et al. (2012) summarized the works into several sections including isolation of gene and promoter, development of transgenic oil palm as well as the production of value-added transgenic oil palm.

13.7.2 Gene and Promoter Isolation

In altering the oil composition, regulatory sequences that control tissue and temporal expression are required for in vitro manipulation. A number of key genes in lipid biosynthesis have been discovered from metabolic studies and subsequently isolated as shown in Table 13.6. For successful targeting of transgenes into specific tissues for oil synthesis, promoters with both spatial and temporal specificities are essential. Meanwhile, promoters with temporal specificity ensure optimal timing of the transgene expression. Both leaf and root-specific promoters have also been isolated to express genes for pest and disease resistance. Table 13.7 lists the promoters isolated from oil palm (Parveez et al. 2015).

13.7.3 Transgenic Oil Palm Development

High Oleic Acid Oleic acid accounts for 39% of the total FAC in oil palm and is mainly used as a feedstock in the oleochemical industry. Prior to any modification in the oil palm to produce high oleic acid, regulation of fatty acid biosynthesis must be well understood. Palmitic acid accounted for 44% of the total fatty acids whereby it is the most dominant fatty acid in palm oil. Hence, silencing of the palmitoyl-ACP

Table 13.6 Lipid biosynthetic genes isolated from oil palm

Genes	Functional analysis	References
Ketoacyl ACP synthase II (KAS II)	*Escherichia coli* and *Arabidopsis thaliana*	Ramli et al. (2012)
Palmitoyl-ACPthioesterase	*A. thaliana*	Abrizah et al. (2000) and Parveez et al. (2010)
Stearoyl ACP desaturases	*A. thaliana*	Safiza et al. (2009a) and Siti Nor Akmar et al. (1999)
Oleoyl-CoA desaturase	Desaturase	Syahanim et al. (2007)
Acetyl-CoA carboxylase (ACCase)		Omar et al. (2008)
Lysophosphatidic acid acyltransferase (LPAAT)	*A. thaliana*	Manaf et al. (2005)
Glycerol 3-phosphate transferase (GPAT)	*A. thaliana*	Safiza et al. (2009b)
b-Ketothiolase		Teen et al. (2008)

Table 13.7 Tissue specific and constitutive promoter isolated from oil palm

Tissue	Function	References
Mesocarp (fruit) specific promoter	Transient expression in oil palm mesocarp and leaves	Siti Nor Akmar and Zubaidah (2008)
Root-specific promoter	*Arabidopsis thaliana*	Zubaidah and Siti Nor Akmar (2005)
Leaf-specific promoter	Transient expression on mesocarp and leaves of *Arabidopsis thaliana*	Chan et al. (2008)
Transient expression in various oil palm and tobacco tissues	Ubiquitin extension protein gene (*uep1*)	Masura et al. (2010)
Translationallycontrolled tumor protein (TCTP)	Transient expression in various oil palm and tobacco tissues	Masura et al. (2011)

thioesterase and improving KASII activities are the likely targets. Enhancing stearoyl-ACP desaturase, however, is not needed as the oil palm already has its active component (Sambanthamurthi et al. 1996, 2002). Biochemical studies conducted to explore the factors causing palmitic acid accumulation in oil palm mesocarp and ß-ketoacyl-ACP synthase II (KAS II) and palmitoyl-ACP thioesterase were identified as the enzymes accountable for palmitic acid accumulation (Sambanthamurthi et al. 2009). Using biochemical information, three strategies are suggested; (i) up-regulate KAS II, (ii) down-regulate palmitoyl-ACP thioesterase, and (iii) down-regulate oleoyl-CoA desaturase (Sambanthamurthi et al. 2000). In order to avoid the spillover of oleic acid to linoleic acid, down-regulation of the oleoyl-CoA desaturase has also been taken into account. The reduction of linoleic acid content from 55% to 3% leads to incremental increase of oleic acid content from 21.5% to 78.9%. The same strategies were applied in cottonseed oil, resulting in higher oleic acid content, from 15% to 78% (Liu et al. 2000).

High Stearic Acid Producing palms with high stearic acid is another trait of interest in genetic engineering. This increases diversity in the use of the oil, such as to generate cocoa butter substitutes and products such as shaving cream, lotions and massage oils (Parveez 2003; Parveez et al. 1999). Oil palm contains an active stearoyl-ACP desaturase enzyme. Hence, the conversion of stearoyl-ACP into oleoyl-ACP will be reduced if stearoyl-ACP desaturase activity was down-regulated. An increment of 2–4% of stearic acid content has been observed in cottonseed oil by down-regulating the same gene (Liu et al. 2000) while in rapeseed, an increase from 1.8–39.8% was reported when using the same approach (Knutzon et al. 1992).

High Palmitoleate Oil Palm Palmitoleic acid is produced by desaturation of palmitic acid. Producing transgenic oil palms with high palmitoleic acid would benefit the pharmaceutical sector as it is estimated to have anti-thrombotic effects which purportedly could avoid stroke (Abraham et al. 1989). Currently, the main source of palmitic acid is from macadamia oil (*Macadamia integrifolia*), which includes ~17% palmitoleic acid. Sambanthamurthi et al. (1996) reported that *Elaeis guineen-*

sis protoplasts can synthesize up to 30% palmitoleic acid in their total lipids which indicates the inheritance ability of the palms to produce high levels of palmitoleic acid. Since it is produced via desaturation of palmitic acid, it is anticipated that the desaturase could also convert palmitic acid for the production of palmitoleic acid. A mutant castor Δ9-stearoyl-ACP desaturase will be overexpressed into oil palm to produce palmitoleic acid.

High Ricinoleic Oil Palm Ricinoleic acid is an economically-valuable fatty acid mostly found in castor oil (Van de Loo et al. 1993). It has active drying properties which has been used in producing cosmetics (~40% lipsticks), plastics (mainly nylon 11, nylon 10–10, nylon 6–10), surfactants, deodorants, plasticizers, diesel lubricants and coatings. The synthesis of ricinoleic acid occurs when oleate 12-hydroxylase converts oleic acid to ricinoleic acid and consequently, introducing this gene into oil palm. In transgenic *Arabidopsis* seeds, hydroxy fatty acids (mainly ricinoleic) content was increased up to 25% when oleate 12-hydroxylase was co-transformed with the castor phospholipid: diacylglycerol transferase 1–2 (*PDAT1–2*) gene (Kim et al. 2011). Thus, introduction of castor oleate 12-hydroxylase into oil palm should also contribute to ricinoleic acid synthesis. According to Masani and Parveez (2008), there were three transformation vectors carrying the castor oleate 12-hydroxylase under the control of CaMV35S, ubiquitin, and mesocarp-specific promoter. Transformation of these constructs into oil palm embryogenic calli and glufosinate ammonium-resistant embryogenic calli are being conducted. Regeneration of transgenic plantlets from these resistant embryogenic calli has been accomplished, and the plantlets have been planted in a biosafety screen house.

13.7.4 Production of Transgenic Oil Palm Synthesizing Biodegradable Thermoplastics

Biodegradable thermoplastics such as polyhydroxybutyrate (PHB) and polyhydroxyalkanoate (PHA) are made as a storage material by bacteria under controlled-growth environments (Senior and Dawes 1973). PHB is synthesized in bacteria from acetyl-CoA (Anderson and Dawes 1990) while PHA is an important polymer used in a wide range of environmentally friendly industrial polymers. In oil palm, besides aiming to synthesize the PHB in the mesocarp and leaf utilizing the three bacterial genes driven by mesocarp- and leaf-specific promoters (Masani et al. 2009; Yunus and Kadir 2008), work to enhance the synthesis of biodegradable plastics by an intervention strategy are also explored.

13.8 Conclusion and Prospects

Biotechnological tools are essential for improving the efficiency of oil palm breeding, clonal propagation and to diversify the application of palm oil. Appropriate tools that can help the palm meet and/or exceed its genetic potential are necessary to satisfy the increasing demands for palm oil, especially considering the diminishing availability of arable land, as well as the increase in cultivation costs.

The integration of several disciplines in oil palm breeding (conventional breeding, molecular breeding, tissue culture) together with the future biotechnology tools and vast genetic resources play a fundamental role in accelerating the improvement of current planting materials. The narrowness of the basic gene pool which delayed progress in oil palm breeding can be solved through the exploitation of the oil palm germplasm as proven by the development of new planting materials (PSes) which meet the needs of industry. The latest advancement in oil palm tissue culture technique will not only be able to facilitate the plant breeding program, but can produce faster, more uniform superior palms as well.

The discovery of mutations controlling the shell gene in oil palm fruits and the establishment of SureSawit™ *SHELL* was a major breakthrough in oil palm genomic studies. It was then followed by the development of SureSawit™ *VIR* kit for the identification of *virescens* (Vir) palms. With the availability of linkage (QTL) and linkage disequilibrium (LD) maps related to specific traits of interest (IV, FAC, *Ganoderma* resistance), relevant information can finally be used to guide efficient MAS in a breeding program.

With genetic engineering program in place, broadening the genetic base of the oil palm, reducing time and cost for desired palms selection as well as increasing precision can be achieved. Through introduction of interspecific genes, it was possible to modify oil composition in the direction of increasing oleic acid through palmitic acid, and also increasing stearic acid, palmitoleic acid, ricinoleic acid and development of biodegradable plastics.

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Appendices

Appendix: I Research Institutes Relevant to Oil Palm Genetic Improvement

Institution	Specialization and research activities	Contact information and website
Malaysian Palm Oil Board	Genetically modified oil palm/ gene function and transgenic technology	Dr. Abdul Masani Mat Yunus Malaysian Palm Oil Board No. 6, Persiaran Institusi Bandar Baru Bangi 43000 Kajang, Selangor, Malaysia Telephone: (6)0387693895 E-mail: masani@mpob.gov.my
Malaysian Palm Oil Board	Improvement and generation of elite oil palm planting materials/breeding and quantitative genetics	Dr. Mohd Din Amiruddin Malaysian Palm Oil Board No. 6, Persiaran Institusi Bandar Baru Bangi 43000 Kajang, Selangor, Malaysia Telephone: (6)0387694482 E-mail: mohddin@mpob.gov.my
CIRAD Agricultural Research for Development	Genomic selection in oil palm	Dr. David Cross CETIC (African Center of Excellence in Information and Communication Technologies), University of Yaoundé 1, Yaoundé, Cameroon E-mail: david.cros@cirad.fr
PalmElit SAS	Plant breeding in oil palm	Dr. Tristan Durand-Gasselin PalmElit SAS Parc Agropolis Bat. 14 2214 Boulevard de la Lironde 34980 Montferrier sur Lez France Telephone: (33) 467457927 Fax: (33) 607301953 Email: tristan.durand-gasselin@palmelit.com

Appendix II: Oil Palm Genetic Resources in Malaysia

Cultivar	Species	Important traits
PS1	*Elaeis guineensis*	Dwarf
PS2	*E. guineensis*	High iodine value
PS3	*E. guineensis*	High kernel
PS4	*E. oleifera*	High carotene content
PS5	*E. guineensis*	Thin shell *teneras*
PS6	*E. guineensis*	Large fruit *duras*
PS7	*E. guineensis*	High bunch index
PS8	*E. guineensis*	High vitamin E
PS10	*E. guineensis*	Long stalk
PS11	*E. guineensis*	High carotene
PS12	*E. guineensis*	High oleic acid
PS13	*E. guineensis*	Low lipase
PS14	*E. guineensis*	High protein kernel

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Chapter 14
Safflower (*Carthamus tinctorius* L.) Breeding

Pooran Golkar and Somayeh Karimi

Abstract As an oilseed source, safflower (*Carthamus tinctorius* L.) also contains natural dyes known for their pharmaceutical properties in the treatment of chronic disorders. The production of this herbaceous plant originated in China from which it was dispersed to the Mediterranean countries. The plant is recognized for its use in herbal medicine, birdseed, animal feed, protein-containing ingredients and cooking oil (full of linoleic and oleic fatty acids). Of the linoleic-acid content of safflower, nearly 75%, is vital for a healthy human diet. Due to its adaptability to simultaneously yield oleic and linoleic oils, it is among the substitutes for the common agricultural products raised in marginal farming lands all over the world. No consensus so far has been reached in studies done on safflower. However, it seems necessary to exploit the potentiality of this underutilized plant. The detection and development of novel safflower ideotypes will enhance the suitability of this plant to various prevailing conditions, thereby enabling it to be incorporated into different intercropping practices. Classic genetic studies have an important role in making an educated guess concerning the action of genes and heritability of various agronomic and pheno-morphologic characteristics. Recently, biotechnological developments have contributed to safflower breeding. These practices, however, have not been well supported molecularly. The present chapter articulates different aspects of safflower breeding including conventional breeding methodologies, agronomic performance and biotechnological tools for improving safflower cultivation.

Keywords In vitro · Biotechnology · Gene action · Genetic marker · Flower · Hybrid · Oil seed

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14.1 Introduction

14.1.1 Botanical Classification

Safflower (*Carthamus tinctorius* L.) (2n = 24) belongs to the Cynareae tribe, Tubulifloreae subfamily and Asteraceae family (Ashri and Knowles 1960). Originally referred to as *usfar* and *saffiore*, it gradually changed name to become known as *safflower*. It is mentioned as *kusumba* in ancient scriptures (Singh and Nimbkar 2006). Presently, it has different local names such as kusumba (India and Pakistan); kardai (in Marathi), kusum (in .Hindi), hing hua (China), alazor (Spain) and golrang (Iran). The genus *Carthamus* is comprised of 25 species classified into 5 sections based on chromosome numbers (2n = 20, 22, 24, 44, and 64), in which *C. tinctorius* is the only cultivated type (Weiss 2000). *Carthamus oxycanthus* M. Bieb., *C. glaucus*, M. Bieb. *C. flavescens* Willd. and *C. lanatus* L. are relatives of *C. tinctorius*, growing as weeds (Singh and Nimbkar 2006). Interestingly, according to Knowles (1969), all these plants evolved from a shared ancestor, possibly from northern Iraq and northwest Iran. The safflower plant can be described as an herbaceous annual and long-season crop possessing a bushy habitat containing different primary, secondary and tertiary branches, each bearing a globular flower bunch, called capitulum or head (Fig. 14.1), enclosed with tightly attached bracts (Dajue and Mündel 1996). An individual capitulum has many flowers, numbering between 20 and 250. In each flower, the stigma is surrounded by 5 fused anthers (Singh and Nimbkar 2006). There can be 3–50 capitula per plant, each measuring 1.25–4 cm in diameter (Bradley et al. 1999).

The primary root system of safflower, i.e. taproot, is a 2–3 m extension into the soil, rendering the plant ideal for farming practices depending upon rainfall for

Fig. 14.1 The intact flower of safflower (*Carthamus tinctorius* L.). (Photo: P. Golkar)

watering, compared with others (Hussain et al. 2016; Kizil et al. 2008). Notably, the stem and branches (with 30–70° angles to the main branch) are covered with leaves bearing numerous spines that act as a defense system against insects. The growth habit of shoots in safflower exhibits a narrow branching habit with the angle to the stem of less than 30°. Also, the flower branches extend over the stem with at an angle of more than 75° (Singh and Nimbkar 2006). White, smooth seeds are produced by this plant, also known as achenes, weighing 0.01–0.1 g (Weiss 2000). The seed consists of the hull (33–45%) and kernel (55–65%) (Jaradat and Shahid 2006). Urie (1986) states that the hulls are greatly fibrous, while the embryo is full of protein and oil. The characteristic safflower seed constituents include moisture (5–8%), oil (27–32%), ash (2–7%), crude fiber (32–34%), and protein (14–15%) (Golkar et al. 2011b).

The developmental stages in safflower ripening sequentially include days to emergence, rosette stage (20–35 days) (slow growing period with tolerance to cold stress), stem elongation, branching (appearance of buds and bolls respectively), flowering and maturity (Golkar 2010, 2011; Singh and Nimbkar 2006). Flowering initiates in a capitulum in the outermost floret whorl and moves toward the center within 3–5 days (Singh and Nimbkar 2006). Safflower is a predominantly self-pollinating crop, but outcrossing has been reported in up to 60% in some genotypes (Claassen 1950). In safflower, the flower color and absence of spines in the flowers become important when the crop is grown for floret usage and ornamental aims (Bradley et al. 1999; Golkar et al. 2010). Spine-free varieties have been grown for manual cultivation of seeds and parts of the flower in specific geographic areas (Singh and Nimbkar 2006). Different flower colors (yellow, orange, red and white) have been observed in safflower depending on the major genes that control color inheritance (C, O, Y or R) by different gene actions.

Worldwide, safflower is the eighth most important oilseed crop following soybean, peanut, rapeseed, sunflower, sesame, linseed and castor (Singh and Nimbkar 2006). Nowadays, the crop is commercially grown in Canada, Mexico, India, USA, Ethiopia, Australia, Kazakhstan, Argentina, China, Uzbekistan, the Russian Federation, Spain, Pakistan, Iran and Turkey (Chapman et al. 2010). Safflower, a crop with an underexploited potential and worldwide adaptability, can possibly be grown in virtually all cultivating areas around the world (Hussain et al. 2016); however, it is not recommended for areas with more than 380 mm of annual precipitation. Its worldwide production is greatly restricted because what is known about the development and management of its derivatives is inadequate. According to recent data provided, the world area harvested, average seed yield and total annual production are 1,140,001 ha, 9546 hg/ha and 948,517 mt, respectively. The top safflower seed producers are Russia, Kazakhstan, Mexico and the USA (Table 14.1) (FAO 2016).

Table 14.1 Seed and oil production of safflower based on FAOSTAT in 2016

Country	Area (ha)	Yield (hg/ha)	Production (mt)
Russia	429,730	6664	286,351
Kazakhstan	223,291	7490	167,243
Mexico	65,755	18,518	121,767
USA	62,480	15,978	99,830
Turkey	39,352	14,739	58,000
India	104,822	5056	53,000
Argentina	79,956	6447	51,550
China	22,754	14,325	32,595
Uzbekistan	46,216	6492	30,003
Tanzania	27,821	5245	14,593
Kyrgyzstan	14,191	7791	11,057
Ethiopia	6738	11,805	7954
Iran	5653	11,050	6246
Spain	4536	8113	3680
Australia	4394	5849	2570
Tajikistan	2023	9476	1917
Other countries	289	7241	161
Total	1,140,001	9546	948,517

14.1.2 Safflower Importance

Safflower is considered a multipurpose crop used for oil, industrial, ornamental and medicinal purposes (Golkar 2014; Hussain et al. 2016). Originally, it was raised because of its floral colorants to dress foodstuffs and prepare textile dyes (Singh and Nimbkar 2006). The most important characteristics indicating the coloring properties (in food and cloth) in safflower are the red (carthamin 3–6%) and yellow (carthamidin, 24–30%) contents of the pigments (Golkar et al. 2010). Safflower carthamin is widely used as a form of a stain, colorant in foods such as ice cream, jelly and soup, and as an additive in beverages and cosmetics (Al-Snafi 2015; Singh and Nimbkar 2006). China manufactures carthamin as a red paint for cosmetic dyes and in medicinal preparations. In recent years, safflower petals have been employed as herbal tea to treat, through infusion therapy, to treat vascular system disorders, hypertension, spondylosis, stomach tumors, stimulate menstruation, relieve abdominal pain and heal wounds in different parts of the world, including China and India (Al-Snafi 2015; Singh and Nimbkar 2006). The flower and leaves have high antioxidant properties (Golkar et al. 2009), which are also rich in carotene, riboflavin, vitamin C and all forms of the amino acids except tryptophan. Safflower leaves show anti-cancer and anti-inflammatory effects (Al-Snafi 2015). Safflower plants can be grazed or stored as hay or silage. Due to its high protein content and value, the meal obtained from oil extraction can be employed to feed animals (Golkar et al. 2011b). Varieties having thicker and whiter hulls are used to feed birds (Johnson et al. 1999). As an oil-based product (25–45%) whose seed composition, in terms of oil, greatly

varies in fatty acids, safflower is similar to olive oil (Fernandez-Martinez et al. 1993; Velasco et al. 2005). Different values of oil yield have been reported in germplasm, including 39–44% in California and 28–30% in Chinese genotypes. The seed oil of safflower conventionally contains a profile of fatty acids composed of 2–3% stearic acid, 6–8% palmitic acid, 71–75% linoleic acid and 16–20% oleic acid (Hamdan et al. 2008). Among the commercial oils, the seed oil of safflower is the richest in terms of linoleic acid (Velasco et al. 2005). There are two types of safflower depending on the content of fatty acids, i.e. oleic type and oleic type (Hamdan et al. 2008). Furthermore, there is no linolenic fatty acid in safflower oil, which can easily be oxidized in oils like canola and soybean. Safflower seed oil is widely used in food, medicine, paint and lubricant industries (Golkar et al. 2011b); because it does not cause any allergic sensitivity, it can best be used in therapies involving injection (Al-Snafi 2015). Agriculturalists in India also make use of this oil for soap making, illumination and water-resistant leather buckets (Johnson et al. 1999; Singh and Nimbkar 2006). Recently, safflower has evolved into a new arena in which secondary metabolites and transgenic products, including insulin, γ-linolenic acid, growth hormones and apolipoprotein are produced (Sujatha 2008). Low values of tocopherols, including (α, β and gamma tocopherols) and sterols (campestrol and stigmasterol) are found in safflower oil (Velasco et al. 2005).

14.1.3 Domestication, Selection and Early Improvements

Safflower was grown as a source of pigment to dress food in the eastern Mediterranean and exported to the Middle East (Iran and Iraq) and other Asian countries, including northern China and India over 4000 years ago (Ashri 1975). It has been called the world's oldest crop. Vavilov assumed safflower originated from three major centers: Ethiopia, the Iran-Afghanistan area and India (Harlan 1992). Later, Knowles (1969), however, stated that the crop was mainly cultivated in what he called *centers of similarity*: (1) The Far East (China and Korea), (2) India-Pakistan, (3) Middle East, (4) Egypt, (5) Sudan, (6) Ethiopia and (7) Europe. Iran is traditionally considered as one of the centers in which safflower is richly represented (Knowles 1969). Safflower was initially cultivated in Mediterranean areas and then dispersed (Dajue and Mündel 1996). Domestication, the process by which wild species are selected for human consumption and use, can be used as a model for understanding the genetics of adaptation in safflower. Since the 1940s, safflower has acquired a new use and has emerged as a modern industrial oil crop (Knowles 1989). Three species: *Carthamus persicus* Desf. ex Willd. (Iran and Palestine), *C. oxyacanthus* M. Bieb. (Afghanistan, Pakistan and northern India) and *C. gypsicola* Ljin are postulated as the progenitor stock of the domesticated plant (Singh and Nimbkar 2006). Chapman et al. (2007) suggested that *C. tinctorius* is most likely derived from wild *C. palestinus* RCL Mech. Safflower was commercialized in the Americas in the 1950s, and since then it has widely been in use there as an oilseed product. During the 1950s, Knowles collected a broad selection of germplasm representative of safflower and

its wild relatives (Knowles 1989). To augment oil levels, the hull thickness was reduced through single genes, particularly those related to partial-hull, reduced-hull and striped-hull (Dajue and Mündel 1996). Safflower domestication improved many of the standard traits (e.g. the time of uniform flowering, seed dormancy loss, an increase in the quality and content of seed oil, and increased seed production). Cultivating sunflower has expanded to regions around the world, particularly developing countries.

14.1.4 Cultivation Practices

Sunflower is thought to be a plant which is day-length neutral and long-day in general (Weiss 2000). The plant spacing (20 × 40 cm) and seeding rate are different worldwide, which is partly associated with cultural method, the habits of variety growth and the growing environment. At different phylogenetic stages, safflower is tolerant of low temperatures during the rosette stage and prior to stem elongation (Dajue and Mündel 1996). The temperature of safflower germination can drop as low as 2–5 °C without seed dormancy (Elfadl et al. 2009). At flowering, it is susceptible to extremely high temperatures frost during kernel filling (Singh and Nimbkar 2006). Safflower gives some specific options to farmers in a dryland crop rotation (with small grains) with respect to weed and disease control (Gupta 2015). In addition, in drought-and-salinity-affected regions, it is possible to cultivate safflower with surplus water from renewal regions, which prevents the expansion of saline seeps and prevents soil erosion (Mündel and Centre 2004). The amount of fertilizer required for safflower depends upon the goal of the product, its position in the rotation and irrigation or rainfed conditions. Deep, fertile, sandy loam soils, which are well-drained, support the highest safflower yields in different climates (Gupta 2015).

14.1.5 Current Challenges

In recent decades, there has been a decreasing trend in safflower production which causes some concern that if undealt with it will remain to some extent a neglected crop. The main reasons that are reducing the pace of safflower breeding can be summarized as follows:

(a) Loss of existent appropriate ideotypes for producing seed and enhancing oil seed safflower.
(b) Manual harvesting problems due to spiny genotypes and no suitable ideotypes for harvest mechanization.
(c) The narrow genetic diversity for genotypes with high acclimatization to different growing conditions and extreme temperatures.

(d) Low harvest index.
(e) Deficiencies in breeding lines containing tolerance to different diseases and pests (Sujatha 2008).

It is important to determine the best application of cultivar practices to improve productivity. Some of the agricultural challenges are as follows:

(a) Use of recommended high-yielding cultivar/genotype treated with a suitable fungicide.
(b) Studies of integrated weed control including: proper cropping systems.
(c) Tillage and herbicides; inter-row cultivation; early-season planting; herbicide application at early growth stages; manual weeding; moisture conservation.
(d) Sowing devices including: timely sowing as per the recommendation for each region; use of recommended seed rate and seedling devices.
(e) Cropping systems: adaptation of recommended crop rotation weed control.
(f) Plant protection: necessity of taking measures against major pests and diseases.
(g) Water management including indication of the most applicable use of water for variant soil-climate environments; protective irrigation at critical stages of crop growth.
(h) Allelopathy: indications of safflower roots having any adverse effects on other crops growth or vice versa.
(i) Thermal and photoperiod reactions: investigations of the reactions of different genotypes to day length under different temperature regimes.

14.2 Genetic Improvement Strategies

Considering the importance of safflower, major breeding aims are planned for crop improvement and sustainability. A great number of breeding practices have been employed in association with crop improvement. The following items are considered appropriate for safflower germplasm enhancement.

14.2.1 Seed Yield Improvement

The degree of success in introducing safflower into new regions is largely dependent on how much the oil content and seed yield is improved (Golkar 2014). The individual safflower cultivar seed yield is dependent on different genetic and environmental conditions, which include: plant height, number of branches per plant, head diameter, head numbers in each plant, seed number in each head and the most significant morphological features of all: oil content and seed weight. The following genetic manipulations could be used to increase seed yield:

(a) Ideotype change: Induction of the suitable changes in different morphologic (branching trait, plant height) phonologic (early flowering and early maturity) and agronomic traits (increase in the number of capsules per plant, capsule diameter and seed weight) could significantly increase seed yield (Dajue and Mündel 1996).
(b) Heterosis: The superiority of progenies to parental genotypes is considered as a main property of hybrid cultivar production in safflower. Hybrid seed production by hand emasculation is very time consuming, so, male sterility is an obvious aim of effective hybrid seed production (Baydar et al. 2003). Production of hybrid cultivars with superior heterosis and/or heterobeltiosis would be facilitated by using cytoplasmic male sterility and genetic male sterility systems.
(c) Interspecific hybridization: This hybridization explores the potential of interspecific hybridization. Species with the same chromosome number as *Carthamus tinctorius,* i.e., C. *oxyacantha*, C. *palaestinus* and *C. persicus,* could produce fully fertile and hybrids in F_1 and F_2 generations (Ashri and Knowles 1960), which could transfer suitable traits to the cultivated safflower.

14.2.2 Oil and Meal Improvement

The focus of safflower breeding should be on improving both oil quality and quantity (Hamdan et al. 2008). The improvement strategies should be aligned with the two present market demands, including enhancement of oleic and linoleic acid and extension of oil shelf life.

Increasing oil yield (more than >30%) has led to the release of such cultivars as NARI-6, NARI-NH-1 and NARI-57 (Singh et al. 2003). Different methods (gene transfer, mutation, crossing with low hull content and high oil content genotypes) could be applied for oil enhancement. In the USA, the increased levels of oil were achieved through reducing hull thickness and also through single genes including those related to reduced-hull, striped-hull and partial-hull (Dajue and Mündel 1996). The following ways are expected to further the oil breeding of safflower:

(a) Evaluation and exploitation of lines possessing oils of essentially different fatty acids for industrial use, as well as food use.
(b) Development of specific fatty acids compositions for specific uses with different short- or long-saturated fatty acids.
(c) Modifying the composition of amino acid in the meal, for example, an increase in lysine content has been tried (Singh and Nimbkar 2006).
(d) Elimination of toxic substances (such as matairesinol monoglucoside and lignan glucoside) in the meal.
(e) Finding new genotypes containing lesser amounts of toxins lignan glucoside and matairesinol monoglucoside detectable in safflower meal.
(f) Development of agro-production technologies for different agroclimatic conditions.

(g) Development of value-added products produced from safflower such as flower and seed color.
(h) Development of enhanced application for hulls, for instance using them as raw material in structural applications or use as fuel.

14.2.3 Oil Improvement of the Resistance to Disease and Insect Pests

Safflower is very vulnerable to foliar diseases generated by dampness and insects, and organisms that cause root rot (Singh and Nimbkar 1993, 2006). If safflower can become more resistant to these diseases its cultivation will be possible in wider areas than it is now. The most limiting factor in its distribution is the safflower fly (*Acanthiophilus helianthi* Rossi), whose habitat is in Asia, Europe and Africa. Attempts to overcome these obstacles requires cooperation between pathologists and breeders. In India, commercial varieties were developed that are somewhat resistant to wilt (NARI-38 and NARI-57) but no single variety with full resistance has yet been found, although some moderate ones have recently been reported. The main required strategies in this field are:

(a) Designing accurate and distinguishable tests which quickly measure the resistance of genotypes.
(b) The necessity to comprehend the resistance/inheritance mechanism in the host and to understand virulence and non-virulence of various physiological races of insects and pathogens.
(c) Improvement of developmental patterns.

14.2.4 Improvement of Suitable Morphological Ideotypes

(a) Angle of branching: production of appressed types would facilitate mechanical and manual harvest of denser stands.
(b) Spininess: spiny varieties are required in the areas where production is nontraditional but flowers are harvested manually.
(c) Seed hull: reductions in hull thickness provide varieties the potential of over 50% oil. An alternative exists between striped or smooth hulls and hulls that lack the melanin pigment.
(d) Safflower with only primary branches: safflower plants showing primary, secondary and tertiary branches with globular capitula, but designing the ideotype with primary branches would produce stronger capitula with increased seed weight, instead of many capitula in secondary and tertiary branches with low seed yield or infertile.

(e) Single-headed safflower: in safflower, each branch usually has 1–5 flower heads, but in some cases, these heads do not have many seeds. It has been postulated that single-headed types with uniform maturity must be developed for yield enhancement.
(f) Lack of dormancy at maturity: germplasm screening for dormancy may prove very beneficial. Germination of mature seed in the safflower capitulum following rain has a negative effect on the quality and quantity of harvested seed.

14.2.5 Improvement of the Resistance to Abiotic Stresses

Drought stress is a major limiting factor for safflower production in arid and semi-arid regions (Nakhaei et al. 2014). Improvement strategies for resistance to salinity, waterlogging, photoperiod insensitivity and frost should be considered. With greater resistance to cold, true winter safflower types may be bred to grow alongside winter wheat. Duration of the rosette stage is a very important factor in cold tolerance. The rosette stage protects the harvest from frost, but weeds frequently overgrow safflower at this stage and diminish yield. Investigating cultivated safflower genotypes, and also the recombination of genes from various crosses with wild species, brings about abiotic tolerance limitations.

14.3 Traditional Breeding Methodologies

Although safflower is categorized as an often cross-pollinated crop, the methods used in breeding self-pollinating crops have been adopted to develop its cultivars. Different breeding methods are described below.

14.3.1 Introduction and Pure Line Selection

This method was established in the beginning of nineteenth century, though the introduction of breeding methods from India, Russia and Turkey, to the USA. A few cycles of adaptation, followed by selection and evaluation of acclimatization tests (over years and locations) are necessary for commercial release of cultivars expressing high seed yield and stability. This method was the most effective for varietal development in safflower. Safflower cultivars developed by this method include: A-2, Bhima, Saffire, N-630, Nagpur-7, N-62-8, A-300, Manjira, S-144, JSF-1, K-1, CO-1, Type-65, APRR-3, Bhima, HUS-305, Sharda, JSI-7, PBNS-12, Nebraska-5 and Nebraska-10. A great number of safflower cultivars have been produced in India via pure line selection because untapped variability is available for various traits of this plant (Singh and Nimbkar 2006).

14.3.2 Mass Selection

This method of selection has been used for disease tolerance in safflower. A number of the major diseases of safflower (such as *Alternaria sclerotina* and *Sclerotinia sclerotiorum*) have been controlled by breeding for resistance by the use of the mass-selection method. For instance, resistance to *Sclerotinia* head was incorporated into the Saffire cultivar (Canadian safflower) (Mundel et al. 1985).

14.3.3 Hybridization

Along with generating variation for different traits in F_2 and subsequent generations, hybridization has greatly contributed to unmasking the genetic make-up of various attributes in safflower (Knowles 1989). Genetic parameters are essential to be estimated in order to develop breeding plans in safflower cultivars that are artificially selected (Golkar 2014). Hybridization with marker-assisted selection (MAS) could be used in the improvement of desired traits linked to molecular markers (Collard and Mackill 2008). Different genetic designs have been carried out to indicate the genetic parameters in safflower, including the dialell cross (Golkar et al. 2012; Gupta and Singh 1988a; Mandal and Banerjee 1997; Ramachandram and Goud 1983), line × tester (Deshmukh et al. 1991), generation mean analysis (Nakhaei et al. 2014; Shahbazi and Saeidi 2007), parental mating design (Naik et al. 2009) and triple test cross (Dhumale et al.1998). The genetic studies for different traits in safflower are summarized in Table 14.2.

14.3.4 Heritability of Traits

Different genetic components (additive gene effects, dominance gene effects and epistasis effects) have been calculated by different genetic designs to identify broad- and narrow-sense heritability of important traits in safflower (Camas and Esendal 2006).

A literature review revealed differences in the estimations of heritability of a trait, that could be compromised by differences in environmental condition, genotype and method of evaluation (Falconer and Mackay 1996). The inheritance and the mode of gene action for stress tolerance (such as disease, insects and abiotic stresses) are not well studied in terms of germplasm improvement. Depending upon the trait to be enhanced, the segregating populations in F_2 and subsequent generations, are conducted using one of the following methods.

Table 14.2 The genetic control of different studies traits in safflower

Trait	Genetic control	References
Phenologic traits		
Days to emergence	Additive	Golkar (2011) and Kotecha (1979)
Days to bolling	Additive	Golkar (2011)
Days to flowering	Dominance	Golkar (2011)
	Partial dominance	Gupta and Singh (1988b) and Singh et al. (2008)
Days to maturity	Additive	Kotecha (1979) and Shahbazi and Saeidi (2007)
	Overdominance	Gupta and Singh (1988b)
Morphologic traits		
Plant height	Additive	Golkar et al. (2012), Kotecha (1979) and Shahbazi and Saeidi (2007)
Stem diameter and leaf length	Nonadditive	Kotecha (1979)
Branches per plant	Additive	Gupta and Singh (1988a)
	Epistatic effect	Narkhede et al. (1987)
Head diameter	Dominance	Golkar et al. (2012) and Singh et al. (2008)
Nutritional traits		
Oil	Additive	Golkar et al. (2011b)
	Dominance	Gupta and Singh (1988a)
Protein	Additive-dominance	Golkar et al. (2012)
Linoleic acid	Additive	Golkar et al. (2011b) and Hamdan et al. (2008)
Oleic acid	Additive	Golkar et al. (2011b) and Hamdan et al. (2009)
Seed yield and its components		
Seed weight	Additive	Golkar et al. (2012)
	Additive-dominance	Shahbazi and Saeidi (2007)
	Nonadditive	Kotecha and Zimmerman (1978)
Pappus weight	Nonadditive	Kotecha and Zimmerman (1978)
Capsule/plant	Additive/dominance	Sahu and Tewari (1993)
	Epistatis	Shahbazi and Saeidi (2007)
Seeds/capsule	Additive	Mandal and Banerjee (1997) and Singh et al. (2008)
Seed yield	Dominance	Mandal and Banerjee (1997) and Singh et al. (2008)
	Additive	Golkar et al. (2012)

(continued)

Table 14.2 (continued)

Trait	Genetic control	References
Morphological markers		
Flower color	Dominance	Claassen (1952) and Narkhede and Deokar (1990)
	Epistatic	Golkar et al. (2010)
Flower spininess	Dominance	Claassen (1952) and Narkhede and Deokar (1990)
Drought stress	Additive (seeds/capsule)	Nakhaei et al. (2014)
	Dominance × dominance epistasis (capsule/plant and dry weight/plant)	Nakhaei et al. (2014)
Salinity stress	Additive, dominance and dominance × dominance (plant height, branches/plant and capsules/plant	Mirzahashemi et al. (2014)

14.3.4.1 Pedigree Method

Selection for attributes that are highly heritable (for instance disease and early maturity) has been carried out by the pedigree method. According to Singh and Nimbkar (2006), some safflower cultivars developed by this method and the year of release are as follows: AC Stirling (1991), AC Sunset (1995), Girard (1986), Girna (1990), Hartman (1980), Leed (1968), NARI-6 (2001), Oker (1984) and Phule Kusuma (2003).

14.3.4.2 Single-Seed Descent Method

This method has been adopted in Spain to develop five safflower cultivars: Alameda, Merced, Rancho, Rinconda and Tomejil.

14.3.4.3 Backcross Method

This method has successfully been used to incorporate disease resistance genes into safflower (Singh and Nimbkar 2006). Recurrent selection and the single-seed descent method have been applied for safflower breeding (Singh and Nimbkar 1993).

14.3.5 Mutation Breeding

Mutation breeding offers the possibility of creating desirable new traits that either have not been found in nature or have been lost during evolution. Mutant germplasm resources have been developed for different crop plants to speed up their development. The mutagenesis method has been suggested to compensate for the

narrow variability existing in safflower germplasm (Rampure et al. 2017). Different methods of mutagenesis have been applied to crops; however, in safflower, seed mutagenesis is commonly applied. A few studies have focused on the use of mutagenesis in safflower, but in vitro mutagenesis has not yet commercially been applied in safflower.

14.3.5.1 Conventional Mutagenesis (Seeds) and Enhanced Traits in Safflower

Different traits have been improved in safflower using seed mutagenesis. Mutation breeding has been applied to different agronomic traits some of which include plant height (semi-dwarf and dwarf), spineless leaf, flowering date (earliness, lateness), seed shape, seed oil quality, seed weight and plant height. The majority of mutation breeding researches are summarized as follows:

Seed Related Traits They include the following traits:

(a) Ebert and Knowles (1966) showed that the most important mutation processes in safflower were thin-hull achene (th) and brown striped-hull achene (stp).
(b) A brittle mutant of safflower emerged in the F_6 of a cross between an introduction from Turkey (PI 175624) and cv. Pacific 7, having a higher content of protein, less raw fiber, and higher amounts of soluble polysaccharides and starch, compared with the parental cultivars (Temple and Knowles 1975).
(c) The examples of mutagenesis in seed to improve safflower traits include black foliage with one recessive gene (*bl*), crinkle with single recessive gene (*cr)* and partial hull with a recessive gene (*par)* (Temple and Knowles 1975).

The effects of gamma irradiation on thick-hulled varieties (A1, S144 and US 104) and two thin-hulled varieties (Th5 and AC1) of safflower were studied by Ramachandram and Goud (1983). Thin-hull varieties were more sensitive to irradiation than thick-hull varieties. Differential radio-sensitivity of varietal groups was attributed to the differences in the proportion of hull in the achene, rather than the quality and quantity of oil.

Agronomic Traits The chief characteristic of using mutagenesis in safflower breeding is a reduction in plant height because of the higher resistance of shorter genotypes against lodging, compared with standard types (Velasco et al. 2000). Ebert and Knowles (1966) found dwarf plants having only a main head; the dwarf plants quickly blossomed since there was no rosette stage. The first mutagenesis study in safflower aimed at plant height reduction was done by Sahu and Kumar (1978) using chemical mutagenesis. Likewise, Muñoz-Ruz et al. (2000) made use of chemical mutagenesis to evolve cv. Enana, the dwarf safflower genetic stock, from Rancho, the Spanish cultivar. By the application of sodium azaid and ethyl methyl solfonate to seeds of Bhima and AKS-207 cvs., Rampure et al. (2017) reported putative mutants for improvement in early bolting, dwarfing, higher

branching, large head, high test weight, thin hull, high oil content and high oleic acid mutants.

Oil Quality New safflower phenotypes can be created by means of induced mutagenesis so as to increase the economic importance of this flower. The inheritance of unsaturated fatty acids of safflower (oleic acid and linoleic acid) are monogenic (Hamdan et al. 2009). Natural mutants whose content of linoleic acid are very high (> 85%) or whose content of oleic acid is great (> 80%) have been identified (Velasco et al. 2005). Mutagenesis have been done for fatty acid improvement. Natural mutants in safflower have been used in different genetic studies. Velasco et al. (2005) used a natural mutant IASC-1 (with high gamma tocopherol content >85%) in crossing with normal genotypes to evaluate its genetic control. Gamma radiation at Giza cv. produced two new safflower mutant lines, i.e. M7 (yellow petals) and hybrid 2 (white petal), having the highest value of oleic acid (41.22% and 39.88%), in comparison with that of the local variety of Giza (13.5% oleic acid) (Ragab et al. 2008). An *oleic acid* type of safflower (>60%) has been proposed in California and Australia as a consequence of the instantaneous mutation. Some chemical mutagenesis changes have been done to increase the level alterations. For example, ethyl methanesulfonate treatment in Bhima cv. increased the frequency of progenies with high levels of oleic acid (2% in M4 generation) (Rampure et al. 2015). Therefore, high levels of linoleic and oleic acid mutants derived from different studies are expected to be employed to improve varieties of oleic safflower desirable for various industrial applications.

14.3.6 Hybridization

14.3.6.1 Conventional Hybridization

The aim of hybridization is to concentrate the favorable characteristics of two or more varieties into a single one. With innumerable wild crosses, the hybridized safflower may possess weedy attributes via interspecific or intraspecific hybridization, developing a potential for competitive hybrid populations (Baydar et al. 2003). Merging safflower cultivar genomes with their wild relatives may seem a natural process. Hybridizing numerous wild varieties of *Carthamus* in Asia is reported to have played a pivotal role in the development of *C. tinctorius* (Ashri and Knowles 1960). Hybridization between *C. tinctorius* and several wild relatives (such as *C. oxyacanthus* and *C. palaestinus)* has been demonstrated to occur both artificially and naturally by open pollination (Rapson et al. 2015) to produce fertile hybrids. Nevertheless, the harvest amount of safflower declines in comparison with other oil products; therefore, it seems essential to boost yield by breeding hybrid varieties through adopting heterosis effects (Baydar et al. 2003). The potential of hybrid safflower has been studied by Claassen (1950), who suggested using low fertility lines as female parents in a hybrid production scheme. The first breeding system in

safflower used the low fertility gene *th* as a female in a hybrid system (Rubis 1969). But, self-pollination problems, along with low yields in hybrid production fields, eventually caused the program to end unsuccessfully. The applied methods were unsuccessful in commercial hybrid production. The first hybrid safflower breeding program was initiated in 1974 by using a cytoplasmic male-sterility system (Hill 1989). Cytoplasmic male sterility (CMS) systems and genetic male sterility (GMS) made safflower an appropriate alternative for adopting hybrid vigor in conventional hybrid production in safflower (Anjani 2008).

The GMS sources in safflower are controlled by the following lines: UC-148 and UC-149 GMS (Heaton and Knowles 1980); GMS lines produced by Ramachandram and Sujatha (1991); MSN and MSV male-sterile lines (Singh et al. 2001) and male-sterile lines associated with dwarfness (Singh 1997).

The use of commercial hybrid seed seems to be greatly limited due to the high expense of hormonal regulation studies necessary for hybrid breeding of safflower (Baydar et al. 2003), revealing that gibberellic acid (GA_3) might inflict male sterility for hybrid production in safflower.

14.3.6.2 Hybrid Cultivars

Diagnosing genetic male sterility sources in safflower (Heaton and Knowles 1980; Joshi et al. 1983; Ramachandram and Sujatha 1991; Singh et al. 2001) and evolution of agronomically superior genetic male-sterile lines in India have led to development and release of spiny safflower hybrids such as DSH-129 and MKH-11 in 1997. NARI-NH-1 was considered the first non-spiny hybrid in safflower, released by Singh et al. (2001).The second variety NARI-H-15 was released in 2005. The safflower hybrids showed an increase in seed and oil yield (20–25%) over the control variety (Anjani 2008). The yield advantage of late hybrids, compared at different sites in California and Arizona, North America, was on average 127% higher than the best parental lines. Hybrid oil levels increased to 45% and higher, and are currently being developed. From the classical breeding point of view, few efforts have been made to develop new F_1 hybrids of safflower through adopting genetic diversity of exotic parents. In order to produce hybrid seed, applying a dwarf (Dw Dw) line either as male-fertile or male-sterile parent could lead to semi-dwarf (Dw dw) hybrids (Velasco et al. 2000).

The cytoplasmic male sterility (CMS) based system could pave the way for hybrid seed production in safflower. Therefore, efforts should be made to develop cytoplasmic male sterility lines in safflower, and subsequently identify suitable maintainer and restorer genotypes to produce highly-productive hybrids. Hybridization accompanied with marker-assisted selection could be useful for applying proper methodologies to make the desired improvement in different traits with different gene effects (Golkar 2014). If biotechnological tools, such as genetic engineering, can be successfully used to incorporate the sunflower mitochondrial

male-sterility factor into the safflower genome, safflower hybrids can be more readily produced (Anjani 2008).

14.4 Germplasm Biodiversity and Conservation

14.4.1 Germplasm Diversity

The genetic variation in a germplasm collection and the heritability of a trait can primarily determine the effectiveness of a selection program (Falconer and Mackay 1996). As stated by Amini et al. (2008), assessing genetic diversity of safflower has so far caught the attention of researchers due to its importance in extending the genetic base, preserving genetic resources and improving practices in breeding programs. Safflower, cultivated for a long period of time in various parts of the Old World, is now genetically more diverse (Knowles 1989). Recently, a number of cases have been observed in Isfahan Province, Iran, indicating natural crossings between cultivated species *Carthamus tinctorius* and its wild relative *C. oxyacantha* whose chromosomes are equal to 2n = 24 (Singh and Nimbkar 2006). Genetic diversity is very important to overcome risks posed by different diseases, rapidly changing environmental conditions and, most importantly, to meet growing consumption demands (Mukta 2012).

The American scientist Paulden F. Knowles, who has been called the father of safflower, in the 1950s–1970s, traveled all over the world exploring for safflower genotypes and collected samples of wild, cultivated and weedy relatives of the *Carthamus* species. His tremendous efforts in collecting and assessing germplasm resulted in to a large collection of germplasm. It comprises some 2383 accessions deposited in the Western Regional Plant Introduction Station (WRPIS), Pullman, Washington, USA (Johnson et al. 2001; Mukta 2012).

The Indian Institute of Oilseeds Research, Hyderabad, India has an even larger collection, comprising 6000 accessions of safflower (Mukta 2012).

In the period 1996–2001, the National Crop Gene Bank, Institute of Crop Germplasm Resources, Beijing, China amassed a collection of 1100 safflower accessions. Other safflower-growing countries also preserve germplasm, including Iran and Turkey. Among the European countries, extensive safflower genetic research has been conducted in Spain. Despite the extensive efforts in germplasm collecting, priority work remains to be done to fully evaluate the collections.

Researchers can enhance the genetic resources of safflower employing multidisciplinary methods, including genetics, plant exploration, cytogenetics, taxonomy, plant breeding, agronomy, plant pathology, distant hybridization, physiology, as well as through molecular biology using genetic transformation and cell culture. Table 14.2 shows the safflower seed yield and oil production in different countries.

14.4.2 Cultivar Characterization and Phylogeny

The domestication of safflower was carried out by dispersal of the plant from its original native area to new locations where it became acclimatized to the new environments (Harlan 1992). Various ecotypes, having diverse physiological and morphological qualities, result from this adaptation to new temporal and spatial environments (Dajue and Mündel 1996; Knowles 1989).

For cultivar classification, and to specify diversity, access to a core collection involving different quantitative elements is possibly the best approach (Fernandez-Martinez et al. 1993; Johnson et al. 2001). Classification in safflower has been carried out using either agro-morphological (Ashri 1975; Jaradat and Shahid 2006; Khan et al. 2009), biochemical traits including isozymes (Bassiri 1977) nutritional traits such as fatty acids (Fernandez-Martinez et al. 1993; Golkar et al. 2011b; Khan et al. 2009) and molecular markers (as mentioned in the previous section). In this respect agro-morphological evaluation is the primary step in the evaluation and categorization of different safflower germplasm. For instance, the germplasm diversity of the core collection compiled by Johnson et al. (2001) was high; apparently, agronomic characters can differentiate regional differences.

Cultivar characterization revealed that among 721 accessions from the world collection, the safflower collected by the USA, could be employed as a source of *Verticillium* and *Alternaria* resistance and high content of seed oil for the sake of breeding (Cervantes-Martínez et al. 2001). Some morphologic descriptors used in germplasm diversity are: primary and secondary branch number, branch location on main axis, branch angle, internode length (short, intermediate, long), leaf shape (linear, oblong, ovate, lanceolate), leaf margins(serrate, entire, dentate), leaf color (light green, dark green, grayish), leaf denotations, leaf spininess, spin number and location on leaf, spin number on other involucral bracts, main head shape (conical, oval or flattened), leaf diameter, flower color (white, orange, yellow, red), corolla color, seed color (white cream, brown, black, grey) and size or shape (hullness or hull). Variations in safflower are mainly because of the spininess and flower color (Bradley et al. 1999; Golkar et al. 2010). Also, different phonologic characters, including days to emergence, rosette period, days to budding, days to flowering, days to maturity (Golkar 2011) and agronomic traits, i.e. plant height, the number of seeds per capsule, capsules per plant seed weight, hull percentage, seed yield per plant, and seed dormancy, and nutritional traits, namely oil and protein content, iodine value of oil, fatty acids pattern. In addition, biochemical markers, i.e. isozymes such as isocitrate dehydrogenase and malic enzyme, and stress susceptibility, including high temperature, drought, salinity, lodging and disease are considered as descriptors for diversity evaluation in safflower germplasm.

14.4.3 Phylogenic Studies

Carthamus comprises 25 species with either 10, 11, 12, 22 or 32 chromosome pairs. *Carthamus. tinctorius* has only $2n = 24$; the rest have a weedy and wild habit (Singh and Nimbkar 2006). Among the wild relatives of *Carthamus*, *C. palaestinus* and *C. oxyacantha* both have $2n = 24$ chromosomes, which are compatible with each other and with cultivated safflower genotypes in terms of crossing ability (Ashri and Knowles 1960). To the extent safflower species are genetically diverse and how they are related, are under investigated by phylogenists. Phylogenetic studies can improve the taxonomic categorization of *Carthamus* to reveal the problematic issues of the present categorization. Phylogenetic relationships are predominantly based on morphological characters, crossing ability success, karyology, plant habit, isozymes and biogeography. Internal transcribed spacer (ITS) sequences and karyology identified descending disploidy as the principal mechanism of karyological evolution in *Carthamus* (Vilatersana et al. 2005). Chapman et al. (2007) suggested *C. palaestinus* as the progenitor species of cultivated safflower. Cultivated safflower is apparently distantly correlated with *C. persicus* and *C. oxyacanthus*. Chapman et al. (2010) emphasized expressed sequence tag-simple sequence repeat (EST-SSR) as an operational marker system because of its five genetic clusters in safflower germplasm: (1) Europe; (2) Turkey-Iran-Iraq-Afghanistan; (3) Israel-Jordan-Syria; (4) Egypt-Ethiopia; and (5) Far East-India-Pakistan. In a phylogenetic study done by EST-SSR markers, *C. palaestinus* had the most genetic similarity with cultivated safflower and *C. oxyacanthus* (Derakhshan et al. 2014). The genetic diversity examined by sequence-related amplified polymorphism (SRAP) markers showed that *C. boissieri* Halácsy, *C. dentatus* Vahl. and C. *glaucus* can be categorized in an individual section, *C. dentatus* in one subsection and *C. glaucus* and *C. boissieri* in another (Mokhtari et al. 2013). Pearl et al. (2014) reported that the genetics of safflower domestication is quite complex, thereby suggesting that the sunflower genome might provide parallel trait transitions within the domestication process.

14.4.4 Genetic Resources Conservation Approaches

Agrobiodiversity is dynamic and hence conservation of genetic resources must to be continues. All over the world, attention is increasingly being paid to previously overlooked crop species such as safflower, especially with regard to germplasm conservation. To successfully plan breeding programs, the genetic diversity of the collected germplasm is vital. Thus, six major objectives can be outlined in terms of the genetic conservation of safflower.

(a) To increase the conservation process by greater use of *Carthamus* resources.
(b) To increase research on the domestication, documentation and evaluation of *Carthamus* species.
(c) To increase preferred selection of useful species of *Carthamus.*
(d) For genetic conservation, developmental processes and food security.
(e) To identify suitable criteria for development and conservation practices that place the use of these genetic resources for sustainable agriculture to broaden the bases of food security.
(f) Core collections of all species of *Carthamus*; this has a major role to play in safflower germplasm conservation (Dwiedi et al. 2005). Various data obtained from different geographic backgrounds should be used to select the core entries so as to make use of the value of each accession in the collection.

14.4.4.1 Seed Conservation

Genetic conservation by seed conservation is the best strategy for maintenance of world safflower germplasm. On behalf of the International Board for Plant Genetic Resources (IBPGR), the two main organizations are the USDA Western Regional Plant Introduction Station, Washington State University, Pullman WA USA and the National Bureau of Plant Genetic Resources, Indian Agricultural Research Institute, India, which handles safflower seed groups from different geographical regions including Southeast Asia, China, Japan, Korea, South Asia (including Bangladesh, Pakistan and India) and East Africa. In the USDA station, every sample was first completely documented and then an Introduction Number (PI number), assigned to each.

14.4.4.2 In Vitro Conservation

The basic technologies comprising in vitro conservation system are collection, disease eradication and indexing, culture initiation, multiplication, storage and distribution. In considering seed-based conservative methods in safflower, in vitro conservation methods have not used for germplasm conservation in safflower, due to the non-efficiency of the vegetative-based breeding methods in it. Tissue culture methods have been applied in safflower for biotechnological purposes, but not for germplasm conservation.

14.5 Cytogenetics

Early cytogenetic studies in safflower were done to determine the number of chromosomes in the various species and to investigate the extent to which interspecific cross pairing can occur so as to create genomic relations among species. It was sup-

posedly not possible to identify further chromosomes in safflower as chromosomes, relatively of the same size, were comparatively low in number (Singh and Nimbkar 2006). A few genes have so far been identified for each chromosome, indicating the lack of knowledge in this respect. One primary and one secondary trisomic were reported by Estilai and Knowles (1980) in the progeny of a triploid plant in safflower. Four chromosome numbers have been identified for safflower, including 2n = 20, 24, 44, and 64. The genus has been classified into four chromosome classes (Singh and Nimbkar 2006).

(a) Class I (2n = 24): includes the annual species *of Carthamus tinctorius, C. palaestinus* and *C. oxyacantha,* in which *C. oxyacantha* is considered to be the wild ancestor of the cultivated safflower (Ashri and Knowles 1960).
(b) Class II (2n = 20): includes *C. glaucus, C. alexandrines* (Boiss & Heldr.) Bornm., *C. tenuis* (Boiss. & Blanche) Bornm. and *C. syriacus* (Boiss.) Dinsm. The Mediterranean Region is where these species can be found.
(c) Class III (2n = 44): Made up of just *C. lanatus*, having 22 chromosome pairs. It is a naturally-occurring species grown in Turkey, Morocco, Portugal, Greece and Spain.
(d) Class IV (2n = 64): Consists of: *C. turkestanicus* Popov and *C. baeticus* (Reuter and Boiss) Nyman.

Correlation analysis of molecular data with morphological and karyological characters reduced the four abovementioned classes into two main classes: *Carthamus* and *Atractylis* (Vilatersana et al. 2005). Three SAT-chromosomes in *C. tinctorius* were found by Kumar et al. (1981). What is still lacking in safflower is the karyological representation of each chromosome and the relationship between genes and specific chromosomes. *Carthamus tinctorius* was analyzed by means of FISH employing pCt*kpn*I-1 and pCt*kpn*I-2 repeated sequences, both, at the same time, indicated that the pCt*kpn*I-1 sequence was absolutely localized at subtelomeric parts on most of the chromosomes (Raina et al. 2005). By the use of ethyl methylsulfonate (EMS) (0.5%) in seed safflower, at various intervals, cytometric variability were also traced with other abnormalities, including precocious movement, bridge, laggards, and non-disjunction of chromosomes (Srivastava and Kumar 2011). EMS has induced cytometric variability in safflower.

14.6 Molecular Breeding

14.6.1 Role of Biotechnology

Safflower breeding programs are not adequately complemented with biotechnological tools. Few research groups are working on the crop and their access to biotechnological tools in terms of techniques, equipment and information are inadequate. The main types of biotechnological tools in safflower development are characterized below.

14.6.1.1 Molecular Markers

Molecular markers have had greater development in genomic research on safflower than other fields like transcriptomics and proteomics (Golkar 2014). The application of molecular markers has been used in germplasm diversity, phylogenetic studies, molecular profiling, genetic mapping and marker-assisted selection (MAS) in safflower (Sujatha 2008). Different molecular markers including simple sequence repeat (SSR) (Ambreen et al. 2015; Lee et al. 2014), expressed sequence tags SSR (EST-SSR) (Naresh et al. 2009), amplified fragment length polymorphism (AFLP) (Johnson et al. 2007), inter simple sequence repeats (ISSR) (Golkar et al. 2011a; Yang et al. 2007), random amplified polymorphic DNA (RAPD) (Amini et al. 2008; Khan et al. 2009; Vilatersana et al. 2005) and sequence-related amplified polymorphism (SRAP) (Mokhtari et al. 2013; Peng et al. 2008), have all been used for genetic variation detection in safflower. All the studies unequivocally establish the existence of wide genetic diversity in safflower germplasm. However, genome base studies of safflower have been restricted because of the inadequate presence of molecular markers in this plant as compared to other major oil crops such as canola and sunflower.

14.6.1.2 Molecular Marker-Assisted Breeding

Molecular breeding, as a general concept, includes improvement and utilization of molecular markers with the aim of increasing the performance of breeding programs. Indeed, marker-assisted selection (MAS) as a complementary tool for classical breeding methods and has provided opportunities for plant improvement via indirect selection. Safflower breeding for yield enhancement, abiotic and biotic tolerance and many other traits was moderate and partial due to the lack of genetics and genomics information about this plant. With the advent of DNA markers, several investigations have evaluated the genetic diversity in safflower genotypes and its wild relatives (Johnson et al. 2007; Sehgal and Raina 2005). Furthermore, phylogenetic analysis by molecular markers increased the knowledge of the history of *Carthamus* (Bowles et al. 2010; Chapman et al. 2007; Sehgal et al. 2009). Chapman et al. (2007) developed universal markers with reproducibility in the Asteraceae family including lettuce, sunflower and safflower. These markers afford the possibility of comparative mapping and phylogenic analysis between taxa as well as within species. In addition, Chapman et al. (2007) generated an expressed-sequence tag (EST) collection resulting in 104 polymorphic markers which exhibited a high level of polymorphism across *Carthamus* species. The largest collection of SSR markers in safflower was developed by Mayerhofer et al. (2010), composed of over 1000 markers. With these markers, the first major linkage analysis of *Carthamus* species and mapping of the flower color gene in *C. tinctorius* was published. A collection of genomic microsatellite (SSR) markers, including 108 unique primer pairs, were designed by Hamdan et al. (2011). The authors utilized the SSR primers to map the high oleic acid content locus (*Ol*) and modify genes associated with oleic

acid content of safflower seed oil. Significant advances in the technology of molecular markers in safflower have been especially advantageous for their implementation in trait mapping. So far, several genetic linkage maps have been constructed for safflower in various research laboratories; their information is summarized in Table 14.3.

Hamdan et al. (2008), first reported the linkage between molecular markers and genes in safflower. The observed *Li* gene, controlling very high content of linoleic acid, was closely linked to the nuclear male sterility gene, *Ms*, both flanked by sequence-characterized amplified region (SCAR) markers.

Mayerhofer et al. (2010) established the first major linkage map of *Carthamus* species using an intraspecific F_2 population of *C. tinctorius* and an interspecific BC1 population derived from a *C. tinctorius/C. oxyacanthus* cross. This map comprised 13 linkage groups with 116 marker loci ranging in genetic length from 1.3 to 170 cM and contained 2 to 27 loci. A single dominant gene was responsible for yellow flower color, *ctfc1*, mapped on linkage group T9. Garcia-Moreno et al. (2011) created a linkage map containing *Tph2* genes for high gamma-tocopherol content with RAPD, SCAR and SSR markers. To map the high oleic acid content locus (*Ol*) and modify genes associated with oleic acid content of safflower seed oil, Hamdan et al. (2012) used 2 populations of F_2 mapping obtained from a nuclear male-sterile line and 2 line crosses of high oleic acid. The constructed map included 15 linkage groups. The *Ol* and *Ms* genes were mapped in the same linkage group, T3, at a

Table 14.3 Genetic linkage maps constructed for safflower

References	Marker number and type	Population	No of linkage group	Length of map (CM[h])
Mayerhofer et al. (2010)	1412 SSR[a] and 75 RFLP[b]	F_2: *Carthamus tinctorius*	11	954
Mayerhofer et al. (2010)	1412 SSR and 75 RFLP	BC_1: *C. tinctorius* × *C. oxyacanthus*	13	580
Hamdan et al. (2012)	47 RAPD[c], 60 SSR and 4 SCAR[d]	F_2: *C. tinctorius*	15	816
Pearl et al. (2014)	244 SNP[e]	F_2: *C. tinctorius* × *C. palaestinus*	12	858
Karimi (2015)	168 SSR	F_2: *C. tinctorius*	11	877
Mirzahashemi et al. (2015)	119 SSR and ISSR[f]	F_2: *C. tinctorius*	24	646
Bowers et al. (2016)	2008196 SNP	F_6 (RIL[g]): *C. tinctorius* × *C. palaestinus*	12	959

[a]Simple sequence repeats
[b]Restricted fragment length polymorphism
[c]Random amplified polymorphic DNA
[d]Sequence-characterized amplified regions
[e]Single nucleotide polymorphism
[f]Inter-simple sequence repeat
[g]Recombinant inbred line
[h]Centimorgan

genetic distance of 68.3 cM (centimorgan). Recently, Ebrahimi et al. (2017) identified 2 marker loci associated with oil content under drought-stress and normal conditions in a worldwide collection of safflower. They believe these markers could be useful in MAS programs for oil yield improvement in arid and semiarid regions.

14.6.1.3 QTL Mapping in Safflower

The construction of molecular maps makes it possible to recognize the genomic regions or quantitative trait loci (QTL) with significant effects on various morphological and physiological traits or performance stability in adverse environmental conditions. QTL mapping coupled with marker-assisted selection speed up the breeding process using the trait-based methods (Collard et al. 2005). Hamdan et al. (2012) mapped one major QTL (Ol3.1) on linkage group T3 explaining phenotypic variation in the F_2 (99.4) and F_3 (96.3%) populations. A QTL having minor effects (Ol2.1) on oleic acid content was also identified for T2. Pearl et al. (2015) constructed a genetic map of safflower with 244 single nucleotide polymorphism markers which coalesced into 12 linkage groups. In the F_2 population derived from *Carthamus tinctorius* and the wild species, *C. palaestinus*, 61 QTLs were identified for 21 instances of the 24 morphological and seed oil traits. Also, in this study, QTL positions in safflower mapping was compared with the same population from sunflower (cultivated × wild) and then 14 new QTLs showed similarity with identified sunflower QTLs having the same traits. Among 61 QTLs mapped in this study, 59 had small to intermediate effects and just 2 QTLs, spininess and flower color, showed significant effects (Fig. 14.2). The QTL residing in linkage group L accounted for 32.7% of phenotypic variation of spininess; the QTL identified on linkage group D for flower color explained 63.4% of the phenotypic variation. Mirzahashemi et al. (2015) reported QTL mapping for yield-related traits in F_2 population under drought stress. Their results showed that 4 major QTLs and 3 linkage groups (2, 4 and 6) had critical effects on drought tolerance in safflower.

In another study, 71 SSR markers were utilized to map a $F_{2:3}$ population of safflower. QTL mapping was conducted under saline and non saline conditions (Karimi 2015). Under normal condition, 2 QTLs with significant effects were mapped for 1000 weight and biological yield (Fig. 14.3). Although under salinity stress 8 QTLs with large effects were identified for seed yield, 1000 seed weight, harvest index, capitula diameter, relative water content, membrane stability index, K^+ content and Na^+/K^+ ratio. The QTLs associated with days to maturity, relative water content, K^+ and Na^+ content, Na^+/K^+ and Ca^{2+}/Na^+ ratios, biological yield, capitula diameter, and H_2O_2 content on linkage group 5 were overlapped. It was concluded that there was a relationship between these traits, which could be controlled by the same gene or linked genes. Indeed, QTLs related to seed yield, harvest index and malondialdehyde (MDA) content (as a final products of lipid peroxidation in the cells) were detected in the same region on linkage group 5. Bowers et al. (2016) constructed a genetic map which was a great sequence-based tool to assemble an inexpensive safflower draft genome. Whole-genome sequencing on F_6 cross recombinant inbred

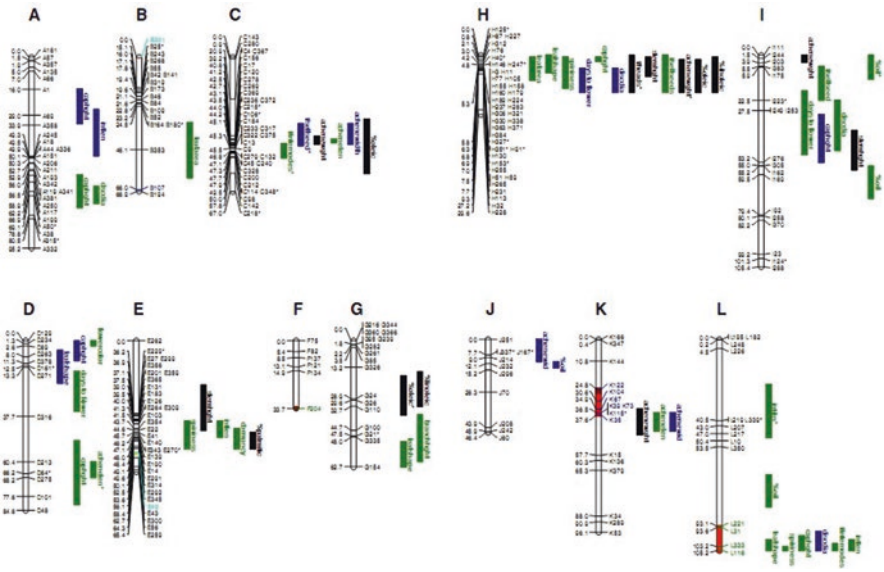

Fig. 14.2 Genetic map provided for safflower genome and positions of identified quantitative trait locus (QTL). On the right are the list of the names of markers for each linkage group; on the left are the list of positions (in cM). Bars represent 1-LOD QTL intervals. (Source: Pearl et al. 2014)

lines (RILs), totalling 96, between *Carthamus tinctorius* and its wild relative, *C. palaestinus*, resulted in a total number of 57,270 scaffolds anchored to the map. This genetic map showed several chromosomal rearrangements when compared with sunflower and lettuce maps.

14.6.2 Functional Genomics

Functional genomics, as a field of molecular biology, converts masses of genome data into gene or protein function data. In other words, functional genomics assigns functions to unknown genes. Gene functions are analyzed by different technological platforms such as transcriptomics, proteomics, metabolomics and phenomics. The number of studies conducted on safflower transcriptomics, compared with those done on other oilseeds, is apparently low. Knowledge of molecular functions and genomic structures of safflower is limited as to the genome sequencing of this diploid flower has not yet been specified. Knutzon et al. (1992) identified two protein species related to the activities of thioesterase (molecular masses: 34 and 40 KD) and then partly made a sequence of them. A cDNA clone (CTOS1) was separated from the genome of safflower encoding a new protein from safflower containing high oleic acid accessions (Mizukami et al. 2000). To sequence the vital and functional proteins in safflower, it seems to be necessary to complete cDNA libraries for

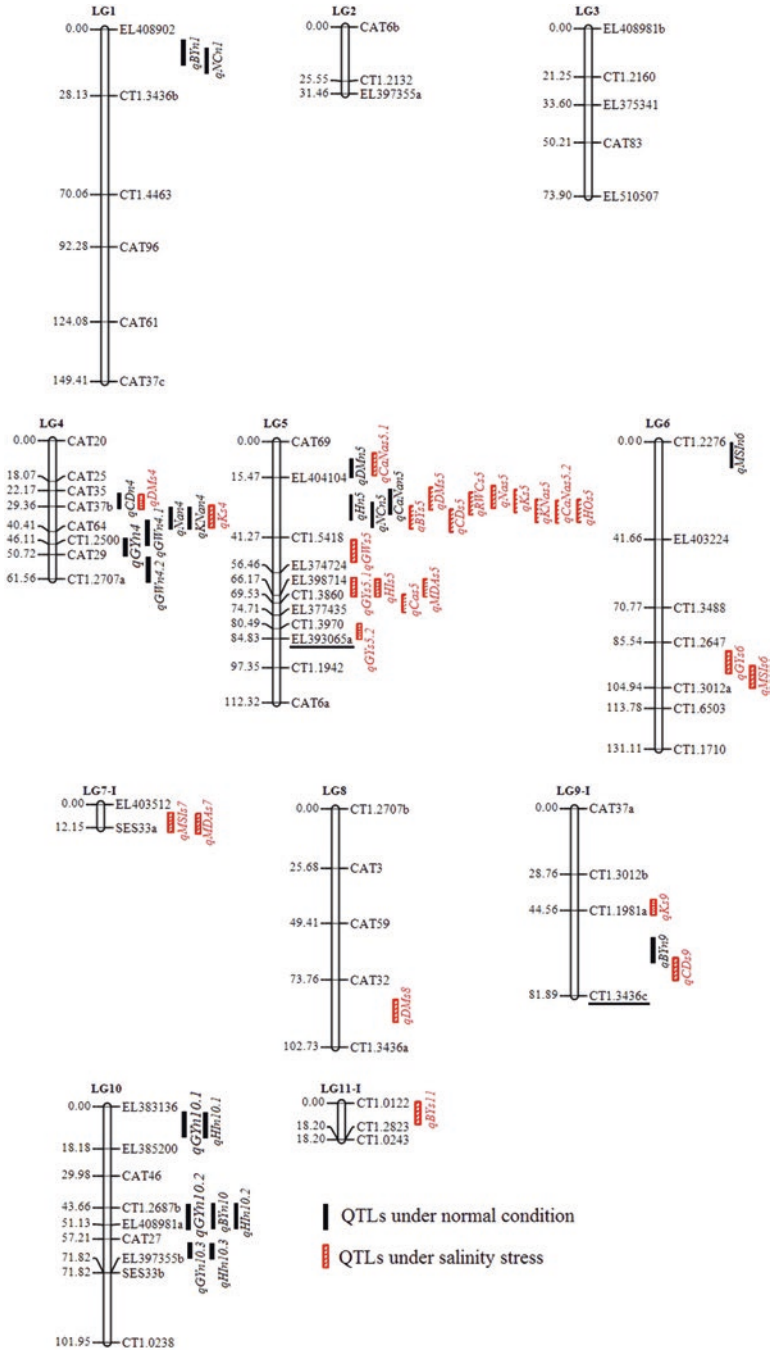

Fig. 14.3 Genetic linkage map of safflower showing the location of QTLs under saline and non-saline condition in a $F_{2:3}$ population. Right: The list of markers; Left: positions (in CM) of every linkage group. Bars indicated the QTLs. (Source: Karimi 2015)

the safflower genome. At least 236 recognized micro RNAs (miRNA) were detected in another study, which were expressed in safflower (Li et al. 2011). In recent years, the technology of high-throughput sequencing, known as next generation sequencing (NGS), has been developed (Lulin et al. 2012). This technology is a powerful, cost-effective and convenient method of transcriptome analysis because it generates direct cDNA sequencing instead of bacterial cloning of cDNA. Lulin et al. (2012) surveyed the poly (A) + transcriptome of safflower flower tissue using NGS technology. For this purpose, they utilized the Illumina Genome Analyzer platform of NGS. Among the assembled unigenes, 70,342 were successfully annotated. In the same year, they found 4 genes and novel pathways controlling the synthesis of secondary metabolites and flavonoids in safflower. They proposed that the given genes could encode other products related to anthocyanidine, which have not yet been recognized in the plant. They believed that this transcriptome dataset could provide an informative platform, facilitating studies conducted about the genomics, functional genomics and gene expression of *Carthamus. tinctorius* (Lulin et al. 2012). Li et al. (2012) also produced a de novo transcriptome using deep sequencing based on Solexa on safflower leaves, seeds, and petals. Results revealed that the identified unigenes were not similarly expressed in the seed, leaf and petal. Unigenes annotated as oleosins were specially expressed in the seed library. Analysis of metabolic pathway showed that 23 unigenes involved in the biosynthesis of flavonoids. Indeed, they found many genes engaged in the secondary metabolite biosynthesis (Li et al. 2012). In seed storage lipids, the microsomal oleoyl phosphatidylcholine desaturase (FAD2) enzyme accounts for the synthesis of linoleic acid from oleic acid by introducing a double bond at the Δ12 position of oleic acid (Cao et al. 2013). To date, the largest FAD2 gene family, in any species, was reported in safflower; Cao et al. (2013) reported the cloning of 11 distinct safflower *ctFAD2* genes, each member of which demonstrated divergent functionality. FAD2 enzymes with novel functionality can be introduced in genetically-engineered crop plants to produce rare fatty acids.

14.7 Genetic Engineering

14.7.1 Methodologies

Genetic engineering techniques are widely used to improve specific traits of crops such as yield and quality. Genetic modification is complementary to conventional plant breeding, accomplished by introduction of a foreign gene into a plant genome. Plant transformation occurs by two principle methods, i.e. vector-mediated and direct gene transfer. Gene transfer can be considered as a potential method for the production of new genotypes, altering fatty acid profiles, and creating novel genotypes tolerant to biotic and abiotic (drought, cold, and salinity) stresses. Transformation methods employed in safflower include callus-mediated regeneration, shoot regeneration and embryo transformation. Safflower is considered a suitable host for the novel technology of transgenes to produce herbal medicines based on vegetable proteins (Carlsson et al. 2014).

14.7.2 Tissue Culture

Different explants (leaf, hypocotyl and seed) are used for callus induction and regeneration (direct or indirect) in safflower. While traditional breeding methods have limited effects on increasing the yield of safflower, in vitro regeneration through tissue culture has the apparent advantage of intermediate propagation. Different plant growth regulators have been applied for success in callus induction which contain high potential for regeneration (Lijiao and Meili 2013). Callus induction from hypocotyls explant in IL.111 cv. is shown in Fig. 14.4. Rarely are studies conducted to improve rhizogenesis of in vitro generated/multiplied shoots. Regeneration of safflower has long been limited due to the lack of an efficient protocol for rhizogenesis, which restricts further genetic modification (Lijiao and Meili 2013; Singh and Nimbkar 2006). Safflower regeneration was achieved through direct shoot bud, hypocotyl and callus-mediated shoot bud procedures (Nikam and Shitole 1998). Applying haploids in breeding cultivars has not been reported in safflower (Singh and Nimbkar 2006). Recently, in vitro research have been carried out solely to investigate abiotic stresses such as salinity tolerance in safflower; callus derived from hypocotyls (Fig. 14.4) from different genotypes showed different ranges of in vitro tolerance to NaCl (Hamedi et al. 2016).

Somaclones for different traits such as plant height, leaf shape, flower color and seed oil were identified in somaclonal variation (Seeta et al. 2000). The detailed effects of biotechnology will be described later in this chapter.

Fig. 14.4 Callus induction from hypocotyls in IL. 111 genotype of safflower (Hamedi 2014)

14.7.3 Enhanced Traits and Transgenic Cultivars

The first attempts to create broad-based genetic improvement of safflower was performed via gene transfer using *Agrobacterium tumefaciens*. In this technique, rooting of shoots in transgenic safflower was difficult; accordingly, recovery of transgenic plants was limited (Sankararao and Rohini 1999). The first transgene mediated via *A. tumefaciens*-mediated transformation was achieved using cultivar Centennial and GUS assay (Ying et al. 1992).

Rohini and Sankara Rao (2000) developed a gene transfer system for safflower that overcame problems of conventional transformation by *Agrobacterium tumefaciens*. They transformed the uidA reporter gene driven by the CaMV 35S promoter and a neomycin phosphotransferase II (nptII) gene driven by the nopaline synthase promoter. They showed that an embryo transformation strategy was successfully applied to every cultivar and genotype of safflower vulnerable to *A. tumefaciens*.

Markley et al. (2006) produced insulin via transgenic oilbody-oleosin technology. In this technique, a transgene encoding an oleosin-insulin fusion protein is introduced into the plant. Plant produced insulin was a cost-effective solution that reduced the unit cost of insulin production. Apolipoprotein Al_{Milano} ($ApoAl_{Milano}$) as a therapeutic substance plays an important role in cardiovascular disease with high LDL cholesterol levels; this is done by increasing HDL cholesterol levels. In transgenic safflower, $ApoAl_{Milano}$ was fused to a single-chain antibody. This fusion was so highly expressed in dry seed, that approximately 7 g $ApoAl_{Milano}$/kg seed was obtained. This transgenic safflower was commercially produced by SemBioSys Genetics Incorporated (Nykiforuk et al. 2011).

Belide et al. (2011) developed a highly efficient *Agrobacterium*-mediated transformation approach in both high oleic acid and high linoleic acid content genotypes. In addition, they have made improvements in in vitro root formation by developing a grafting method (Fig. 14.5). This method of genetic transformation generated stably-transformed plants and progeny (Belide et al. 2011). Gamma linolenic acid (GLA) is a fundamental ω-6 fatty acid which is very important for metabolic pathways. Overexpression of Δ6-desaturase in high oleic and high linoleic safflower cultivars was conducted by Nykiforuk et al. (2011).They showed that transformed safflower accumulated the highest levels of fatty acid just introduced into a transgenic product. This transgenic safflower was commercially produced by Arcadia Biosciences, Davis CA, USA, under the trademark name SONOVA™400 (Nykiforuk et al. 2011). These achievements demonstrate the potential of safflower to produce high-quality oil and protein through genetic engineering for medical and therapeutic purposes.

Fig. 14.5 Regeneration of transgenic safflower via grafting method. (**a**) Principle seedling with strong root system, (**b**) Transgenic scion prepared v-shaped for grafting, (**c**) Supporting the seedling and scion with a ring, (**d**) Parafilm guarded the graft union, (**e**) Establishment of union, (**f**) Flowering of grafted safflower. (Source: Belide et al. 2011)

14.8 Conclusions and Prospects

Research on safflower is incomplete and scattered so it seems vitally important to explore the uncharted territories of safflower potential. A literature review showed that there are some deficiencies in safflower breeding programs that are summarized below.

Nowadays, there is a growing demand for safflower flowers as a source of natural herbal tea for medicinal use. Unfortunately, there is a widening gap in medicinal use between what is known and what seems necessary to discover.

The lack of genetic research on the various floral and physiological traits, flower yield, pigment content (carthamin, carthamidin) and luteolin and different bioactive

compounds is obvious. An important gap exists in the identification of gene action in disease resistance (bacterial and fungal) in safflower. Only a few studies of molecular markers, trait mapping and mapping populations are reported for safflower. Further genetic studies in this regards would be of help to breeders to pinpoint the QTLs position of significant agronomic attributes and oil quality-related characteristics to develop cultivars with enhanced productivity and resistance against biotic and abiotic stresses. Evidently a gap exists in the research carried out on the proteomics of safflower.

Safflower research can be broadly categorized into five sectors. First, the spotlight should fall on the wild germplasm genetic diversity as a proper measure to transfer desirable genes to cultivated genotypes. Techniques of embryo rescue are essential to be improved in order to surmount the genetic and cytologic obstacles in the way of combining safflower cultivars and their wild relatives.

Second, development of agroproduction technologies for different agroclimatic conditions, and management of pests and diseases is essential. Regarding the development of value-added products derived from safflower, progress in molecular farming and diagnosing prominent genes with transcriptomic research such as including genes in enzymatic and nonenzymatic antioxidant biosyntheses, are highly recommended to breed safflower.

Third, the development of molecular markers contributes to the study of map-based cloning, marker-assisted selection and hybrid production by identifying the heterozygous maintainer line in genetic male sterility (GMS).

Fourth, production of mapping populations via double haploid lines in safflower would enable breeders to identify the gene position of important agronomic and oil quality-related attributes to develop cultivars with enhanced productivity, that are tolerant to biotic and abiotic stresses. Accordingly, optimizing the protocols for the production of haploid lines (microspore and anther culture) and efficient regeneration are proper fields of research targeted at producing new lines.

And fifth, unified collaboration is vital among research institutes engaged in modern activities of plant breeding, conservation of germplasm and biotechnology to improve safflower breeding projects.

Appendices

Appendix I: Research Institutes Relevant to Safflower

Institution	Specialization research activities	Contact information and website
Directorate of Oilseeds Research (DOR)	It is now known as Indian Institute of Oilseeds Research (IIOR), coordinate and monitor the research programs of oilseed crops from all over the India.	Rajendra Nagar, Hyderabad, India http://www.icar-iior.org.in

(continued)

Institution	Specialization research activities	Contact information and website
FAO	Food and Agriculture Organization of the United Nations is a specialized agency of the United Nations that leads international efforts to defeat hunger.	Rome, Italy http://www.fao.org
National Crop Gene Bank, Institute of Crop Germplasm Resources, Chinese Academy of Sciences	It is the long-term preservation center of crop germplasm resources and the research center of germplasm preservation technologies in China.	Beijing, China http://ics.caas.cn
USDA	United States Department of Agriculture is the U.S. federal executive department responsible for developing and executing federal laws related to farming, agriculture, forestry and food.	Washington, D.C., United States https://www.usda.gov/
Western Regional Plant Introduction Station (WRPIS)	It has the responsibility of maintaining seed and clonal germplasm of over 2600 plant species from 376 genera.	Pullman, Washington, United States http://grbio.org/institutional-collection/western-regional-plant-introduction-station-collection

Appendix II: Genetic Resources of Safflower

Cultivar	Important traits	Cultivation location
A1	Suitable under scanty and assured moisture regions	India
A-300	Moderately salt tolerance	India
AC Stirling	Early maturity and *Sclerotinia* head rot resistant	Canada
AC Sunset	Early maturity and *Sclerotinia* head rot resistant	Canada
AC1	early maturing, high linoleic content, wilt resistant	United States
AKS-207	High yield and high oil content	India
Alameda	High oleic acid	Spain
APRR-3	Resistant to rust	India
Bhima	High yield, moderately tolerant to aphids	India
Centennial	Resistance to *Alternaria* leaf spot and *Pseudomonas* bacterial blight	United States
CO-1	Non-spiny	India
DSH-129	High yield, resistant to wilt, moderately tolerant to *Alternaria* and aphids	India
Girard	High oil, high oleic acid, *Alternaria* resistance	United States
Girna	Moderately resistant to wilt	India

(continued)

Cultivar	Important traits	Cultivation location
Hartman	Resistance to leaf blight, high oleic content	United States
HUS-305	Moderately tolerant to wilt	India
JSF-1	Resistant to pest infestation	India
JSI-7	High yielding spineless variety	India
K-1	Moderately high yield	India
Leed	The first high oleic variety	United States
Manjira	High oil content	India
Merced	High linoleic acid	Spain
MKH-11	High yield, moderately tolerant to wilt, *Alternaria* and aphids	India
N-62-8	High yield	India
N-630	High yield	India
Nagpur-7	Salt tolerance	India
NARI-38	Spiny variety, tolerant to wilt	India
NARI-57	High oil, high yield, highly resistant to wilt	India
NARI-6	A non-spiny hybrid, moderately resistant to wilt	India
NARI-H-15	Moderately tolerant to aphids	India
NARI-NH-1	Non-spiny hybrid, moderately tolerant to *Alternaria* and aphids	India
Nebraska-10 (N-10)	Early maturity, high yield	United States
Nebraska-5	High yield	United States
Oker	High oil, high oleic acid, *Alternaria* resistance	United States
Pacific 7	Lower crude fiber, higher protein content	United States
PBNS-12	Moderately tolerant to aphids	India
Phule Kusuma	High yield	India
Rancho	High linoleic acid	Spain
Rinconda	High oleic acid	Spain
S-144	Tolerant to aphids	India
Saffire	Early maturity, high yield, rot resistance	Canada
Sharda	Moderately tolerant to aphids and wilt	India
Th5	High oil yield, early maturity	Canada
Tomejil	High linoleic acid	Spain
Type-65	Non-spiny	India
UC-148	Male sterile safflower line	United States
UC-149	Male sterile safflower line	United States
US 104	High yield and high oil content	United States

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Chapter 15
Advances in Sesame (*Sesamum indicum* L.) Breeding

Swapan K. Tripathy, Jayashree Kar, and Dibyabharati Sahu

Abstract Sesame is a high value and important oilseed crop owing to its dietary uses, health benefits and industrial applications. Sesame oil maintains a balanced fatty acid composition with more or less equal and higher percentages of unsaturated fatty acids. In spite of its several merits, it is behind in genetic improvement as compared to other commercial oilseed crops. Narrow genetic base, less attention to genetic improvement and cultivation in marginal lands with poor management practices are the major constraints for increased yield potential. Sesame has ample scope to breed cultivars with greater yield, as the gap between the potential and realized yields in this crop is enormous. Capsule shattering leads to heavy loss of seed yield and the crop is sensitive to a wide array of biotic and abiotic stresses. Innovative breeding approaches such as mutagenesis, somaclonal variation, interspecific hybridization, somatic hybridization and genetic transformation can be used to restructure the plant's ideotype. In addition, identification of candidate genes/quantitative trait loci (QTL) and their monitoring in succeeding breeding cycles using molecular markers can pave the way for genetic improvement in sesame. In this pursuit, the authors present a detailed outline of the importance of sesame as a potential oilseed crop, its biosystematics, floral biology, genomics, breeding goals, present status of breeding strategies and attention to prospects for sustainable production and productivity in future.

Keywords Achievements · Breeding constraints · Breeding strategies · Future prospects · Importance · Sesame

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15.1 Introduction

Sesame (til or gingelly) (*Sesamum indicum* L.) is recognized as the *queen of oil seeds* owing its high value oil quality and plethora of nutritive substances. Sesame is an important vegetable oil and its consumption is forecast to be 100 million mt by 2030 (Troncoso-Ponce et al. 2011). Traditionally, sesame seeds are used for making confectioneries, cookies, cake, margarine and breads. It is also used in paint formulation due to its unique semidrying property (Bedigian 2003a). It acts as one of the components for the manufacture of soaps, cosmetics, perfumes, insecticides and pharmaceutical products (Warra 2011). The oil is highly resistant to oxidative rancidity due to the presence of antioxidant lignans (Mohamed and Awatif 1998). Moreover, it promotes health and may provide relief from hypertension, oxidative stress and neurodegenerative diseases (Nakano et al. 2002).

Sesame is ranked ninth among the top 13 oilseed crops which make up 90% of the world production of edible oil (Adeola et al. 2010; http://www.statista.com/statistics/267271/worldwide-oilseed-production-since-2008/). India has the largest area under sesame which makes it the largest producer of sesame seeds (15%) in the world with an estimated production of 636,000 mt and productivity of 3419 kg/ha in 2013 (FAOSTAT 2013). India's total edible oil consumption is projected to increase 5.5–6% per annum. Therefore, there is an urgent need for enrichment of oil content in sesame seeds. A gene family comprising 34 lipid transfer protein type 1 (LTP1) genes are confirmed to have a major role in lipid biosynthesis (Wang et al. 2014) by strengthening the transport of fatty acids, acyl-CoAs and other lipid molecules (Kader 1996). Variation and selection are the two basic requirements of genetic improvement in any crop. Without variation, selection becomes ineffective. There exists a wide array of variation in oil content (43.3–51.7%) among the available genotypes (Spandona et al. 2013) and this can be increased by conventional breeding methods. Compared to other edible oil crops, sesame harbors a comparatively higher oil content (up to 55% of dry seed), and is thus an attractive potential model for studying lipid biosynthesis (Ke et al. 2010). However, the pattern of inheritance of oil content in sesame is not clear. Sesame oil is more variable in terms of quantity of oil than quality (fatty acid composition) (Baydar et al. 1999a). Erucic acid content remained unaltered while stearic, linolenic and arachidic acid contents were reported to be least affected over changing environments (Were et al. 2006a, b). There is no conclusive information on the number of genes controlling oil content and the exact nature of the gene (s) meant for biosynthesis of lipids although many researchers (Aladji Abatchoua et al. 2015; Vekaria et al. 2015) have addressed this problem using different base materials. In this pursuit, the authors reviewed the present status of breeding and suggested future prospects to increase yield potential and oil quality in sesame.

15.1.1 Cultivation Area, Production and Productivity

The major cultivated areas of sesame (90%) are in Asia and Africa. World sesame production was estimated at 6.1 million mt in 2016, with Tanzania, Myanmar, India, China and Sudan as the largest producers (https://www.tridge.com/intelligence) and FAOSTAT 2017) (Fig. 15.1). India ranked third in sesame production (866 million mt) with the largest area (1947 million ha) among different countries in 2015–2016 (http://agritech.tnau.ac.in/demic/pdf/2016/Price%20Forecasting%20for%20sesame-english.pdf). Despite its unique position, productivity in India is extremely low (413 kg/ha), below the world average yield (535 kg/ha), and about one-third the productivity of China (1234 kg/ha) (Figs. 15.2 and 15.3).

15.1.2 Uses and Nutritional Composition

Sesame seeds and their high quality oil are traditionally used as key ingredients in food products, salad preparation, pharmaceuticals and for making margarine, confectioneries, cookies, cake, breads, cosmetics (skin softener and in massage), antibacterial mouthwash and perfume (Bedigian 2003a). Nutrient-rich sesame seeds are mixed with cereals, rice, noodles and other dishes at mealtime as condiments. The seeds are often mixed with warm jaggery and sugar to make sweet balls eaten as a snack.

Low-grade oil is used locally in soaps, paints, varnishes, insecticides, lubricants (Blal et al. 2013), and as source of illuminants and biodiesel (Ahmed et al. 2010). The meal after oil extraction contains 30–35% protein that makes a rich feed for poultry and livestock. Sesame oil has both dietary (edible oil) and therapeutic applications. Sesame oil is also used as laxative, a solvent for intramuscular injections and as an ingredient in Ayurvedic medicine. In addition, sesame oil is used as a coating on stored grains to prevent weevil attacks.

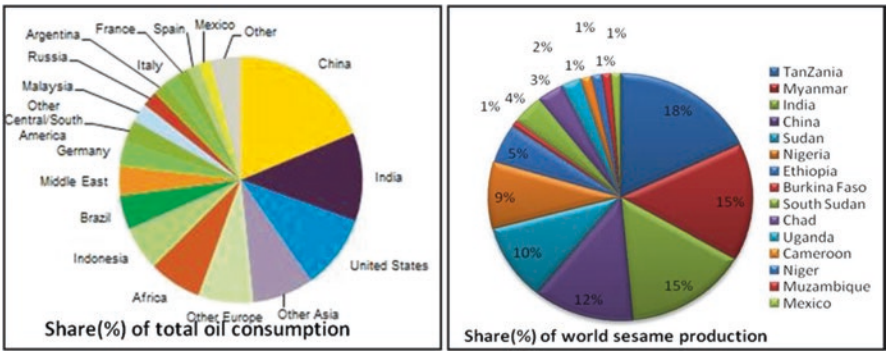

Fig. 15.1 Global scenario of total oil consumption and sesame production (2016). (Sources: https://ihsmarkit.com/products/fats-and-oils-industry-chemical-economics-handbook.html; https://www.tridge.com/intelligence)

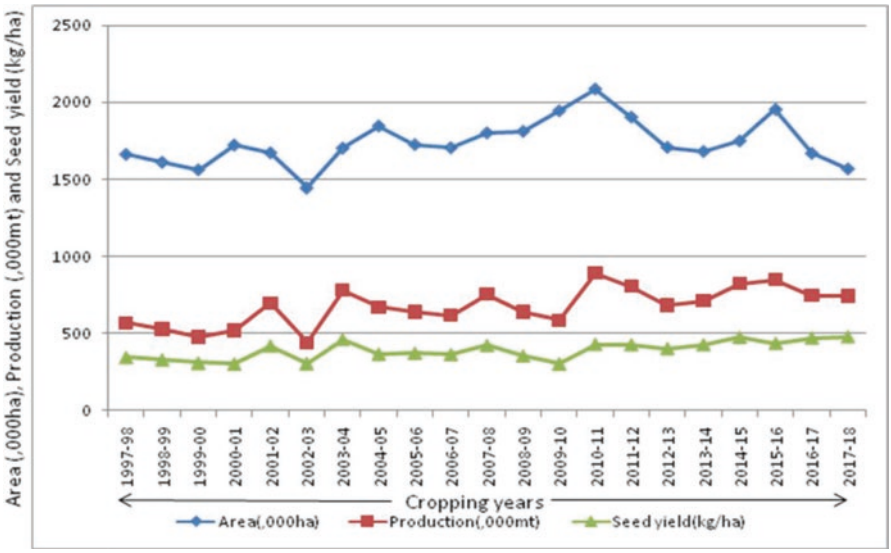

Fig. 15.2 Area, production and productivity of sesame in India. (Source: Ministry of Agriculture, Govt. of India)

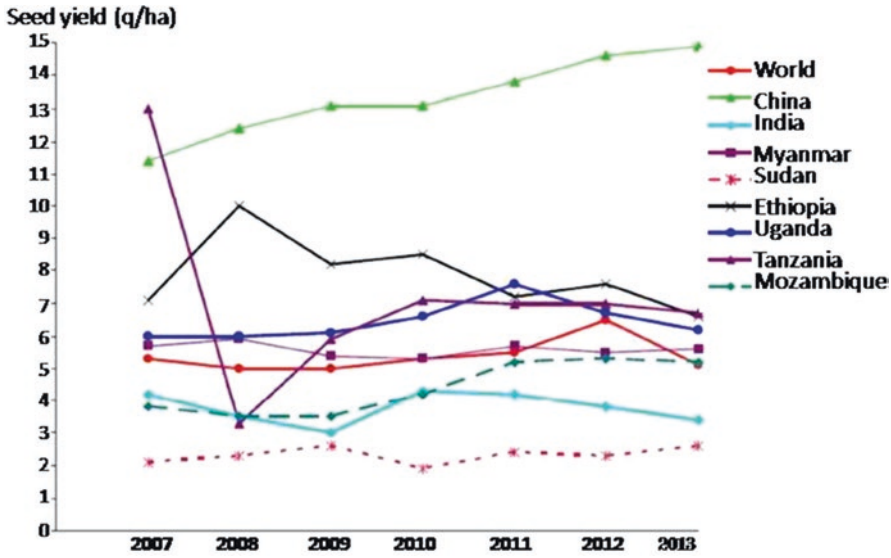

Fig. 15.3 Productivity of sesame in leading countries. (Source: FAOSTAT 2015)

Table 15.1 Nutritional value per 100 g dried sesame seeds

Constituents	Content
Moisture	4.7 g
Energy	573 kcal (2400 kJ)
Carbohydrate	23.4 g
Sugar	0.3 g
Dietary fiber	11 g
Protein	17.7 g
Fat	49.7 g
Saturated	7 g
Mono-unsaturated	18.8 g
Poly-unsaturated	21.8 g
Minerals	
Calcium	975 mg
Phosphorous	629 mg
Iron	14.6 mg
Zinc	7.8 mg
Magnesium	351 mg
Sodium	11 mg
Potassium	468 mg
Vitamins	
Vitamin A	9 IU
Thiamine (B1)	0.79 mg
Riboflavin (B2)	0.25 mg
Niacin (B3)	4.52 mg
Pyridoxin (B6)	0.79 mg
Folate (B9)	97 μg
Vitamin C	0.00 mg
Vitamin E	0.25 mg

Source: Link to Full USDA Database Entry (https://ndb.nal.usda.gov/ndb/foods/show/3620)

Sesame seeds are rich in oil and protein, and have high dietary energy value 5730 kcal/kg. Chemical composition of seed reveals that it contains 49.7% oil (fat), 17.7% protein and 23.4% carbohydrate (Table 15.1). Oleic acid (43%), linoleic acid (35%), palmitic acid (11%) and stearic acid (7%) are the most abundant fatty acids present in sesame oil, which together contribute 96% of total fatty acid content (Elleuch et al. 2007). Seeds are rich in iron, magnesium, manganese, copper and calcium, and important vitamins B_1 (thiamine) and E (tocopherol). Sesame fat is preferred in the food industry due to its delicate flavor and stability as well as high-quality cooking value. The nonglycerol fraction of sesame oil contains sesamin and sesaminol lignans that contribute to its oxidative stability and antioxidative property (Wu 2007). These antioxidants restrict oxidative damage to cells by detoxifying oxidative radicals. In addition, the above lignans serve as dietary supplements purported to prevent cancer and heart disease.

Sesame serves as the cheap source of high content dietary protein; vitamin B-complex which helps to improve the metabolism and nervous system, organs, eyes, muscles, skin and hair; magnesium, calcium, iron and copper – useful for red blood cell production, bone mineralization, enzyme synthesis and hormone production; high fiber content and high in mono-unsaturated fatty acid, oleic acid, lower bad cholesterol and increased good cholesterol in the blood and purported prevention of coronary artery disease and strokes. In addition, sesame proteins are rich in lysine, tryptophan and methionine.

15.1.3 Quality Features of Sesame Oil

Sesame seeds harbor oil in the cotyledons regarded as one of the highest oil content (up to 55%) among oil crops. It is used as edible oil and also in the pharmaceutical and chemical industries (Blal et al. 2013). The oil is colorless with distinct sweet flavor and quality is similar to olive oil (Kapoor 1990). It harbors a wide array of phytochemicals with antioxidant, antifungal, hypolipidaemic and hypoglycemic properties. Oil content and its chemical composition vary with genotype, color and size of the seed. Oil content in sesame varies from 35% to 63% among the available world collection of germplasm lines. Sesame oil maintains a balanced fatty acid composition with more or less equal or higher proportion of unsaturated fatty acids e.g., oleic acid (up to 39%) and linoleic acids (up to 46.26%) (Liu et al. 1992). The major saturated fatty acids are palmitic acid (7.9–12%) and stearic acid (4.8–6.1%) along with small quantities of vaccenic, linoleic, arachidic, behenic and eicosenoic acids. Cultivars with exceptionally high oleic or linoleic acid are rare. The stearic, oleic and linoleic acid contents differs between determinate and indeterminate cultivars. Determinate cultivars generally have higher stearic and oleic acids, and lower linoleic acid compared to indeterminate ones. Capsule position on the plant also affects the relative quantities of the fatty acids. Palmitic, stearic and oleic acids tend to increase up the stem while linoleic acid decreases. The fatty acid composition is strongly influenced by environmental factors (year, season). Among fatty acids, erucic acid content does not vary with the year, while stearic, linolenic and arachidic acid are least affected. Linoleic acid content has been reported to increase under cool growing conditions. The peroxide value and free acidity increases during storage over 5 weeks. The iodine value of the sesame oil decreases over a period of storage suggesting the loss of unsaturation in the fatty acids. Sesame oil is rich in essential fatty acids needed for normal growth and development.

Sesame oil is high quality semi-drying oil suitable for use in paint formulation (Bedigian 2003a). The presence of natural antioxidants such as sesamin, sesamolin, gamma tocopherol and sesamol, prevent rancidity of oil and, therefore, sesame oil is blended with less stable vegetable oils to improve their stability and longevity.

Usually, light colored and thin-coated seeds are higher in quality and content of oil than dark-colored seeds. In contrast, black sesame seeds are abundant in fiber, calcium, zinc, iron and vitamin B (Saha 2017).

Sesamin has bactericide and insecticide properties, but sesamolin has only insecticidal properties for which it is used as a synergist for pyrethrum insecticides. The oil elicits formation of higher concentration of lecithin, a phospholipid that acts as a powerful emulsifier (facilitating the dissolution of saturated fatty acids in an aqueous medium) to control blood pressure.

15.1.4 Health Benefits

Sesame is used for many reputed health promoting and anti-ageing benefits. The seeds and oils are widely used around the globe for the following health benefits. Sesame seeds are rich in magnesium which regulates the insulin and glucose levels in blood, lessening the chances of hypertension by reducing blood pressure and to manage diabetes effectively (Miyahara et al. 2001). High fiber content, phytosterol content and polyunsaturated fatty acids in seed also displace blood cholesterol. Fermented sesame often exerts anti-allergic effects (Jung et al. 2018). In addition, high zinc, calcium and phosphorus content in sesame purportedly prevent osteoporosis (Onsaard 2012). It also said to reduce inflammation due to the high copper content in sesame seeds. Intake of sesame seeds in the form of various food products is reported to clear up worms in the intestinal tract and also improve digestion due to its high fiber content (helps in making food ball up and move through the digestive tract) and hence, reduces constipation and diarrhea. Sesame seeds are rich in antioxidants and reduce the effects of free radicals (Nupur et al. 2010). This purportedly reduces the chances of colon cancer.

Sesame oil is also used as carrier oil for many cosmetic products. It serves as a good moisturizer and massage oil (making the skin soft), and is said to improve blood circulation and joint pains. Sesame oil may improve blood circulation due to high copper content required to produce red blood cells. It is an anti-aging of skin due to sesamol (an antioxidant) and vitamin E (gamma-tocopherol-an isomer of vitamin E) that prevent the skin cells from oxidation, repairs damaged skin cells and is claimed to avoid the appearances of wrinkles (Weldemichael and Juhar 2018). Antibacterial properties of linoleic acid and palmitic acids of sesame oil prevent bacterial infections of skin wounds. The high magnesium and phytate content of sesame oil makes it a natural sunscreen as it serves as a natural UV protector.

High zinc, copper and calcium content in sesame oil may improve bone growth. In addition, high iron content in sesame oil may act as a natural cure of anaemia. Nutritionally, it is rich in vitamin B-complex and vitamin D; while high zinc and magnesium content may boost metabolism. Sesame oil purportedly prevents diabetes by stabilizing blood glucose level (Miyahara et al. 2001) and may keep hepatitis and migraines at bay. In addition, use of sesame oil may help in treating premature graying of hair and boost dental health. Consumed as part of a balanced diet, sesame may have health benefits; however, there is scant scientific evidence to substantiate the various claims of its medicinal properties.

15.2 Biosystematics

15.2.1 Origin and Distribution

Sesame is one of the oldest oilseed crops in the world. It belongs to the genus *Sesamum* and the family Pedaliaceae, which contains more than 38 species (Kobayashi 1991). Most of these species are distributed in the tropics and subtropics of Africa and some are common in both Asia and Africa. Archaeological surveys demonstrate domestication of sesame in South India about 1500 years ago and the sesame trade with other countries (Mesopotamia) dates back to 2000 BC (Fuller 2003). There exists high morphological and cytogenetic homology (Bedigian 2003a, b) and close molecular affinity (Nanthakumar et al. 2000) between cultivated sesame (*S. indicum* L.) and the south Indian native species *S. mulayanum* (progenitor). In addition, genetic variation is enormous among the cultivated forms of Indian origin (Ganeshan 2001). Uncu et al. (2015) revealed a common pattern of gene flow between *S. indicum* and *S. mulayanum.* Therefore, it is believed that the wild form *S. mulayanum* is the progenitor and the Indian subcontinent the origin of sesame (Ganeshan 2001).

15.2.2 Taxonomy and Cytogenetic Elucidation of Sesame Evolution

Taxonomically, *Sesamum* is related to *Utricularia gibba* as revealed by high throughput genome sequencing; it is estimated to have diverged from *U. gibba* approximately 98 million years ago. The 38 species are classified into three groups on the basis of chromosome numbers. A few important species are indicated below (Table 15.2).

Table 15.2 Important *Sesame* species with chromosome number variation

Group	Species	Chromosome number	References
1	*Sesamum indicum* *S. latum* *S. capense* *S. malabaricum* *S. mulayanum* *S. schenckii* *S. africanum*	2n = 26	Ram (2011)
2	*S. prostratum* *S. laciniatum* *S. angolence* *S. angustifolium*	2n = 32	Ram (2011)
3	*S. radiatum* *S. occidentale* *S. schinzianum*	2n = 64	Ram (2011)

The basic chromosome number in sesame is x = 8 and 13. Giemsa banding allows identification of all the 13 chromosomes. The most closely related genera *Cerathotheca* and *Pedalium* contain the basic chromosome number x = 8; while chromosome number of the wild form *Sesamum mulayanum* (progenitor) is the same (x = 13) as the cultivated species *S. indicum*. A number of researchers have reviewed the evolutionary relationship within and among the chromosome number groups (2n = 26, 32 and 64) (Nayar and Mehra 1970; Prabhakaran 1996). The detailed taxonomic hierarchy of the present-day cultivated sesame (*Sesamum indicum* L.) is as follows:

Kingdom: Plantae – Plant kingdom
Sub-kingdom: Viridiplantae
Infrakingdom: Streptophyta
Superdivision: Embryophyta
Division: Tracheophyta
Sub-division: Spermatophytina
Class: Magnoliopsida
Superorder: Asteranae
Order: Lamiales
Family: Pedaliaceae
Genus: *Sesamum*

15.3 Botany, Floral Biology and Crossing Techniques

15.3.1 Botany

Sesame plants are generally tall and branched with an indeterminate growth habit. Some nonbranching/shy branching types are also found. Plants are annual or biannual herbs, the stems bear ridges, the leaves are sessile at the bottom and petiolate from middle to top, with entire margins; sometimes with lobed leaves. Leaves are arranged in opposite phyllotaxy and carry oil glands on the dorsal side. Flower color is mostly white, but pink, purple or various shades of purple-white flowers also occur.

15.3.2 Floral Biology

Flowers are solitary, axillary, shortly pedicilate and zygomorphic with pendulous tubular corollas 3–4 mm in length (Fig. 15.4). They are hermaphrodite and borne (singly or in groups) in the leaf axils. Each flower bears 4 stamens in didynamous condition (2 long and 2 short), filaments are dorsifixed, epipetalous and anthers dehise longitudinally. The gynoecium is multicarpelar with a superior ovary (hypogynous) with a long style and bifid stigma. The flower produces nectar in a nectary

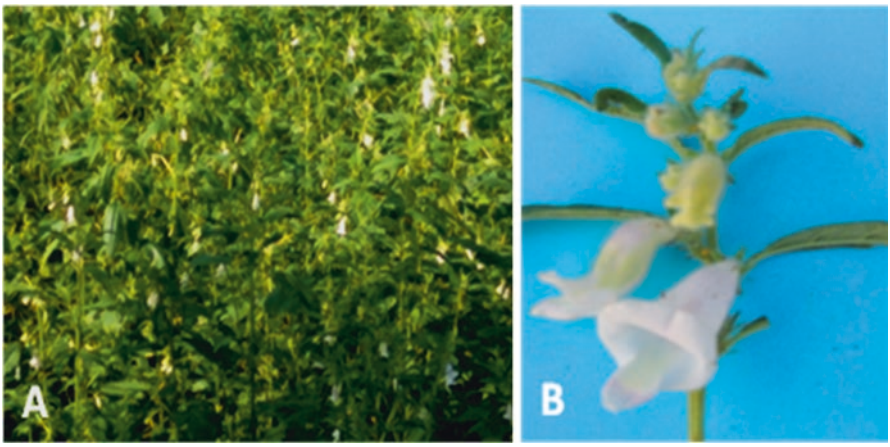

Fig. 15.4 Sesame: (**a**) Field view, (**b**) Floral morphology

disk-like structure surrounding the ovary. The calyx bears 5 fused sepals. The corolla is tubular with a lobe upwards. One of the petals is extended and serves as a landing platform for the visiting insects.

Stigma receptivity varies with genotype (Langham 2007) and it usually lasts 24 h after flower opening (Abdel et al. 1976), although loss of viability within 14 h is also reported (Yermanos 1980). In addition, some reports state that the start of stigma receptivity is from 2 h after anthesis (Free 1993) and duration of pollen viability for 24 h at 24–27 °C after dehiscence of the anther (Yermanos 1980).

15.3.3 Mode of Pollination

Sesame is predominantly a self-pollinated crop although outcrossing occurs and may vary from 5% (Langham 1944) to as high as 68% (Ashiri 2007) in field-grown crops under tropical condition. Therefore, required isolation distance is approximately 180–360 m. Flowers open at morning; anthers dehisce very shortly and stigma remain viable for about 24 h. Pollination must occur between 7 and 11 am to ensure a greater fruit set; the flower withers after 4–6 h of anthesis.

15.3.4 Selfing and Crossing Techniques

Crossing between parent varieties differing in flowering and maturity duration are staggered and sown thrice at 10-days interval. The varieties are grown in 3 rows each of 4 m length on every sowing. Any off-types are rouged out. Hybridization is initiated as soon as about 50% of plants of a variety start flowering.

Selfing in sesame can be ensured by tying the corolla tips of mature flower buds with threads in the evening. For emasculation, the epipetalous corolla is pulled out

leaving the gynaecium intact before the opening of the flower. A plastic straw with one side bent, is used to cover the emasculated flower bud at evening. Pollination is carried out the following morning and the plastic straw is placed in position. Alternatively, the corolla (with stamens intact) of the desired male parent, which is about to open is placed on to the emasculated flower using a speck of fevicol (Das 1990) for pollination and follow-up fertilization. A speck of fevicol is to be placed on the top of the corolla in late afternoon hours to prevent its opening.

15.4 Factors Affecting Sustainable Production and Quality of Sesame

Genotype Farmers prefer high yielding stable sesame cultivars with inherent resistance to major pests and diseases, good end use quality, and exportable quality (white seed color, large seed size and good flavor). The cultivar must be resistant to bacterial blight in high rainfall areas, early maturing in low rainfall areas and resistant to phyllody under irrigated condition.

Adaptability The cultivars and breeding lines so developed have poor adaptability to production systems in the area of cultivation. Selective mid-early genotypes with tolerance to biotic and abiotic stresses can cope best with the changing environments across different locations.

Yield Potential Important components traits e.g. number of capsules per plant, seeds/capsule and 1000-seed weight are the important consideration for improvement of productivity. Eight-loculed plants have more seeds per capsule but they are not necessarily the highest yielding cultivars as they bear a comparatively fewer number of capsules.

Harvest Index Modification of plant architecture is needed to improved the harvest index. This can be achieved by selection of medium plant height (around 1.0 m) with high density capsule bearing starting from 15 to 20 cm above the ground.

Crop Management Sesame is mostly grown under low-input conditions. Global average productivity of the crop is around 340 kg per ha, but it may go up to 2250 kg per ha using advanced management practices (Brigham 1987). Selective sesame genotypes with a specific ideotype and wider adaptability can endure in diverse environments and respond better to intensive management practices.

Biotic Stress Resistance Sesame is sensitive to bacterial blight, *Fusarium* wilt (*Fusarium oxysporum* Schlecht. emend. Snyder & Hansen) and charcoal rot (*Macrophomina phaseolina* (Tassi) Goid.) in high rainfall areas and phyllody (caused by *Phytoplasma*) under irrigated condition (Ojiambo et al. 1999). Similarly, the crop may be drastically affected by major insect pests such as the leaf webber/roller and capsule borer (*Antigastra catalaunalis* Duponchel), sphinx moth

(*Acherontia styx* Westwood), aphids (*Aphis gossypii* Glover) and gall-midge/gall fly (*Asphondylia sesami* Felt). There is a need to develop cultivars with multiple resistance to the above biotic factors.

Abiotic Resistance Sesame is generally cultivated in marginal lands. Therefore, cultivars with inherent tolerance to drought stress and better water use efficiency are mostly preferred. In addition, sesame is sensitive to salinity, waterlogging and chilling which limit sustainable production.

Shattering Resistance Almost all cultivars are of the shattering type and 99% of the fields are harvested manually, leading to 60–70% yield loss under dry weather (Georgiev and Stamatov 2005). There is a need to reorient breeding strategy to alleviate the high costs of manual harvesting and yield loss due to shattering. Development of new high-yielding cultivars with semi-indehiscent capsules is a possible option to fit mechanized farming.

Oil Content and Fatty Acid Composition Sesame is a high value high yielding oil crop. Oil yield can be increased by improving mature seed yield and oil content (˃50%). Light seed color cultivars harbor higher oil content than the dark seeded cultivars. A balanced fatty acid composition with proportionately higher percentages of unsaturated fatty acid (linoleic and oleic acids) as compared to saturated fatty acid (stearic and palmitic acids) together with higher amounts of antioxidants (sesamol, sesaminol) and tocopherols is needed for high quality export value.

Confectionery Quality For confectionery uses, the breeding lines should have favorable seed color, size and shape (Fig. 15.5). White-seeded types are most preferred for export. In addition, these should be screened for desirable texture and seed coat thickness, and oil flavor using specific descriptors. The seeds should be readily dehulled by processors for use in sweets, halva and bakery products or milled to produce high-grade sesame oil.

Fig. 15.5 Seed color development at physiological maturity. (**a**) White seed, (**b**) biscuit color seed, (**c**) black color seed

15.5 Genetics

15.5.1 Genetics of Qualitative Traits

The knowledge of inheritance pattern of various traits is a priori to any genetic improvement in sesame. Pioneering works on sesame genetics date back to Langham (1945–1947). Thereafter, a number of studies were published on the mode of inheritance of various traits using diverse sesame germplasm resources. Subsequently, these were reviewed by Joshi (1961), Weiss (1983) and Ram (2011) in detail. The information is summarized in Table 15.3.

Table 15.3 Inheritance pattern of qualitative traits in sesame

Traits	Mode of inheritance
Growth habit	Branched dominant over unbranched (Nb, nb)
Stem characteristics	Normal vs fasciated stem – single or duplicate genes involved (F_1, f_1; F_2, f_2)
Flowering trait	Solitary is dominant over multiple (2–3) flowers and capsules/ leaf axil (T, t)
	Indeterminate is monogenic dominant over determinate habit (Dt, dt) (Cagirgan et al. 2009)
Capsule trait	Bicarpellate is dominant over quadricarpellate (Tc, tc)
Male sterility	Fertility is dominant over male sterility (Ms, ms)
Capsule dehiscence	Dehiscent vs indehiscent are monogenic with pleiotropic effect (Id, id) or two genes with complementary gene action
Capsule hairiness	Capsule hairiness is dominant over hairless
Capsule number per leaf axil	One capsule/leaf axil is dominant over three capsules/axil
Phyllody resistance	Single dominant gene (Singh et al. 2007), or two dominant genes with complementary gene action (Vanishree et al. 2013)
Powdery mildew	Susceptibility is dominant over tolerance and is controlled by two independent recessive genes with complementary epistasis (Rao et al. 2011)
Alternaria leaf spot	Polygenic inheritance with significant additive effect
Stem rot	Polygenic inheritance with equal proportion of positive and negative alleles in the parents
Fusarium wilt	Polygenic inheritance with equal proportion of dominant and recessive genes in the parents
Seed coat color	Brownish black is monogenic dominant over white seed color. In some crosses, F_2 plants segregated into black, brownish white, brown and white-seeded types indicating complex nature of inheritance (Falusi 2007). Zhang et al. (2013) identified two major genes with additive-dominant – epistatic effects; and four QTLs (QTL1-1, QTL11-1, QTL11-2, QTL13-1) with additive-dominant-epistatic effects

15.5.2 Genetics of Agro-economic Quantitative Traits

Gene action for agro-economic quantitative traits has been studied by different researchers. Some also reported the importance of both additive and nonadditive components of variation. Usually these follow the inheritance pattern as below, although they may vary depending upon different sets of materials. They include the traits listed in Table 15.4.

The presence of both additive and nonadditive gene action operates for all morpho-economic traits (Mungala et al. 2017) although their magnitudes proportionately differ. Singh (2004) reported higher variance due to general combining ability (GCA) than specific combining ability (SCA) effects for days to maturity, plant height and 1000-seed weight, indicating a greater role of additive gene action in the inheritance of these characters. In contrast, Solanki and Gupta (2003) analyzed combining ability for yield and its components in a 6-parent half diallel cross and reported that GCA and SCA effects were similar in magnitude for seed yield indicating equal importance of both additive and nonadditive gene action for productivity. In another set of materials, variance due to the SCA effect was greater than that of GCA for number of capsules per plant and seed yield indicating greater role of nonadditive gene action in the inheritance of these traits (Saravannan and Nadarajan 2003; Singh 2004). Preponderance of nonadditive gene action was also revealed for days to maturity, plant height, number of primary branches per plant, capsule length, seeds per capsule, 1000-seed weight, yield and oil content except days to flowering where GCA was greater than SCA (Prajapati et al. 2006). Balaram et al. (2018) reported the preponderance of additive effects for days to flowering, seeds/capsule and oil content. Differential magnitudes of GCA and SCA effects for above various traits could be due to different composition of experimental materials used. Mishra et al. (2016) reported additive gene action for days to maturity, number

Table 15.4 Inheritance pattern of agro-economic quantitative traits in sesame

Traits	Mode of inheritance
Flowering duration	Additive gene action
Leaf chlorophyll content	Nonadditive
Capsule length	Both additive and nonadditive
Seeds per capsule	Both additive and nonadditive
Capsule bearing nodes	Additive gene action
Main fruiting stem length	Both additive and nonadditive
Number of capsules/plant	Both additive and nonadditive
Photoperiod response	Polygenic inheritance
Photosynthesis rate	Additive gene action
Leaf area index	Nonadditive
Height to first branch	Nonadditive
Effective branches/plant	Nonadditive
Harvest index	Additive
Seed yield	Both additive and nonadditive

of primary branches/plant and capsule breadth for which pedigree selection would be an appropriate method to have higher selection response for these traits. In contrast, heterosis breeding may be exercised for period of flowering, number of capsules/plant, oil content and seed yield/plant owing to their greater magnitude of nonadditive variance. However, number of days to cessation of flowering and seeds/capsule exhibit more or less equivalent GCA and SCA effects. In this context, diallel selective mating along with recurrent selection would be useful for recovery of desirable homozygotes in later generations. Tripathy et al. (2016a) reported that a number of groups of genes or loci having recessive alleles with increasing effect might be involved in realization of high seed yield and involvement of modifiers in the background genotype could not be ruled out. Pratap x RT103, CST785 x E8 and BS 5-18-6 x Phule Til-1 revealed high SCA effect for seed yield in which at least one parent in each case was reported to have high GCA (Tripathy et al. 2016b). On the other hand, a few of the crosses e.g. B67xE8, B67xRT 103 and RT 103xT13 being good specific combiner for oil content, did not involve any parent with high GCA indicating role of dominance and epistatic gene interaction in these crosses. Such above crosses are likely to be useful for genetic improvement of seed yield and oil content. Balaram et al. (2018) reported a preponderance of additive effects for days to flowering, seeds/capsule and oil content.

15.5.3 Genetic Basis of Oil Content and Fatty Acid Composition

Comparative genomic and transcriptomic analyses have revealed the mechanisms of lipid biosynthesis in sesame. Lipid biosynthesis is associated with tandem duplication in Type 1 lipid transfer genes, truncation of genes related to lipid degradation and tissue specific expression of genes in the triacylglycerol biosynthesis pathway during early stage of seed development (https://phys.org/news/2014-03-genome-sesame-oil biosynthesis.html). Thus, most of the candidate genes related to lipid biosynthesis are involved in one of the three pathways: (a) fatty acid and TAG (triacylglycerol) synthesis and elongation, (b) TAG degradation or (c) fatty acid dehydrogenation (e.g. Stearoyl-ACP desaturase, determining the ratio of saturated and unsaturated fatty acids). Li et al. (2014) performed the association mapping of oil content and found it varied from 27.9% to 58.7% and protein content from 16.7% to 27.8% among 369 worldwide germplasm accessions under 5 environments using 112 polymorphic SSR markers. Among these, 19 and 22 SSR markers were linked with oil content and protein content, respectively, with high phenotypic variation. In addition, a genome wide association study revealed a total of 13 significant associations for oil content (Wei et al. 2015).

Usually, oil content maintains a weak positive correlation with oilseed yield but, negatively with the protein content in sesame. Modern cultivars more or less show lower nucleotide diversity than landraces. However, there exist no significant asso-

ciations between the allelic variation for the seed oil content and the yield traits, suggesting that it would be possible to generate sesame cultivars with both high yield and oil content.

Fatty acid composition displays less genetic variability among cultivars than the oil content. The genetics of oil content indicated importance of both dominant and additive gene action. A group of genes with *dominant* and *increasing* alleles and some modifying genes are likely to be involved in the biosynthesis of oil in seeds. Whole genome scanning can help to gain insight into the oil biosynthesis to understand oil content in the seed. Recently, a gene family consisting of 34 lipid transfer protein type 1 (LTP1) genes was identified to have key role in lipid biosynthesis. LTP1 family help in oil accumulation by strengthening the transport of fatty acids, acyl-CoAs and other lipid molecules. However, until now, there is no conclusive information on the number of genes controlling oil content and fatty acid composition. As many as 46 candidate causative genes encode the enzymes involved in fatty acid biosynthesis and oil content. Lignification and black pigmentation in the seed coat are controlled by two major candidate genes and these are also associated with large variation in oil content. SNP analysis using genome wide scanning revealed a 100 bp 'A' in the low-oil allele of cv. Mishuozhima. This allelic variant has a very high expression level in seeds 8 days after pollination. The genes strongly associated with oil content in sesame also have a major role for sesamin and sesamolin content. Fortunately, such allelic variation for the seed oil content is not linked to yield traits, suggesting the possibility to breed high yielding sesame varieties with high oil content. Weak negative correlation exists for oil content with palmitic and linoleic acids, and feeble positive association with stearic and oleic acids. This indicates no overlap(s) between the associated loci for the traits. However, the content of different fatty acids is often correlated and the shared associations are possibly due to common candidate genes (*SiACNA, SiDGAT2, SiFATA, SiFATB, SiSAD*) being involved.

15.6 Genomics of Sesame

The sesame genome size is about 350 Mb and it is largely unexplored. The phenotypic and genome sequence information is available from the data base SesameFG (http://ncgr.ac.cn/SesameFG/) which can serve as an important tool for functional genomic research and molecular breeding of sesame (Wei et al. 2017). Dossa et al. (2017) developed a SSR marker-based sesame genome map. As many as 27,148 genes have been annotated in a sesame reference genome which has a relatively low proportion of repetitive sequences (28.5%) (Wang et al. 2014). Dinucleotide repeat motifs are the most common (84.24%), followed by 13.53% trinucleotide, 1.65% tetranucleotide, 0.3% pentanucleotide and 0.28% hexanucleotide motifs in the ses-

ame genome sequence (Wei et al. 2014). Candidate genes represent loci that encode components of metabolic or signaling pathways known to be related to the corresponding phenotypes or based on expression profile. Using whole sesame genome analysis, Supriya and Bhat (2018) revealed 8244 functional genes, 58 transcription factors and 25,069 transposable elements in the sesame genome. Multiple loci that related to several agronomic traits such as plant height (Ding et al. 2013), disease resistance (Zhang et al. 2012), drought tolerance (Li et al. 2013), waterlogging tolerance (Zhang et al. 2014), seed coat color (Zhang et al. 2013) and oil and protein content (Li et al. 2014) had been identified in sesame land races (cvs. Baizhima and Mishuozhima). Next generation sequencing (NGS) revealed a total 1,332,025 SNPs (single nucleotide polymorphisms) and 506,245 indels (insertion-deletions) by genome comparison with Zhongzhi 13 (white seeded). Indels are more polymorphic than SSRs but these are comparable in terms of deciphering genetic diversity (Wu et al. 2014). Seed color varies from black, intermediate colors to white. Black-seeded types usually harbor less oil but more protein and lignin content (Zhang et al. 2013) than black-coated seeds. The polyphenol oxidase (PPO) gene produces black pigments is the key regulatory gene of sesame seed coat color (Wei et al. 2017). Sesame was domesticated 5000 years ago, then dispersed to quite different environment along trade routes and thereby underwent natural selection to adapt to new conditions (Bedigian 2003b). During the domestication of land races, some of the variations (indels) may have come from the natural selection. Rare SNPs and indels can serve as useful markers to assist genetic improvement and delineate status of variation across genomes (Ohmido et al. 2000). The regions saturated with high SNPs and indels contain genes with elevated localized mutation rates or recombination hot spots (Lercher and Hurst 2002). High stringency filtering resulted in the identification of 420 SNPs distributed among 13 linkage groups. These markers will be useful to tag QTLs of metabolic and agronomic traits (Uncu et al. 2016).

15.7 Genetic Diversity

In any crop breeding program, the existence of adequate genetic variability is the prerequisite for selection to be effective to create the desired genotype. In general, the genetic variability in available *Sesamum* germplasm is limited. Sesame has a long history of domestication. Owing to continuous cultivation in marginal lands under rainfed situation, some plant types have adapted to survival through natural selection. Therefore, in the course of sesame evolution, most of the valuable genes associated with high yield have been eroded. In addition, modern plant breeding with limited use of land races (as parents), has presumably narrowed the genetic basis of cultivated sesame. Genetic variation was subsequently reduced by genetic drift and selection. Therefore, broad-based genetic resources have become more

important for genetic improvement in sesame. A complete array of sesame germplasm consists of: (a) wild distant relatives and closely-related species, (b) local land races, (c) obsolete breeding lines including mutants and (d) newly developed improved varieties.

Knowledge of genetic variation, inheritance pattern and interrelationship of plant traits is a priori for effective use of germplasm in any genetic improvement program (Ganesh and Thangavelu 1995). Germplasm banks are reservoirs of the valuable genes essential for improvement of crop species. Despite high nutritional value, sesame is still lagging behind in the achievement of breeding perspective. No international agency has yet come forward to work on sesame (Bedigian 2003a, b). Centers for sesame genetic diversity are found in India, China, Central Asia and Ethiopia (Hawkes 1983). However, information on their genetic diversity is not explicitly documented. Such a stock of information is of immense importance for planning genetic conservation strategies and for use of elite germplasm lines in breeding programs.

Crop genetic diversity can be determined with the aid of morphological, biochemical and molecular markers (Stuber 1992) or in combination. Several studies have exploited the morphological genetic diversity in sesame populations (Arriel et al. 2007; Bisht et al. 1998). However, characterization of cultivars for genetic diversity by molecular markers is of great value to assist phylogenetic relationships, parental line selection, population structure and allele distribution among germplasm lines and to design breeding strategies. Presently, molecular techniques including isozymes (Isshiki and Umezaki 1997) and DNA profiles based on amplified fragment length polymorphism (AFLP), simple sequence repeats (SSRs), intersimple sequence repeats (ISSRs) and random amplified polymorphic DNA (RAPD), all widely used to study genetic diversity in sesame (Abdellatef et al. 2008; Erkan et al. 2004; Kim et al. 2002; Kumar et al. 2012; Pham et al. 2009; Salazar et al. 2006; Uncu et al. 2015). Dossa et al. (2016) studied the genetic relatedness among 96 sesame accessions collected from 22 countries and found that the genetic diversity observed in African accessions was lower than that in Asian accessions. Indels are more polymorphic than SSRs; hence, they can comparatively reveal maximum degree of allele diversity in a set of genotypes. Wu et al. (2014) detected 325 alleles including some unique alleles among 130 Chinese sesame accessions. Morphological and molecular markers help scientists and plant breeders in screening and selection (Pham et al. 2011) of germplasm and their use in breeding program. However, little progress has been made in this regard in sesame (Bhat et al. 1999). Development of genetic maps (Dossa et al. 2017) and availability of few closely-linked molecular markers (using the AFLP technique) have made it possible for reliable screening of sesame germplasm. Uzun et al. (2003) detected closely-linked molecular marker for the closed-capsule mutant trait and it helped to introgress shattering resistance in sesame.

15.8 Germplasm Conservation

Germplasm serves as the source of valuable genes. Conservation of genetic resources has had an impact on today's cultivated crops (Ishaq and Falusi 2008). Wild related species and local land races are primary sources of genetic variation due to the evolution of the crop over time. The preservation and protection of genetic resources may be done in their natural habitat (in situ) e.g. national parks and government reserves (Ford-Lloyd and Jackson 1986). However, ex situ conservation (collection and maintenance by research institutes/universities) ensures safety, proper characterization, documentation and ready accessibility of materials. Genetic stocks are also preserved in sealed test tubes over nutrient media in the form of cells in a culture room, calli or tissue (in vitro conservation) or cryopreserved for more than 10 years at ultra low temperature (cryopreservation). In addition, DNA banks are most preferred for those species that do not produce seeds and are not possible to conserve in situ due to high risk factors.

India has a rich diversity of sesame and a large collection of landraces maintained at the National Bureau of Plant Genetic Resources (NBPGR). The Ethiopian Institute of Agricultural Research collected 221 germplasm lines during 2002–2004 and preserved these lines at the Werer Agricultural Research Center (Teshome et al. 2015). A core collection of world sesame germplasm accessions (over 2100) is held in Israel, and now also maintained in gene banks in Kenya, Korea and India. These were developed to facilitate efficient germplasm management and effective use in crop improvement (Mahajan et al. 2007).

15.9 Breeding Goals

Sesame breeding objectives are primarily focused on improved seed retention in the capsule, increased oil content, uniform maturity and disease resistance. For more clarity, the following breeding goals deserve special mention:

(a) Ideal plant type: Determinate plant type, higher numbers of leaf axils for more capsule bearing/plant, high yield and stability of performance;
(b) High oil content, improved fatty acid composition and other quality traits;
(c) Low or zero antinutritional factors (oxalic and phytic acids) for value addition;
(d) Development of CMS lines for ease in hybrid seed production;
(e) Resistance to biotic stress: insect pests (leaf eating caterpillar, gall fly) and diseases such as phyllody (virus, mycoplasma), bacterial leaf spot (*Pseudomonas sesami*), powdery mildew, wilt and leaf curl;
(f) Semi-indehiscence of capsules;
(g) Resistance to abiotic stresses: drought, waterlogging and salinity, particularly under the scenario of global climate change.

15.10 Distinctness, Uniformity, Stability Characterization and Prebreeding

It is in vogue to characterize cultivars for DUS (distinctness, uniformity, stability), characteristics based on morphological expressions (Goodrich et al. 1985). However, often the morphological markers are not quite enough to reveal the genetic diversity between the morphological overlap cultivars and the morphological identical accessions. DNA and seed storage protein profiling of genotypes can provide an analytical tool for genome probing and reliable cultivar identification (Gilliand 1989). In addition, this serves as a useful tool to eliminate duplicates and/or very similar genotypes resulting in a core germplasm stock (prebreeding) without reducing the existing genetic diversity.

Sesame displays contrasting genetic variation in morpho-economic traits, oil content, quality features (seed shape, color, length) and resistance to biotic and abiotic stresses. This helps to identify segregants in a crossing program and accelerate crop improvement (Weiss 1983). Mathur et al. (2016) characterized 16 *Sesamum* cultivars for DUS testing. Similarly, Singh et al. (2017) carried out DUS testing of 83 cultivars of sesame using 18 morphological descriptors. They revealed maximum variation in seed coat color, capsule shape, leaf lobe, leaf size, leaf serration, capsule hairiness, capsule arrangement, stem hairiness, petal color, seed size, days to 50% flowering, branching pattern and petal hairiness. The standard evaluation system using DUS characteristics as per the Protection of Plant Varieties and Farmer's Rights (PPV & FR), Govt. of India for sesame are given in Table 15.5.

15.11 Breeding Approaches

Creation or existence of genetic variability is a prerequisite for selection of better plant types and follow-up establishment of a pure breeding line in sesame. A general scheme of different breeding approaches followed in sesame breeding is shown in Fig. 15.6.

15.11.1 Plant Introduction

Useful new sesame seed are in vogue, introduced from their place of origin to new areas of cultivation across geographical boundaries and as such used directly or recommended for release for commercial cultivation after minimum selection. Two new outstanding cultivars, cv. Dangur (EW 013) and cv. Chalasa (EW023), in Ethiopia are such introductions. The Mexican cv. Yori 77 was introduced to Northern Australia where it has performed well. The Venezuelan cv. Morada was a selection from introduced materials from Republic of Congo and also performed well in Tanzania. Similarly, cv. Early Russian was introduced from Texas and released directly in South Korea (https://books.google.co.in/books?isbn=1420005367). In

Table 15.5 Distinctness, uniformity, and stability (DUS) characteristics of sesame

Characteristics	States	Score (1–9)	Example cvs.	Observation stage	Assessment type
Days to 50% flowering	Early <36 Medium 36–45 Late >45	3 5 7	RT 125,RT 54 TKG 21, JCS94 Rajeswari	45	VG
Flower petal color	White Light purple Dark purple	1 2 3	Kalika RT 54 RT 103	45	VS
Flower petal hairiness	Absence Sparse Dense	1 3 5	– RT 125 Rajeswari	45	VS
Plant height of main stem (cm)	Short <75 Medium 75–125 Tall >125	3 5 7	RT 125, JTS 8 GT 1, N 32 Rajeswari	65	MS
Plant branching	Absent Few 1–2 Medium 2.1–4.0 Profuse branching >4	1 3 5 7	N32 GT 1, JCS 94 TKG 55, RT-T25,T13, RT 46	65	VS
Plant branching pattern	Basal branching Top branching	1 2	RT 127 AKT 64	65	VS
Stem hairiness	Absent Sparse Dense	1 3 5	Rajeswari T 12 B67, RT 46	65	VS
Leaf lobes	Slight lobed Deeply lobed	1 2	GT 10, N 32 Rajeswari	65	VG
Leaf size	Small Medium Large	3 5 7	VRI 1, Gauri TKG 22 Rajeswari	65	VG
Leaf margin serration	Weak Strong	3 5	TKG 21 Sweta Til	65	VG
Capsule hairiness	Absent Sparse Dense	1 3 5	Rama, T 78, Chandana JCS 94, GT 2	65	VS
Capsule locule number	4 6 8	3 5 7	TKG 22 – Adarsh 8	75	VS
Capsule shape	Tapered Narrow Oblong Broad oblong	1 2 3 4	GT 10 TKG 21 Phule Til-1 –	100	VG
Capsule number per leaf axil	1 More than 1	1 9	Thilak G.Til-1, GT 2	100	VG

(continued)

Table 15.5 (continued)

Characteristics	States	Score (1–9)	Example cvs.	Observation stage	Assessment type
Capsule arrangement	Alternate Opposite Cluster	1 2 3	RT 46 TKG 22, N32 G.Til-1, GT 2	100	VG
Capsule length (cm)	Short <1.5 Medium 1.5–2.5 Long >2.5	3 5 7	– Adarsh 8, GT 2 AKT 64	100	MS
Days to maturity	Early <75 Medium 76–85 Late 86–95 Very late >95	3 5 7 9	RT 54 RT 125 Swetha Til Rajeswari	100	VG
Seed coat color	White Grey Light brown Dark brown Black	1 2 3 4 5	TKG 21 Uma Rama Thilak Krishna	100	VS
Seed:1000 seed weight (g)	Low <2.5 Medium 2.5–3	3 5	Kalika TKG 55	100	MG
Seed oil content (%)	Low <45 Medium 45–50 High >50	3 5 7	Tilottama Krishna TKG 21	100	MG

Source: Guidelines for DUS testing on sesame, PPV&FR Authority, Govt. of India
VS & VG: Visual assessment of individual and group of plants, respectively
MS & MG: Measurement of individual plants and group of plants, respectively
Growth stage code: 45–50% flowering, 65% complete flowering, 75% complete capsule formation, 100% maturity stage

addition, the new introduced cultivars/breeding lines serve as valuable starting material for intensive breeding programs.

15.11.2 Mass and Pureline Selection

Mass and pureline selection is the most ancient and basic breeding approach in which desired plants are selected from genetically variable populations. Local land races are the target for such a selection process. A land race may be a domesticated, locally adapted or genetically heterogeneous traditional variety cultivated over time in a certain ecogeographical area (Casanas et al. 2017). At times, farmers and breeders made unconscious or conscious selection of similar looking plants (mass selection) to improve the locally adapted genetic stocks. Alternatively, a few plants distinctly different from the local land race are often selected and grown plant on a row basis to assess their merit based on progeny performance (pureline selection). In

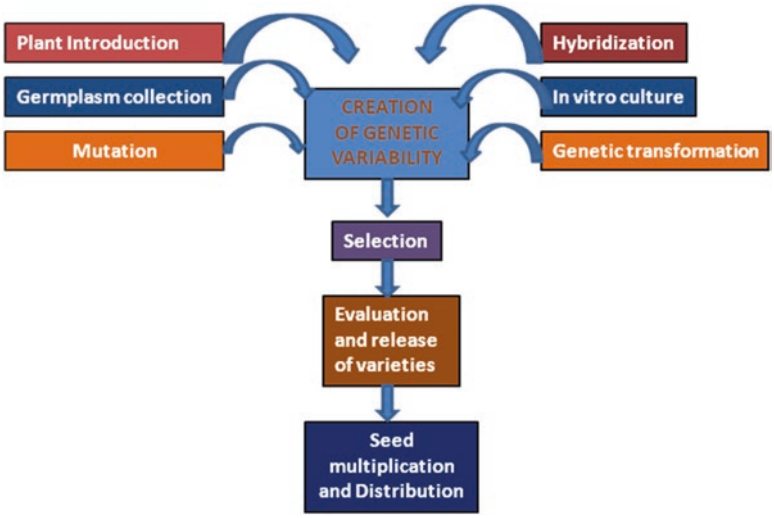

Fig. 15.6 Breeding approaches in sesame

self-pollinated crops, varieties developed through mass selection are in fact composed of a mixture of purelines (https://www.britannica.com/science/plant-breeding/Breeding-self-pollinated-species). Hence, these cultivars show wider adaptability and stability of performance over environments than individual purelines. A number of sesame varieties have been developed using the above selection process.

15.11.3 Hybridization

Most of the national production of sesame comes from landraces which are less productive than exotic genotypes due to a low level of resistance to biotic and abiotic stresses. Therefore, it is often necessary to combine desirable traits from different parental lines into a single plant by hybridization. Selection of desirable segregants with suitable gene combinations is of practical value for development of new varieties. Bisht et al. (2004) successfully recovered high-yielding sesame plants from progeny of 103 crosses. Pungsankkae is an example of a high-yield mutant variety (Islam et al. 2016), which was released in Korea by crossing a Korean variety with the Israeli determinate mutant dr-45.

15.11.4 Mutation Breeding

Well-adapted, high-yielding varieties which are popular in a locality but lack one or two important quality traits are selected for mutation induction. Low doses of mutagens (gamma-rays 150–800 Gy and fast neutrons 30–80 Gy) are more suitable for

inducing desirable mutations (Ashri and van Zanten 1994). For chemical mutagenesis, seeds are first pre-soaked in water for 24 h at 4 °C and then treated with chemical mutagens such as ethyl methane sulfonate (EMS: 0.4–1.0% v/v) solutions with phosphate buffer (pH = 7) for 2–4 h or sodium azide (NaN_3) solution (4–6 mM) with phosphate buffer (pH = 3) for 4–6 h at 18–24 °C (Ullah et al. 2012).

15.12 Breeding Strategies

15.12.1 Breeding for Ideal Plant Type

15.12.1.1 Determinate Type

Ashri (2007) identified the first determinate sesame mutant dt-45 from a M_2 population of Israeli cv. No-45 irradiated with gamma-rays (500 Gy). In addition, three true-breeding determinate mutants (dt-1, dt-2, dt-3) were derived from cv. Muganl 1-57 and another three such mutants (dt-4, dt-5, dt-6) were isolated in the Çamdibi cv. following irradiation of seeds with gamma rays (150–750 Gy) Cagirgan (2006).

15.12.1.2 Formation of Increased Number of Leaf Axils

In sesame, flowers are produced in the leaf axils and later develop into capsules. Therefore, the extent of capsule bearing depends upon the number of leaf axils per plant (Fig. 15.7). Theoretically, a greater number of leaf axils and capsules/leaf axil per plant would result in higher seed yield. This can be achieved by a plant type with short internodes and determinate flowering.

Fig. 15.7 Capsule bearing in sesame. (**a**) Poor capsule density, (**b**) Medium capsule density, (**c**) Very high capsule density per leaf axil

15.12.1.3 High Yield and Wide Adaptability

Tall-stature plant types with longer duration are not suitable for crop rotation; the ideal plant type of sesame for rice-fallow would be: (a) short stature and profuse fruit set, (b) short duration and high seed set and (c) moderate basal branching and high productivity (Ganeshan 2001).

Preferably, genotypes that exhibit medium plant height, moderate branching, determinate habit, high capsule density with moderately long capsule bearing starting from 15 to 20 cm above the ground, reach physiological maturity before the first few capsules dry, release seeds upon sun drying after harvest and are expected to yield better over environments. The number of capsules and dry-weight per plant are reported to have a significant positive correlation with seed yield and these also exert a higher magnitude of direct effects on seed yield (Abdalla 2017). In addition, increased initial vigor, a deep rooting system, non-lodging and lanceolate hairy leaves are favorable for plant establishment and higher seed yield. A monogenic recessive determinate growth habit mutant, termed dt45, with a very unique plant architecture and with clustered capsules was induced by gamma rays (500 Gy) in the Israeli cv. No. 45 (Ashri 1988, 1995). Also, short flowering period mutations (synchronous maturity) were induced by EMS and gamma rays in Thailand (Wongyai et al. 1997).

15.12.2 Breeding for Oil Content and Fatty Acid Composition

Sesame oil is highly preferred due to its exceptional quality. Sesame seeds harbor 44–57% oil (Borchani et al. 2010), but Baydar et al. (1999a, b) claimed oil content can vary from 34% to as high as 63%. Estimation of oil content of African sesame showed also wide variability of oil (29–51%) (Were et al. 2006a, b). In addition, Azeez and Morakinyo (2011) reported wide variation in fatty acid composition among Nigerian sesame accessions. This envisaged enough scope for improvement of oil content and fatty acid composition in sesame. Higher proportion of linoleic and linolenic acid content in seeds drastically affects oil stability and other quality features. Conventional breeding methods in vogue are effectively used to increase oil content in sesame and, successively, have produced sesame varieties with high oil content (Baydar et al. 1999b). In addition, genetic modification can be an alternative way to achieve high oleic acid and low linoleic and linolenic acids content.

15.12.3 Induction of Novel Variants

The induction of novel variants involves induction of novel sesame plant types by treatment with mutagens (chemical/physical) and thereby avoids the use of wild/related species for genetic improvement. The mutants induced include higher yield

(Wongyai et al. 2001), improved seed retention, determinate habit, modified plant architecture (Cagirgan 2006), synchronous maturity, earliness, resistance to diseases, genic male sterility, larger seed and changes in seed coat color (Hoballah 2001), higher oil content and modified fatty acid composition (Arslan et al. 2007). Mutation breeding has been practiced to induce a number of useful morphological and physiological mutants (Ashri 1985; Micke et al. 1987). A number of mutants (Kalika, Uma, Usha) have been released as improved cultivars in India. Some mutants are useful in physiological, genetic and molecular studies.

15.12.3.1 Disease Resistance

Currently, sesame breeding for disease resistance is the main focus in several countries. An induced mutation with moderate resistance to *Fusarium* and *Rhizoctonia* and resistance to *Corynespora* and *Phytophthora* has been reported (Lee and Choi 1985); it was released in South Korea as cv. Ahnsan. Similarly, Pathirana (1992) successfully isolated induced mutations for resistance to *Phytophthora* following irradiation with gamma rays; and one of these mutant lines was released as cv. ANK-82 in Sri Lanka.

15.12.3.2 Pest Resistance

Thus far, no efforts have been made to induce mutations for pest resistance, but identifying lines with tolerance or resistance to devastating pests in sesame such as webworm/leaf webber and capsule borer (*Antigastra catalaunalis*), sphinx moth (*Acherontia styx*), aphids (*Aphis gossypii*) and gall-midge/gall fly (*Asphondylia sesami*) would be very helpful.

15.12.3.3 Shattering Resistance

A spontaneous indehiscent mutant (id) was recovered by Langham (1946), but could not be exploited due to low yield and other undesirable side effects. Van Zanten (2001) reported eight gamma ray (300–750 Gy) induced closed capsule (indehiscent) mutants from four different Turkish cultivars. Three shattering resistant mutants, all out yielding their respective parent varieties were induced by gamma ray (500 Gy) in two Thailand local landraces. In addition, seven delayed shattering and shattering resistant mutant lines were obtained following treatments with EMS (0.5–1.0%, 4 h) in Thailand. Wongyai et al. (1997) reported a delayed shattering mutant and Maneekao et al. (1997) found semi-shattering mutants in sesame.

15.12.3.4 Hairy Capsule Mutants

The hairy capsule mutant trait is dominant over smooth capsules; the hairy trait may be related to drought tolerance (Cagirgan 2001).

15.12.3.5 Oil Yield and Fatty Acid Composition

Baydar (2005) found the highest oil content in low-yielding types, while the reverse is the case in the high-yielding types. The lowest content of oleic acid (41.3%) and the highest content of linoleic acid (43.1%) are associated with low-yielding types, while the best high-yielding type harbor a lower amount of tocopherol. However, induced mutations can bring about changes in the fatty acid composition in sesame lines, with high oil content (>50%). Variation for fatty acids content was induced by gamma-rays (Murty and Bhatia 1990) and by dodium azide (Kang 1997) in South Korea.

15.12.3.6 Antioxidants

Induction of mutations could be attempted for increased content of lignans such as sesamin, sesamolin and similar products. These substances have a wide applications in the production of pharmaceuticals, pesticides and other industrial products. In addition, total tocopherol content has been reported to vary from 175.6 to 368.9 mg/kg in the seed oil of sesame pedigree mutant lines (Baydar 2005).

15.12.3.7 Morphological Marker Traits

Marker mutant traits are always useful in genetics and breeding. Some of these display distinctive traits such as narrow, elongated, thick leaf types, ovate, ternate elongated petiole types and white, pigmented flower types. Out of these, the thick leaf mutants are most preferred. They possesses superior agronomic traits such as plant height, primary and total branches per plant, number of capsules on main axis, distance from base to first branching, total capsules per plant, seed yield and seed protein content, than the parent variety (Najeeb et al. 2012). Similarly, Mary and Jayabalan (1995) reported EMS induced mutations affecting leaf morphology in sesame at M_2.

15.12.4 Breeding for Disease and Insect Resistance

Plants inheriting the resistance (r) gene express a defense response as soon as a pathogen having the corresponding avirulence (avr) gene invades and express the gene (avr) in the plant tissue (Flor 1971). Disease resistance is usually pathogen species-specific or pathogen strain-specific. Pathogens evolve continuously and natural selection in the pathogenic population may lead to emergence of more virulent strain(s) leading to resistance breakdown (boom & bust cycle).

Biotic stresses such as diseases and insect pests affect sesame crops adversely resulting in huge losses in productivity and production. Thus, improved cultivars need to be resistant to diseases and insect pests to attain sustainable production. Sesame yield is drastically reduced by diseases such as, *Alternaria*, *Cercospora* leaf spot (Nahunnaro and Tunwari 2012), stem rot, bacterial diseases, powdery mildew and wilts (Nyanapah et al. 1995; Ojiambo et al. 1999). Farmers typically control these diseases through fungicide application and location-specific cultivation techniques. However, breeding a cultivar resistant to diseases and insect pests seems to be cost effective. Natural genetic variation for disease resistance in a core germplasm does exist. A varying degree of resistance to white and angular leaf spot has been observed in Kenyan sesame cultivars. SIK 031 and SIK 013 have shown resistance to white leaf spot, while SIK 031 and SPS 045 have been reported to have field resistance to angular leaf spot (Nyanapah et al. 1995).

In general, disease infestation is controlled by additive gene action with appreciably high heritability estimates, and consequently it becomes easy to effectively select disease-free sesame plants. But, there exists no cultivar with absolute resistance to bacterial leaf spot, stem rot, wilt and phyllody in sesame (Naqvi et al. 2012), although a number of Indian and Nigerian wild species are resistant to the above biotic stresses. Efforts to incorporate these traits into commercial cultivars is scanty. A few crosses involving *Sesamum indicum* with wild sesame species resulted in recovery of resistant lines to root rot. El-Bramawy and Abd Al-Wahid (2009) claimed resistance of S2 and H4 cvs. to *Fusarium* wilt out of 28 sesame genotypes screened under field conditions. In addition, Sanliurfa-63,189 was also identified to be the most resistant genotype (Kavak and Boydak 2006) for the disease. Wang et al. (2014) constructed a SSR based genetic linkage map and identified QTLs for charcoal rot resistance in sesame.

Different researchers attempted intra- and inter-specific crosses in sesame for phyllody-resistance and it revealed that disease resistance is governed by one dominant (wild species) and one recessive (cultivated species) gene (Singh et al. 2007). Naqvi et al. (2012) identified a few elite bacterial blight (*Xanthomonas campestris*) resistant germplasm lines (SG 22, SG 55, SG 72, SG 33) for use as donors in breeding program.

15.12.5 Breeding for Abiotic Tolerance

Cultivated sesame is sensitive to salinity (Koca et al. (2007), drought (Boureima et al. 2011), waterlogging (Ucan et al. 2007) and chilling (Levitt 1980). Koca et al. (2007) found that cv. Cumhuriyet was relatively more salt tolerant (at 50 and 100 mM salinity stress) than cv. Orhangazi which was linked to high-proline level. Proline plays a crucial role in plants by stabilizing proteins, regulating cytosolic pH, and scavenging hydroxyl radicals by superoxide dismutase, ascorbate peroxidise, catalase, peroxidise (Matysik et al. 2002). In addition, the growth parameters, lipid peroxidation and proline accumulation are reported to be positively correlated with the salt tolerance in sesame (Matysik et al. 2002).

Sesame cultivated in marginal uplands becomes drastically affected by drought stress which hinders plant metabolism, growth and seed yield (due to decreased seed setting and reduced seed weight) (Kim et al. 2007). A sesame cultivar with an extensive rooting system is able to sustain drought stress. Development of drought tolerant cultivars with enhanced water use efficiency will benefit both sesame cultivation and production.

Sesame is highly sensitive to chilling stress (0–5 °C). Its growth is considerably reduced below 20 °C, while seed germination and growth is completely inhibited below 10 °C (Oplinger et al. 1990). Low temperature reduces the efficiency of ROS (reactive oxygen species) scavenging enzymes produced in response to chilling stress (Beroza and Kinman 1955). Cold stress causes cellular injury, plant senescence and degrades oil quality by reducing the lignin content of seeds (Beroza and Kinman 1955). Progress towards breeding for cold tolerance in sesame is indeed limited. Understanding the pathway for cold acclimation and searching for molecular changes (Dong et al. 2006) can help to achieve success.

Sesame experiences a reduction in growth and yield after 2–3 days of submergence in water or under excessive irrigation. In fact, stagnate water considerably reduces plant growth, leaf axils per plant, biomass, seed yield and net photosynthesis at various stages of growth (Sun et al. 2009). A number of genes involved in energy metabolism and those related to flavone and flavonol biosynthesis, steroid biosynthesis, photosynthesis, are reported to be down-regulated under standing water in the field (Wang et al. 2012).

15.12.6 Heterosis Breeding and Development of Heterotic Hybrids

Despite many efforts, classical breeding methods of selection, pedigree breeding, backcross and induced mutations have not achieved a major yield breakthrough in sesame seed yield. Heterosis breeding exploits the advantage of heterozygotic performance. In addition, it exploits a greater extent of instant genetic variation as

compared to conventional breeding techniques. Recently, a number of research reports have indicated significant heterosis in certain hybrid combinations. Therefore, there is a need to intensify hybrid development programs to maximize sesame yield and it may be remunerative, as in case of other autogamous crops and as such may be profitable for oil production.

Male sterile lines provide an opportunity to facilitate the cross-pollination process for hybrid seed production, and to exploit sesame heterotic vigor. A naturally-occurring *split corolla* recessive genic male sterile mutant (msms) was first found in Venezuela (Langham 1947) and it was induced later with gamma rays by Murty and Oropeza (1989). In addition, male sterile mutants were induced by Rangaswamy and Rathinam (1982), and Ramanathan et al. (1992) with lower gamma-ray doses and with fast neutrons by Murty and Bhatia (1990) in India. Liu et al. (2013) recovered two spontaneous male-sterile plants in a Chinese sesame cv. Zhuzhi 4 in 2006. They developed a new high yielding GMS line, D248A, by consecutive sib mating with fertile plants from Zhuzhi 4. Its yield potential was higher than other GMS lines (95 ms-2A, 95 ms-5A). GMS is maintained by hybridizing male sterile lines (ms/ms) with isogenic heterozygous plants (Ms/ms) which segregate into 1 male fertile: 1 male sterile. This would require early rouging of the fertile Ms./ms progenies which is labor intensive and not cost effective. This becomes a major hindrance for production of commercial hybrid using GMS.

Fortunately, it was possible to develop cytoplasmic male sterile (CMS) lines in sesame by transferring the male sterility factor from the wild relative *Sesamum malabaricum* to the cultivated sesame *S. indicum* (Bhuyan et al. 1997). This CMS system was later used to develop 36 hybrid combinations of diverse origin (Bhuyan and Sharma 2003). Many of these experimental hybrids exhibited high heterotic performance for capsule number per plant, seed yield and oil content. Cultivars in suitable cross combination resulted in high-yielding sesame hybrids that exhibited 77–540% heterotic effect (Yadav et al. 2005). Similarly, heterosis in sesame hybrids for seed yield may be as high as 100–500% (Uzun et al. 2003). Mubashir et al. (2009) reported 40.3–255.1% heterosis in yield-contributing components in a set of ten crosses comprising of five diverse parental lines. However, Pandey et al. (2018) revealed a weak association of genetic distance based on SSR markers with F_1 performance.

Popular sesame hybrids show fast growth rates and a high leaf area index (LAI). Banerjee and Kole (2006) also showed that increased LAI in sesame plays a crucial role in oil production. However, Banerjee and Kole (2011) opined that the increased oil content in sesame hybrids is the result of the combined heterosis effects of different physiological traits. This demonstrates the great potential of hybrid sesame plants for higher oil yield. China is the first country to develop sesame hybrids for commercial cultivation. In India, seven experimental hybrids (AHY TIL 5, AHY TIL 12, RTH 1, AHYT 13, RTH 3, TKG-HY 5, TKG-HY 4) have been developed which exhibited superiority of 31.0–44.3% in seed yield and 13–48% in oil yield over cv. TKG 22.

15.12.7 Breeding for Mechanized Farming

Seed shattering in sesame before and during the harvest causes considerable losses (30–40% or even more) (Fig. 15.8). Recently, sesame with an indehiscent trait became a breeding target to produce high-yielding cultivars and to achieve profitability (reduced labor cost) and sustainability in production (Langham and Wiemers 2002). In fact, capsules with a thinner endocarp layer retain seeds better, as the tension built up between the mesocarp and endocarp during capsule drying would decrease leading to less shattering of capsules.

Medium plant height of at least 25–30 cm to the first branch, upright stem with a limited number of branches, strong and lodging resistant stem, determinate flowering habit, synchronous maturity, senescence and/or drop of leaves before maturity and maturity of seed before capsule opening, placenta attachment of seeds (better seed retention), capsule constriction (better seed retention) and closed capsule at the time of harvesting are all desirable for mechanized harvesting. The capsules should open easily by slight pressure during threshing and release all seeds without visible damage. This would prevent a deterioration of product quality and decrease in germination of seeds. Georgiev (2002) developed 22 indehiscent lines from 6 crosses. The total number of capsules/plant correlated positively with the capsule number on the branches, but negatively with capsules on the main stem. In addition, plant height was shown to be negatively correlated with branch number. In order for the capsules to be more densely positioned on the stem, the stem top should be shorter. This means that tall plant types are usually sensitive to lodging, associated with shy branching and show less fruiting density which is unwanted in mechanized harvesting, as well as undesirable for high yield. Therefore, restructuring of plant architecture is needed for its complete adaptation to the requirements of mechanized harvesting (Georgiev et al. 2008). The extent of shattering may be scored, as shown in Table 15.6, to assist the selection process in different breeding cycles.

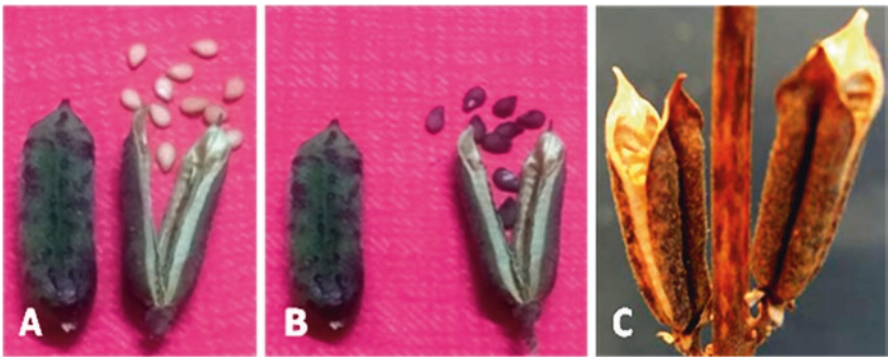

Fig. 15.8 Capsule shattering in sesame. (**a**) and (**b**) Green capsule before physiological maturity (left) and capsule shattered at maturity stage (right), (**c**) Capsule severely shattered due to overmaturity

Table 15.6 Shattering types based on percentage of seed retention

Shattering types	Percentage of seed retention
Super-shattering (SUS)	Less than 10% seed retention
Shattering (SHA)	10–50% seed retention
Non-shattering (NSH)	50–80% seed retention
Direct combine (DC)	Greater than 80% seed retention
Indehiscent (ID)	Id/id genotype, retains all seeds

15.12.8 Innovative Breeding Strategy

In certain instances, conventional plant breeding fails to achieve desired success and requires working in conjunction with biotechnological approaches. With the advancements in biotechnology, sesame breeding can be hastened with reliable selection of desired plant types. A few possible avenues worth mentioning are discussed below.

15.12.8.1 In Vitro Screening

Somaclonal variation among regenerants and their progeny is the result of hereditary changes, mostly induced in the process of callus induction and proliferation (Hoffman et al. 1982). A repeated and prolonged period of subculture of calli increases the frequency of gene mutations and gross chromosomal aberrations (Sanal and Mathur 2008). Regenerants derived from such long-term callus cultures are most likely to bear one or more heritable changes (Bairu et al. 2006). In addition, in vitro mutagenesis due to a mutagen added to the medium can be a step forward to enhance genetic variability. In vitro screening of cell lines, somaclonal variants and somatic mutants is possible using various selection agents such as pathogenic fungal toxins, antinutritional factors (phytate, trypsin inhibitor, tannins), herbicides, PEG (polyethelene glycol) and minerals (Abd El-Himed and El-Bramawy 2011; Maluszynski et al. 1995; Tripathy 2015).

In vitro culture has been extensively studied in sesame, but with limited progress toward genetic improvement primarily due to inefficient selection of induced variants. In sesame, response to callus induction and plantlet regeneration was reported to be better using cotyledon (Yadav et al. 2010) and hypocotyl and shoot tips (Baskaran and Jayabalan 2006) as explants. Nutrient media supplemented with 6-BAP (6-benzylaminopurine) elicited rapid shoot induction and plantlet regeneration (Yadav et al. 2010). Baskaran and Jayabalan (2006) studied the effects of plant growth regulators on callus induction in hypocotyls and cotyledon explants of sesame. Good callusing was achieved at 2, 4-D (2,4-dichlorophenoxyacetic acid) (3 mg/l) with 100 ml of coconut milk followed by 2, 4-D (3 mg/l) with casein hydrosylate (0.1 mg/l). However, MS media (Murashige and Skoog 1962) supplemented with 1 mg/l IAA (indole-3- acetic acid), 1–1.5 mg/l BAP and 1.25 mg/l Kn (Kinetin) resulted in significantly higher shoot multiplication ratio. Callus cultures derived

from cotyledons and hypocotyl segments produced somatic embryos and plants regenerated from such cultures showed morphological variations for seedling growth, vigor, placental thickness, capsule dehiscence, seed size, seed dormancy, yield, oil content and oil quality (Ram et al. 1990).

15.12.8.2 Genetic Transformation

Sesame plants regenerated from primary cultures via somatic embryogenesis or direct regeneration from shoot apical meristems and hypocotyl segments (George et al. 1987) reveal minimal or no genetic variability and such regeneration systems provide an opportunity for *Agrobacterium*-mediated gene transfer (Xu et al. 2009).

Some wild species of sesame are resistant to biotic and abiotic stresses, but post-fertilization barriers restrict transfer of their resistance genes to cultivated *Sesamum indicum* through conventional breeding. However, Were et al. (2006a, b) devised the protocols for gene transfer of sesame by optimizing hormonal concentration and macronutrients for plant regeneration. Application of 20 mM TDZ (Thiodizuron) along with 2.5 mM IAA was the best for successful plant regeneration. The major drawback is that sesame is sensitive to *Agrobacterium tumefaciens* infection (Taskin et al. 1999). However, Yadav et al. (2010) successfully produced fertile trasformants by using cotyledon explants for plant regeneration via multiple shoot organogenesis on MS basal medium containing 25 mM BAP, 25 mg/l kanamycin and 400 mg/l cefotaxime.

15.12.8.3 Interspecific Hybridization

Tissue culture methods can be used to facilitate interspecific crosses using embryo and ovule culture techniques. Crossing was unsuccessful between cultivated *Sesamum indicum* (2n = 26) with either of the wild species *S. alatum* Thonn (2n = 26) or *S. radiatum* (2n = 64). However, there was normal fruit development and seed setting for crosses involving cultivars of *S. indicum* with *S. malabaricum* (2n = 26) indicating high genomic homology of sesame with such wild species (Kumara and Ganesamurthy 2015). Kulkarni (2006) revealed poor pollen tube growth finally reaching to the micropylar end in case of cross *S. indicum* × *S. prostratum* using aniline blue fluorescent microscopy. In contrast, there was good pollen tube growth having micropylar penetration in two crosses e.g. *S. radiatum* × *S. indicum* and *S. occidentale* × *S. indicum*. However, none of the above crosses produced any viable seed, confirming the presence of post-fertilization barriers. However, a standardized ovule culture protocol recovered the above interspecific hybrids. In another study, Kulkarni et al. (2017) also confirmed non-existence of pre-fertilization barrier between wild (*S. mulayanum* and *S. malabaricum)* with cultivated species of sesame. Rajeswari (2001) observed cessation of pollen tube growth in the mid-stylar region in the direct crosses of *S. indicum* and *S. alatum*. The reciprocal cross combinations (*S. alatum* × *S. indicum*) resulted in fertilized embryos, but such

embryos degenerated after 48 h of pollination. However, ovule culture of the crosses made it possible to recover plantlets through direct organogenesis in MS medium supplemented with growth regulators such as BAP (2 mg/l) + IAA (0.5 mg/l) + glutamine (250 mg/l).

In vitro culture and plant regeneration was optimized for wild species of genus *Sesamum* by Dasharath et al. (2007a, b). This paved the way for recovery of successful interspecific hybrids between cultivated *S. indicum* and its wild relatives *S. radiatum* and *S. occidentale* through ovary and ovule culture. Use of 8 mg/l Kn along with BAP was shown to the best combination among different levels of BAP and Kn applied. Ram et al. (1990) cultured zygotic embryos at various developmental stages, and plants were regenerated from 15-day-old embryos after pollination. MinMin et al. (2017) attempted interspecific hybridization of cultivated sesame with *S. indicatum* to introgress charcoal rot (*Macrophomina phaseolina*) resistance from the later using the immature embryo culture technique. Rajeswari et al. (2010) produced interspecific hybrids between *S. alatum* and *S. indicum* through ovule culture to introgress phyllody disease resistance.

15.12.8.4 Doubled-Haploid Breeding

Sesame being self-pollinated, doubled haploid (DH) breeding offers a rapid method of genetic improvement as it significantly reduces the breeding period due to early fixation of homozygosity. Callus induction and androgenic plantlet regeneration from F_1s have been successful (Fig. 15.9). At present, more than 200 crop varieties have been developed by utilizing the doubled-haploid approach (Thomas et al. 2003). Variation among anther culture derived DHs is due to unlocking new genetic variation. It ensures production of stable desirable recombinants with high efficiency stacking of specific target genes (without masking effects) in a homozygous state.

Production of DHs using anther culture of intervarietal/interspecific heterotic hybrids has been reported in rice (Baisakh et al. 2001), wheat (Chaudhary et al. 2015), barley (Weyen 2009) and *Brassica* species (Alam et al. 2009). In sesame, anther-derived callus induction was first reported in Korean cultivars using MS with 25 mg/l 2,4-D and 1 mg/l BAP (Anonymous 1986). Ranaweera and Rathirana (1992) reported better callusing response of anthers from flower buds collected 36–48 h before anthesis and pre-treated at 8 °C for 24 h in the dark. MS media with

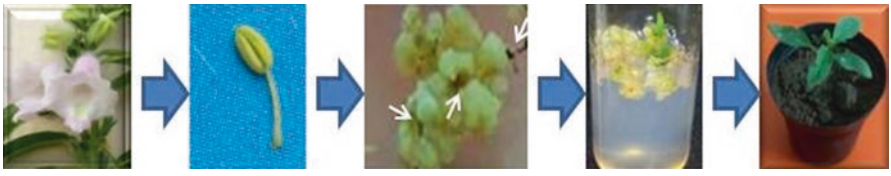

Fig. 15.9 Callus induction and androgenic plantlet formation derived from a sesame cross: Vinayak (reddish brown seed) × TC 25 (white seed). (Source: authors' unpublished research)

10 mg/l 2,4-D, 2 mg/l IAA and 2 mg/l BAP induced calli (46%) after 2–3 weeks of inoculation and the above medium with 5 m/l IAA and 3 mg/l BAP resulted in better response in sub-culturing. In addition, Yifter et al. (2009) were successful to induce anther derived calli in MS media with 2 mg/l 2,4-D + 1 mg/l BAP and recovered regenerants in MS with 1 mg/l NAA + 2 mg/l BAP in four Ethiopian varieties of sesame. The plantlets were rooted in MS with 0.25 mg/l IBA + 0.5 mg NAA. After hardening, the survival percentage of plantlets was as high as 66.7% and 50% in coco peat and soil mixture, respectively.

15.12.8.5 Marker-Assisted Selection (MAS)

Sesame is a promising target oilseed crop for marker-based studies. Its genetic improvement relies on the search for and utilization of desirable alleles present in the gene pool. Use of MAS seems to be an appropriate option to detect the presence of allelic variation in the genes underlying the agronomical important traits (shattering, abiotic and biotic resistance). Precise phenotyping coupled with high throughput next generation sequence information in the form of web-based database SesameFG (http://ncgr.ac.cn/SesameFG/) is now available.

Construction of genetic maps is the foundation of the genome research in any crop. The first comprehensive genetic linkage map in sesame was developed on a F_2 population using EST-SSR (expressed sequence tags-simple sequence repeat), AFLP (amplified fragment length polymorphism) and RSAMPL (random selective amplification of microsatellite polymorphic loci) markers (Wei et al. 2009). It serves as a starting point to tag traits of breeding interest and further aid in sesame molecular breeding. In this context, marker-aided selection allows rapid introgression of a target trait into the recurrent parent by identifying plants carrying the target allele even at early vegetative stages.

Molecular markers are in vogue and used in genetic diversity analysis (Fig. 15.10), construction of genetic maps, gene mapping and cloning and marker-aided selection in crop improvement. Molecular markers have been applied for the study of genetic

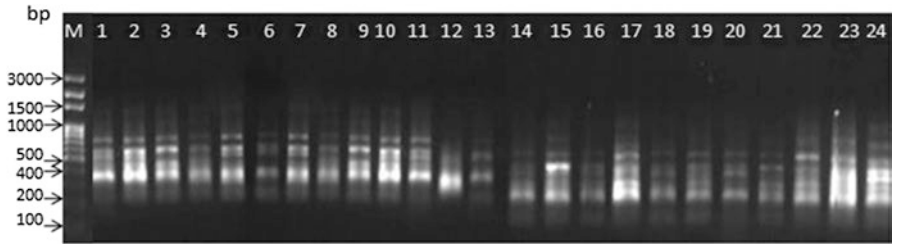

Fig. 15.10 ISSR profile of 24 genotypes of sesame amplified with primer OUAT 10. M = DNA ladder, Lane 1-24: Vinayak, TC 25, B67, CST 785, Pratap, BS 5-18-3, BS 5-18-5, BS 5-18-6, BS 5-18-10, T 46, RT 103, RT 346, RT 351, TMV 5, Phule Til 1, T13, Madhabi, E8, B7-11, IS 309, RT 54, TKG 22, Kanpur local Sel. 1 and Kanpur local sel. 2. (Source: authors' unpublished research)

diversity by using diverse *Sesamum indicum* accessions and Abdellatef et al. (2008) suggested the usefulness of the RAPD (random amplified polymorphic DNA) technique in sesame breeding, conservation and maintenance of germplasm banks and in efficient parental line selection. A limited number of reports are available on molecular markers such as isozymes (Isshiki and Umezaki 1997), ISSR (Kim et al. 2002), AFLP (Ali et al. 2007), SSR (Dixit et al. 2005) and SNP markers (Libins et al. 2014) for sesame improvement and study of genetic variability. Only a few studies are available for tagging of desired genes to assist in the selection process for genetic improvement (Wei et al. 2009). Using marker assisted selection, Uzun and Cagirgan (2009) identified two ISSR markers linked to the dt gene regulating determinate growth habit in sesame using bulk segregant analysis (BSA) in F_2 population of a determinate mutant line (dt1) x indeterminate (cv. Muganlı-57) cross. Yield loss due to capsule shuttering may be greater than 50%. Uzun et al. (2003) reported an AFLP marker linked to closed capsule mutant trait in a cross of cc3 x Muganly-57 using BSA. SSR molecular markers (ZMM0913, ZMM3752, ZMM5636, ZMM5775) closely linked to the major candidate gene/locus for sesame stem rot resistance are now available (https://patents.google.com/patent/CN107058518A/en) which can be suitably used in sesame breeding. The primer sequences of the above molecular markers are:

(a) ZMM0913 (F: 5′-ctcatgtggaacgaggcata-3′, R:5′-atggccaccacctaacattc-3′),
(b) ZMM3752 (F: 5′-caacgatgagatggctttga-3′, R:5′-tcttgcacgcacagtagtcc-3′)
(c) ZMM5636 (F: 5′-ctgctcatcacctctggaaag3′, R:5’tgacctatgatgtgataacagttgg-3′)
(d) ZMM5775 (F:5′-ttcactttgcttttgttgcc-3′, R:5′-gcccattccatgagtttttg-3′).

Wang et al. (2017) identified 14 QTLs for charcoal rot resistance using SSR markers. Yan-Xin et al. (2014) detected an effective SSR marker ZM 428 closely linked with the major QTL qWH10CHL09 for waterlogging resistance in sesame. A SNP survey in the sesame genome revealed 30 SNP markers associated to three important fatty acid (oleic, linoleic, linolenic acids) compositions (Mondal and Bhat 2015) which can be used to enrich unsaturated fatty acid composition in sesame.

15.13 Achievements

A large number of sesame cultivars have been released and reported from time to time for commercial cultivation in different states of India. A state wise list of these cultivars with specific features is provided in Table 15.7. In addition, a detailed list of sesame genetic resources arranged by specific traits and cultivars developed through international sesame breeding is presented in Appendix II.

Table 15.7 Sesame varieties released and notified for different states of India

Variety	Year of notification	Duration (days)	Seed yield (q/ha)	Oil content (%)	Salient features
Andhra Pradesh					
Gouri	1974	85–90	675	43–49	Biscuit color seed, MR to major diseases and pest, MT to water stress and lodging
Madhavi	1978	70–75	700	50–53	Pale white seed, MR to stem/root rot, leaf spot, phyllody, PM, *Antigastra* MT to water stress
Rajeswari	1988	85–90	Kh– 450, Rabi 750	50.5	White seeded, few branches, T to *Macrophomina*, stem rot and powdery mildew, for late kharif (autumn) and rabi (winter)
Varaha	1993	80–85	950	53.0	Dark brown seeded, uniform maturity for kharif and rabi-summer
Gautama	1993	75–80	925	53.0	Light brown seeded, uniform maturity, tolerant to *Alternaria* leaf spot, kharif and rabi-summer
Sweta Til	2014	75–80	900	44–49	Determinate white seeded, tolerant to gall fly, capsule borer, phyllody, leaf curl and powdery mildew for kharif
Bihar					
Kanke White	1965	85–90	550	50.0	Dull white seeded, for kharif
Krishna	1986	85–90	775	46.0	Black seeded, tolerant to *Alternaria* leaf spot and capsule borer, for kharif
Gujrat					
Gujurat Til 1	1979	85	630	49.2	White seeded, branched, smooth green stem, pink flower, multicapsule for kharif
Gujurat Til 2	1994	85	790	50.2	White seeded, branched, multicapsule for kharif
Gujurat Til 3	2009	86	1200	47.3	White bold seeded suitable for export
Gujurat Til 4	2012	85	770	50.8	White bold seeded, resistant to gall fly, capsule borer and mites,suitable for export, kharif
GJT–5	2015	93	1000	46.9	High yielding, summer irrigated condition

(continued)

Table 15.7 (continued)

Variety	Year of notification	Duration (days)	Seed yield (q/ha)	Oil content (%)	Salient features
Gujurat Til 10	2004	92	807	50.0	Black seeded, good for export, kharif
Haryana					
Haryana Til 1	1978	80–85	500	52.0	White seeded, early maturing, resistant to major diseases, tolerant to phyllody and leaf curl
RT 46	1990	76–85	500	49.2	White and bold seeded, for kharif
RT 125	1994	71–83	500	49.0	Seed with brown tinge for kharif
HT–9713 (HT–2)	2013	–	700	48.2	White seeded, T to phyllody and leaf curl
Karnataka					
E 8	1991	100–110	550	53.0	White bold seeded, MR to powdery mildew, R to bacterial leaf spot
DS 1	1997	85–90	450	51.0	White seeded, non–branching, T to bacterial blight and *Alternaria* leaf spot
DSS–9	2009	85–90	625	48–50	White bold seed, early maturing
DS–5	2012	–	650	49–51	White bold seed, kharif
Kerala					
Kayamkulum1	1980	90–100	550	50.0	MT to phyllody, for summer rice fallows.
Thilothama	1982	80–85	650	50.0	Brownish black seeded, for summer rice fallows.
Soma	1985	80–85	750	51.2	Black seeded for kharif
Surya	1985	87–90	750	51.4	Gray seeded, T to leaf spot and phyllody disease, eight loculed, small capsules for semi-rabi
Thilak	1998	80–85	650	51.0	Dark brown bold seeded
Thilathar	1999	78–85	850	51.5	Brown bold seeded
Madhya Pradesh					
N 32	1970	100	770	53.0	White seeded, MR to leaf spot, R to gall fly and capsule borer, kharif
Kanchan	1981	85	880	54.0	Medium sized white seeded, for kharif
JT 21	1993	78	600	55.9	White seeded, T to *Antigastra*, bacterial and *Cercospora* leaf spot, for kharif

(continued)

Table 15.7 (continued)

Variety	Year of notification	Duration (days)	Seed yield (q/ha)	Oil content (%)	Salient features
TKG 22	1994	76–81	800	53.3	White seeded, T to *Phytopthora* blight, for kharif
TKG 55	1998	76–78	630	52.6	White seeded, T to *Phytopthora* blight, R to Macrophomina stem rot for kharif
Jawahar Til −12	2004	82–85	775	48–52	White seed, MR to *Macrophomina* stem/root rot
TKG–306	2007	86–90	750–800	49–52	White seeded, R to *Phytophthora* blight and MR to *Macrophomina*, *Cercospora*, powdery mildew, *Alternaria* leaf spot
Jawahar Til −14	2008	82–85	700–750	50–52	For summer
TKG–308	2010		700–750	48–50	MR to *Macrophomina*, *Cercospora*, bacteria leaf spot, leaf curl, T to capsule borer, kharif
Maharashtra					
Phule Til1	1978	85	500	50.0	White and bold seeded, for kharif
N–8	1982	130	500	50.5	Seed with brown tinge for kharif
Tapi	1987	85	600	50.0	White and bold, early maturing, for kharif
Padma	1991	75	700	50.0	White seeded, early maturing, for kharif
RT 54	1992	83	800	44.1	Brown seeded, R to leaf blight, *Macrophomina* stem rot, tolerant to *Antigastra*, kharif
RT 103	1994	88	900	48.0	White seeded, T to *Macrophomina* stem rot, *Alternaria*, bacterial leaf blight and phyllody and R to insect pest, for kharif
PKV–NT–11	2009	88–92	800–850	50–53	White seed, MR to root rot, bacterial blight, summer
JLT–408	2010	80–85	700–800	51–53	High yielding, moderately tolerant to major diseases & pests

(continued)

Table 15.7 (continued)

Variety	Year of notification	Duration (days)	Seed yield (q/ha)	Oil content (%)	Salient features
Odisha					
Vinayak (Sel–14)	1989	90	500–600	47–48	Light brown seed MR to major diseases and pest
Kanak (BS–6–1)	1979	80–85	600–650	46–48	Deep brown color seed, MR to leaf spot resistant to lodging
Kalika (BM–3–7)	1985	85–90	600–650	45–48	Dark brown seed, less S to CLS, stem/root rot R to lodging
Usha (OMT–11–6–5)	1992	85–90	650–700	43–49	Biscuit color seed, MR to major diseases and pest, MT to water stress and lodging
Uma (OMT–11–6–3)	1992	70–75	650–750	50–53	Pale white seed, MR to stem/root rot, leaf spot, phyllody, PM, *Antigastra* MT to water stress
Nirmala (OS–Sel–164)	2003	80–85	750–800	42–44	Gray white seed T to phyllody, wilt, BLS, PM MR to stem/root rot and ALS
Prachi (ORM 17)	2004	85	700–750	42–45	Black seed, MT to leaf spot, PM, stem/root rot and *Antigastra*, T to water stress and lodging
Amrit	2007	75–80	750–850	43–46	Light brown seed, T to leaf spot, PM, stem/root rot, R to lodging
Smarak	2014	75–80	800–1000	44–49	Golden yellow seed, MR to major diseases and pest
Shubhra	2014	75–90	900–1000	46–52	White seed MR to major diseases and pest, R to water stress
Punjab					
Punjab Til 1	1966	85	700	50	White seeded, T to phyllody, for kharif
TC 25	1978	85	700	48.4	White seeded, MT to disease insect pest, for kharif
TC 289	1986	100	600	51.6	White seeded, kharif
TH–6	2008	105	700	50	Edible bold seed, R to charcoal rot and phyllody
Rajasthan					
RT 46	1990	85	700	49.2	White seeded, R to oozing complex, T to *Macrophomina* stem rot, capsule borer and gall fly, for kharif

(continued)

Table 15.7 (continued)

Variety	Year of notification	Duration (days)	Seed yield (q/ha)	Oil content (%)	Salient features
RT 54	1992	83	800	41	Brown seeded, R to leaf blight, *Macrophomina* stem rot, T to *Antigastra*, kharif
RT 125	1994	83	700	49	White seeded, T to *Macrophomina* stem rot, *Alternaria, Cercospora* and bacterial leaf spot and phyllody, for kharif
RT 103	1994	88	750	48	White seeded, T to *Macrophomina* stem rot, *Alternaria*, bacterial leaf blight and phyllody and R to insect pest, for kharif
RT 127	1999	85	800	50.6	Bold, white seeded, drought, hardy, T to *Macrophomina* stem rot, phyllody, bacterial leaf spot, powdery mildew, gall fly and mites, for kharif
RT 346	2009	84	629	50	White seed, R to leaf curl, MR to *Macrophomina*, *Alternaria*, *Cercospora*
RT 351	2011	85	650	49.7	White seeded, T to *Macrophomina*, root rot, *Cercospora*, phyllody
Tamil Nadu					
TMV 1	1939	85	300 (rainfed) 600 (irrigated)	50	Erect, fairly bushy with moderate branching, 4-loculed reddish brown to black seeds
TMV 2	1942	80	300 (rainfed)	52	Open, moderate branching 6–8 loculed, cylindrical big sized capsules dark brown to black seeds. Suitable for rabi
TMV 3	1948	82.5	600	52	T to leaf miner
TMV 4	1977	87.5	650	50	Light brown seeded for summer
TMV 5	1978	80	750	51	Erect with moderate branching, 4 locule, brown seeds.
TMV 6	1979	87.5	700	54	Brown seeded, T to drought, for kharif
Co. 1	1983	87.5	730	50	Black seeded for kharif
SVPR 1	1992	80	800	50	White seed, 4locule, high yield, suitable for irrigated condition

(continued)

Table 15.7 (continued)

Variety	Year of notification	Duration (days)	Seed yield (q/ha)	Oil content (%)	Salient features
TSS 6	1994	77.5	800–900	54	White seeded, T to phyllody, *Alternaria* leaf spot, 4 loculed capsules, for kharif
Paiyur 1	1997	92.5	600–700	50	Black seeded, bushy, capsules 4 loculed, for kharif
VRI 1	1997	72.5	600–700	51	Dark brown seeded, short erect, 4 loculed capsules, for kharif
VRISV2	2005	82.5	750	51.9	MR to shoot webber, suitable for both rainfed and irrigated condition
TMV (SV7)	2009	85.0	800	51	Suitable for kharif
VRI 3	2017	77.5	700	49	High yielding, moderately tolerant to major diseases & pests
Uttar Pradesh					
T 12	1963	87.5	600	47	White seeded, T to phyllody and leaf curl, kharif
T 13	1968	92.5	600	47	White seeded, T to lodging, for kharif
Sekhar (T78)	1994	82.5	700	50	White seeded, R to lodging, T to phyllody and leaf curl, kharif
West Bengal					
Tilottama (B 67)	1981	77.5	900	40	Blackish brown seeded, R to *Macrophomina* stem rot, phyllody and Bihar hairy caterpillar, for rabi/summer
Rama	1989	87.5	1200	45	Reddish brown seeded, R to *Macrophomina* stem rot, phyllody and Bihar hairy caterpillar, for rabi
SWB–32–10–1	2008	86.0	1200–1500	50	Light brown seed, T to *Macrophomina* stem rot

Sources: Ram HH (2011) and URL: https://sites.google.com/a/tnau.ac.in/cpbg/oilseeds/sesame-cultivars

R resistant, *MR* Mod. resistant, *T* tolerant, *MT* Mod. tolerant, *S* susceptible

15.14 Limitations and Challenges

Sesame is a highly valued oilseed crop owing to its high percentage of oil recovery of superior quality. A large number of cultivars have been developed using conventional breeding. However, productivity (325 kg/ha) in India is far below the world average (535 kg/ha), primarily due to poor adaptability, high capsule shattering and sensitivity to salinity, waterlogging, low temperature and biotic stresses including phyllody, stem rot, *Cercospora* leaf spot, bacterial leaf blight, powdery mildew and wilts. The progress of genetic improvement in sesame has been inadequate as much attention is directed toward food crops (rice, wheat, maize). Fertile plain lands are the choice for food crops while; the left-out, undulating and marginal lands are in vogue and diverted to sesame cultivation with minimal input use. In addition, the narrow genetic base of the sesame gene pool may be an explaining factor for the slow pace of genetic improvement. A number of reports on novel induced variants for agro-economic traits are available. However, in most of cases, research initiatives become stalled at some midpoint without carrying forward materials to establish new cultivars. Similar is the case for breeding materials generated through innovative breeding approaches such as in vitro culture, genetic transformation, protoplast fusion and marker-aided backcrossing.

15.15 Conclusion and Prospects

Among the oilseed crops, sesame offers a range of dietary and purported health benefits, as well as industrial applications. A genotype with a higher number of capsule bearing leaf axils, longer capsules, determinate and semi-shattering habit, resistant to major diseases and having good end use exportable quality (white color, large seed size and good aroma/taste) are preferred. This can be achieved by harnessing genes present in novel mutants and locally-adapted cultivars through crossing programs. In vitro induced variation (somaclonal variation), in vitro mutagenesis (somatic mutation), interspecific hybridization (embryo rescue), wide hybridization between distant species (somatic cell hybridization) may be adequately attempted to re-structure the sesame ideotype. In addition, development of doubled haploids (DH) has immense potential in sesame improvement to short-cut breeding cycles without sacrificing the success rate expected in pedigree selection. Identification of candidate genes/QTLs underlying tolerance to drought, salinity, chilling, waterlogging and heavy metal stresses using molecular markers and pyramiding the genetic

factors for the above abiotic stresses using MAGIC (multi-parent advanced generation inter-crosses) populations seems to be a possible option to adjust against vagaries of environmental conditions.

A variety of targeted gene editing methods such as RNA interference (RNAi), site directed mutagenesis, zinc finger nucleases (ZFNs), transcription activator like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats/Cas9(CRISPR/Cas9) are available that bring about knock-outs (deletions) or knock-ins (insertions) for a gene of interest to generate altered phenotypes in vitro. The most popular and current précised gene editing technique is the CRISPR/Cas9 system where, gRNA sequences (about 20 bases long) direct the Cas9 protein to induce a site-specific double strand break in the genomic DNA. However, none of the currently available editing platforms is foolproof to guarantee a pure population of cells. For gene knock-outs, single cell cloning will typically require a few hundred cells to be plated. For knock-ins, these occur at further lower frequencies, and may need over 1000 target cells per edit. The desired gene edit event can be identified by a typical amplified product by PCR or absence of the protein due to gene knock-outs and such gene edited clones can be maintained in vitro. This is a most versatile and precise method of genetic manipulation and it can be extended to sesame genome editing to offer disease resistance.

Oil content is a very complex trait which varies depending upon genetic background and environmental conditions. Therefore, identification of wide adaptable sesame genotypes maintaining high oil content is warranted. A number of candidate genes/QTLs determine the oil content and its fatty acid composition. Little information is available about the number of such genetic factors and their mode of action. There is an urgent need to explore candidate genes underlying biosynthesis of sesame oil for selection of suitable parents for convergent breeding to improve oil content and/or its quality. The study of molecular marker-trait association is still at infancy in this crop as compared to other commercial oilseed crops (peanut, mustard). Identification of QTLs and their monitoring in succeeding breeding cycles using molecular markers can pave the way for increase in seed oil content.

Acknowledgement The authors are grateful to all researchers for their valuable contributions included in this pursuit.

Appendices

Appendix I: Research Institutes Relevant to Sesame

Institute	Specialization and research activities in sesame	Contact information and website
Bose Institute, Kolkata	Genetic diversity	Ranjana Prasad https://www.researchgate.net/profile/Ranjana_Prasad
C. S. Azad University of Agriculture & Technology, Kanpur, India	Screening for phytoplasma disease	Prabhakaran Kumar Singh, CSAUAT, Kanpur, India http://csauk.ac.in/
Chinese Academy of Agricultural Sciences, China	Sinbase and ssr based-genome mapping	Wang Linhai http://www.sesamebioinfo.org/PMDBase.
Crane Global Solutions Ltd, India	New approaches for crop improvement	Raghav Ram https://www.zoominfo.com
Henan Academy of Agricultural Sciences, Nanjing Agricultural University, China	Association mapping of seed oil and protein content	Chun Li Email:zhy@hnagri.org.cn http://www.caas.cn/en/index.shtml
Indian Agricultural Research Institute, New Delhi	Breeding for disease resistance	Sajadun Nabi https://www.researchgate.net/publication/284097386
Indian Institute of Oil-seeds Research (IIOR), Hyderabad-30, India	Oil seeds research including sesame breeding	A. Vishnuvardhan Reddy email: director.iior@icar.gov.in http://www.icar-iior.org.in/
Institute of Plant Genetic Resource, Bulgaria	Mechanized harvesting	St. Georgiev, IPGR, BG – 4122 Sadovo, Plovdiv, Bulgaria http://ipgrbg.com/en/
Izmir Institute of Technology, Turkey Georg-August-Univ., Germany	SNP identification for GBS analysis	Sami Doganlar, Ayse Ozgur Uncu Email: samidoganlar@iyte.edu.tr http://www.iyte.edu.tr/AnaSayfa.aspx?d=ENG
JNKVV, Jabalpur, India	DUS testing	Rajani Bisen Email: rajanitomar20@gmail.com http://jnkvv.org/
Joint FAO/IAEA Divi-sion of Nuclear Techniques in Food and Agriculture	Sesame improvement by induced mutations	IAEA-TECDOC-1195 https://www.iaea.org/
Louisiana State University, USA	Powdery mildew resistance	P. Venkata Ramana Rao https://www.facebook.com/public/P-Venkataramana-Rao

(continued)

Institute	Specialization and research activities in sesame	Contact information and website
Modibbo Adama University of Technology, Nigeria	Disease resistance	H. Nahunnaro Email: hycenth.nahunnaro@yahoo.com. http://mautech.edu.ng/new/index.php/en/
Nanjing Agricultural University, China	Genetic linkage map construction	Li-Bin Wei Email: moelab@njau.edu.cn, http://english.njau.edu.cn/
National Bureau of Plant Genetic Resources, India	SNP markers for study of functional polymorphism	Nupur Mondal Email: nupur.mondal84@gmail.com http://www.nbpgr.ernet.in/
National Crop Experiment Station, Korea	Disease and Shatter resistance	C.W.KANG, https://www.researchgate.net/profile/Cw_Kang
Odisha Univ. of Agril. & Tech, Odisha, India	Gene action, heterosis, combining ability	Swapan K Tripathy Email: swapankumartripathy@mail.com http://www.ouat.nic.in/
Oil Crops Research Institute of Chinese Academy of Agricultural Sciences	Interspecific hybridization	Yang MinMin E.mail: nc.saac@nimnimgnay; moc.361@mmgnaybh http://en.oilcrops.com.cn/
Oil Crops Research Institute, China	Sesame genomics	Linghai Wang E.mail: wangnuo@dlmu.edu.cn http://en.oilcrops.com.cn/
Pir Mehr Ali Shah Arid Agriculture University, Pakistan	Bacterial blight resistance	S. Farah Naqvi Email: dr.inam@uaar.edu.pk http://www.uaar.edu.pk/
PPV & FRA, Govt. of India	Guidelines for DUS Testing	PPV & FRA, Govt. of India http://plantauthority.gov.in/
Punjab Agricultural. University, India	Genetic diversity using ISSR markers	Hitesh Kumar Email: hiteshkmr25@gmail.com http://www.pau.edu/
Suez Canal Univ., 41522 Ismailia, Egypt	Nature of gene action, screening for antinutritional factors	M.A.S. El-Bramawy Email: el_bramawy71@hotmail.com http://scuegypt.edu.eg/en/
Tamil Nadu Agricultural University, India	Interspecific hybridization	S. Rajeswari Email: rajisundar93@gmail.com http://www.tnau.ac.in/
Universidade Federal do Ceará, Brazil	Floral biology	Patrícia Barreto de Andrade Universidade Federal do Ceará, Brazil http://www.ufc.br/

(continued)

Institute	Specialization and research activities in sesame	Contact information and website
University Goettingen, Germany	Relationship between metabolic and genomic diversity	Petr Karlovsky Email: pkarlov@gwdg.de http://www.uni-goettingen.de/en/1.html
University of Agricultural Sciences, Dharwad, India	Mechanized harvesting	Vikas V. Kulkarni Email: VikasVKulkarni@VikasVKulkarni3 http://www.uasd.edu/
University of Ruhuna, Sri Lanka	Selection procedure for breeding	Ranjith Pathirana ranjith.patirana@plantandfood.co.cn http://www.ruh.ac.lk/ruh/
University of Suleyman Demirel, Turkey	Breeding for ideal plant type	H, Baydar Email:baydar@ziraat,sdu,edu,tr https://w3.sdu.edu.tr/international
Izmir Institute of Tech., Turkey	Genome sequencing and SNP based characterization of the high oil crop	Ayse Ozgur Uncu Email: samidoganlar@iyte.edu.tr http://www.iyte.edu.tr/AnaSayfa.aspx?d=ENG

Appendix II: Sesame Genetic Resources and Varieties Developed Through International Sesame Breeding

Cultivar	Important traits	Cultivation location
Zhongzhi13	High oil content, improved variety	China
Baizhima and Mishuozhima	Land races and both contain multiple loci for several agronomic traits	China
Morada	Moderate branching	Venezuela
dr-45	Determinate, mutant of 'No. 45'	Israel
dt-4, dt-5 and dt-6	Determinate, mutants of Çamdibi	Israel
dt-1, dt-2 and dt-3	Determinate, mutants of Muganl 1-57	Israel
SIK 031 and SIK 013	Resistance to white leaf spot,	China
SIK 031 and SPS 045	Resistance to angular leaf spot	China
'S2' and 'H4'	Stable resistant to *Fusarium* wilt.	Egypt
Sanliurfa-63189	Resistant to *Fusarium* wilt	Turkey
SG 22, SG 55, SG 72 and SG 33	Resistant to bacterial blight	Pakistan
Zhuzhi 4"	High yielding	China
D248A	High yielding MS line	China
95 ms-2A and 95 ms-5A	GMS lines	China

(continued)

Cultivar	Important traits	Cultivation location
Zhonghi 11,12,14	High yielding	China
Ezhi 1, 2, 4	High yielding	China
Zhu 0J3, 9-4155, Hangzhi 2, 98-6204, Zhonghi 18 and 01-2658	High yielding restorer lines	China
AHY TIL 5, AHY TIL 12, RTH 1, AHYT 13, RTH 3, TKG-HY 5 and TKG-HY 4	All are experimental hybrids, 31.0–44.3% in seed yield and 13–48% in oil yield over TKG 22(Check).	India
IC-204078	Moderate branching, very early maturing, low plant height, small capsules, low yield potential	Andhra Pradesh, India
IC-204099	Less branched, early maturing, moderate	Andhra Pradesh, India
C-204337	Less branched, early maturity, medium tall, bold seeds, moderate yield potential, susceptible to phyllody	Rajasthan, India
C-204524	Moderate branching, medium maturity, relatively longer capsules, low yield potential, susceptible to phyllody	Gujrat, India
C-204628	Highly branched, medium tall, medium to late maturity, moderate yield potential	Karnataka, India
C-204653	Highly branched, medium to late maturity, tall and moderate to high yield potential	Kerala, India
C-204681	Highly branched, multilocular (6–8), early maturity, moderate yield potential	West Bengal, India
IC-204773	Highly branched bushy type, late maturity, photosensitive, resistance to phyllody and leaf roller	Nagaland, India
C-204814	Highly branched, medium to late maturity	Mizoram, India
C-204843	Medium branching, multilocular capsules, early	Bihar, India
C-205000	Highly branched bushy type, late maturity	Assam, India
C-205209	Moderate branching, late maturity,	Andhra Pradesh, India
IC-205314	Moderate branching, relatively long capsule	Uttar Pradesh, India
IC-205479	Less branched, early maturity, bold seeds	Himachal Pradesh, India

(continued)

Cultivar	Important traits	Cultivation location
C-205509	Moderate branching, medium maturity	Odisha, India
C-205595	Moderate branching, late maturity, tall, low yield potential	Odisha, India
IC-205730	Unbranched, medium maturity, relatively long capsules	Rajasthan, India
IC-205817	Moderately branched, relatively long capsules	Tamil Nadu, India
EC-346125-1	Moderately branched, tall, medium-sized capsules, early	Greece
EC-346489	Late maturity, small capsules, white seeds	Afghanistan
EC-346987	Moderately branched, medium-sized capsules	Unknown
EC-377025	Unbranched, glabrous stem, long capsules, low yield potential	Somalia
DLH-2 (S. mulayanum)	Branched, thin glabrous stem, tall, purple flower, black seeds, low susceptibility to phyllody	Delhi, India
SVPR 1	White seed, selection from Western Ghats White	Srivilliputhur, India
VRI 1	Brown seed, Pureline selection from Tirukattupali local	Vridhachalam, India
VRI 2	Reddish brown seed	Vridhachalam, India
VS07023	Landraces, brown seed	Vridhachalam, India
MD 1	Landraces, white seed	Madurai, India
MD 2	Landraces, white seed	Madurai, India
MD 3	Landraces, white seed	Madurai, India
MD 4	Landraces, white seed	Madurai, India
MD 5	Landraces, white seed	Madurai, India
NIC-7943	Shy branching	India
SP-41, VOSI-5846, VOSI-8458, NIC-8202, IS-101,IS-92-2,SI-3265-5	Moderate branching	India
TKG-22, SI-2973, GRT-83125, IS-56, NIC-16268, IS-355	Moderate branching	India
SI-2940, IC-382-2, GT-10, DSK-1, 49-E-SPS-6, ES-29, NIC-8559	Moderate branching	India
KANPUR LOCAL, KMR-77, ES-28, MT-6262, SI-2174, B-7-11	Moderate branching	India
KIS-357-A, KIS-297-2, RJS-29, EC-14121, EC-334952, IC-132408	Profuse branching	India

(continued)

Cultivar	Important traits	Cultivation location
IS-172, IS-136, IS-750-1-84, IS -184-1, IS-146, SI-44, SI-3275, PjCU-36	Profuse branching	India
S-0448, IC-14160-1, S-0337, KMR-17, C-96128, S-0434,NIC-16236	Profuse branching	India
KIS-357-A, KIS-297-2, VOSI-5846, VOSI-8458, EC-14121, EC-334952,	Basal branching	India
IC-132408, NIC-8202, IS-101, IS-136, IS-146	Basal branching	India
NIC-8984, MT-67-25, MIC-8526,NAL/28/27/31/4, IC-14093, IS-351-2	Basal branching	India
SI-199-2-84, SI-3275, SI-982, PCU-37, PCU-38	Basal branching	India
IS-750-1-84, IS -184-1, IS-92-2, RJS-29, SP-41	Top branching	India
13,598, NIC-8559, NIC-8202, NIC-10622	Top branching	
KIS-297-2, KIS-357-A, VOSI-5846, VOSI-8458, SP-41, EC-334952, IC-132408, NIC-8202	Dense hairiness of flower petal	India
EC-14121, RJS-29, MT-6262, NIC-8984	Sparse hairiness of flower petal	India
GT-10, TKG-22, RT-54	Light purple petal of flower	India

In addition, a detailed list of Indian sesame improved varieties with specific traits has been mentioned in Table 15.7

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Chapter 16
Breeding Strategies for Sunflower (*Helianthus annuus* L.) Genetic Improvement

Saeed Rauf

Abstract Sunflower is well known as an important oilseed crop and also consumed roasted, as a confectionary and bird feed. The plant has been subjected to the improvement by plant breeders resulting in the *yellow* revolution in many countries. Russian plant breeders have improved the oil content of sunflower seed that converted this crop from a roadside plant to a world famous oilseed crop. The cultivated germplasm retains 50% of genetic diversity present in crop wild relatives. This may be threatened due to worldwide hybrid cultivation which shares common parentage and a source of cytoplasmic male sterility. Therefore, there is a need to use the available genetic diversity within cultivated and wild germplasm to develop pre-breeding lines and elite breeding material with good combining quality. Sunflower breeding involves development of breeding lines suitable for hybrid breeding, diseases, abiotic stress and herbicide resistance. These objectives are fulfilled by recurrent selection for population improvement. Wide crosses were made to transfer cytoplasmic male sterility, diseases, abiotic and *Orobanche* resistance. Moreover, induced mutations were used to create new genetic variability for diseases and herbicide resistance and reduction of plant height. Marker-assisted selection has been validated for rust resistance, downy mildew resistance, and oleic acid content and fertility restorer genes. Transgenic sunflower development could be used to enhance oil content and quality. Sunflower breeding will be greatly facilitated by genomic tools such as CRISPR/Cas and whole genome association mapping.

Keywords Breeding objectives · Crop wild relatives · Combining ability · Cytoplasmic male sterility · Resistant genes · Stability

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16.1 Introduction

Sunflower is the fourth largest seed crop worldwide after the oil palm, soybean and rapeseed (Fig. 16.1). Total sunflower edible oil production was 15.84 million mt (FAO 2014) which represented 10% of the total edible oil production (Fig. 16.1). Sunflower is grown in 72 countries with the Russia being the largest grower in terms of its harvested area followed by Ukraine and Argentina. These three countries contributed about 56% of the total harvested area. World average achene yield was 1806.7 kg ha^{-1}in 2014. Achene yield ha^{-1} showed a growth rate of 2.6% $year^{-1}$ (Fig. 16.2). Total trade value of the sunflower oil was USD 1864.24 billion while the trade value of sunflower seed was USD 705.76 billion in 2016. This represented 17 and 21% share of the total export and import values among the major oilseed crops, respectively, and was the second major oil crop after the palm oil in the total major edible oil trade of the world (FAO 2016).The world average sunflower oil annual per capita consumption of 152 nations was 2.56 kg. Kazakhstan had the highest per capita (17.98 kg) consumption of sunflower oil. Sunflower seed was consumed as snack food in 58 countries, and the consumption was the highest (2.2 kg per capita) in Tanzania (FAO 2013).

This chapter describes the importance of sunflower, germplasm resources, breeding achievements and objectives in sunflower breeding programs as well as breeding methodologies.

There are three types of sunflower i.e. oilseed, confectionary and bird food. The confectionary and bird food sunflower contain high protein content (>40%) and low oil content (≤30%). The confectionary and bird food sunflower are large seeded and

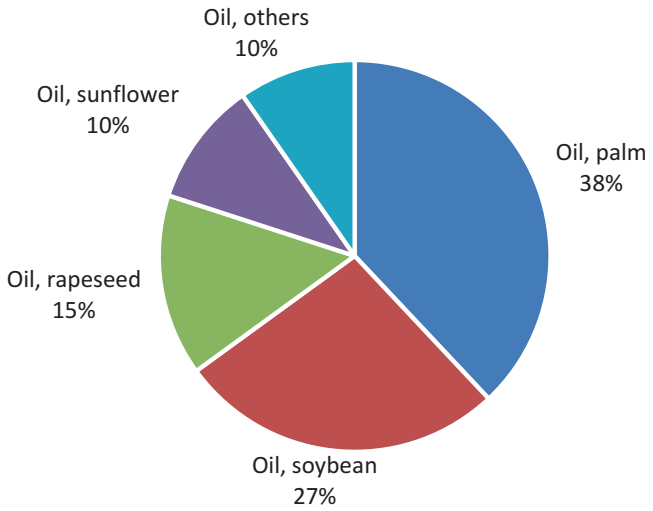


Fig. 16.1 Contribution of various oilseed crops to the total world edible oil production. (Data source FAO (2014). The figure was prepared from public data available at FAO (www.fao.org))

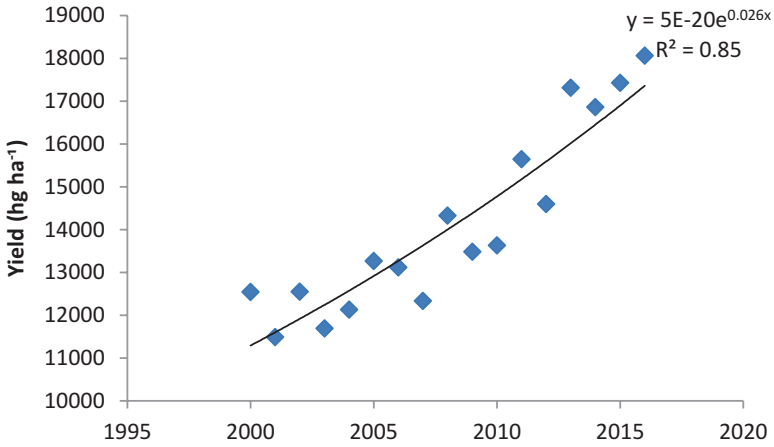


Fig. 16.2 Change in the sunflower achene yield (hg ha^{-1}) over the years. (Data Source: FAO (2016). The figure was prepared from public data available at FAO (www.fao.org))

striped with 100-seed weight greater than 10 g (Fig. 16.3). Oil content types are small seeded and black in color.

Cultivated sunflower plant is determinate with a single floral head called a *capitulum*. The head size range of 6–40 cm in diameter contains two types of flower discs and ray florets. Ray florets are sterile and generally yellow in color but exhibit different colors (crimson red to white) and shades (Fig. 16.4).The plant initiates a floral bud about 40–45 days after planting depending upon the cultivar and growth conditions (Fig. 16.5). The involcural bract (phyllaries) surrounds the floral bud. The number of disc florets range from 100–2000 per head arranged in various rows. Disc florets open from outside to inside rows. Two to four rows open daily and floral anthesis is completed within 5–8 days. Each disc floret is hermaphroditic, comprised of a single inferior ovary, tubular corolla form by the fusion of 5 petals, 5 anthers united to form tube with separate filaments, and upon fertilization, it bears a single large achene enclosing a kernel. The sunflower is a cross-pollinated species, and disc florets are protandrous i.e. male part matures ahead of the female part. The pollen is large, sticky and is carried by insect pollinators which are vital for hybrid seed production. Cross-contamination between breeding lines is prevented by enclosing the heads in net bags or net cages.

Sunflower seed kernels are a favorite snack food, consumed as a roasted and salted product in several countries. Dried seed kernels of sunflower contain 584 calories and 5% water per 100 g. They are a rich source of vitamin E, Vitamin B such as niacin and folate and also contain appreciable amount of magnesium, phosphorous, potassium, selenium and small amounts of iron and zinc. Dry sunflower seed kernels contain the highest amount of folate (227 µg10 0 g^{-1}) as compared with other popular snack nuts such as hazelnut, sesame, pistachio and almonds. Similarly, they are also rich in vitamin E (total tocopherol 36.74 mg10 0 g^{-1}), chlorogenic acid (antioxidant and purported anticarcinogenic 1003.7 mg 100 g^{-1}) and total choline

Fig. 16.3 Diversity in seed coat color of various types of sunflower: (**a**) Black seed oil type sunflower, (**b**) Light brown high seed coat, (**c**) Albino seed for confectionary and animal feed, (**d**) Striped sunflower for roasting. (Photos by Saeed Rauf)

(purported protection against heart diseases 55.1 mg 100 g^{-1}). The range of tocopherol content was 314.5–1024.5 mg kg^{-1} in seed kernels and 562.8–1872.8 mg kg^{-1} in oil (Velasco et al. 2004). Phytosterol content is known to lower low density lipid which ranged from 2100–4540 μg g^{-1} (Vlahakis and Hazebroek 2000). Breads (300 g) enriched with high oleic acid sunflower seed can provide 40% of the daily protein, 90% of copper, 20% of the zinc and 5 times the daily fiber requirement. A handful of sunflower seed consumed daily is reputed to appreciably improve the body requirements for fat, fiber, alpha tocopherols and linoleic and oleic acids.

Fig. 16.4 Diversity in ray and disc floret color of sunflower. (**a**) Crimson red ray floret with brown disc floret, (**b**) Yellow with crimson red pattern ray floret with purple disc floret, (**c**) Yellow wide ray floret with yellow disc floret, (**d**) Yellow narrow ray floret with green disc floret, (**e**) Yellow ray florets with purple disc floret, (**f**) Crimson red with yellow tip ray floret and purple disc florets. (Photos by Saeed Rauf)

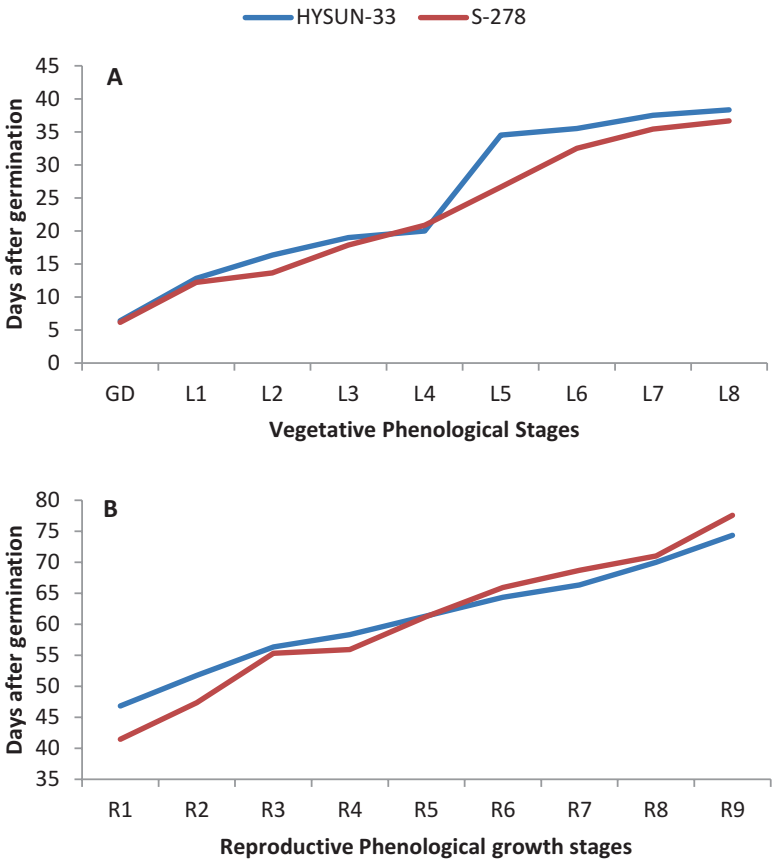


Fig. 16.5 Days to various phenological growth stages. Response during the vegetative stage (**a**) and reproductive stage (**b**) in two popular hybrids of sunflower (*Helianthus annuus* L.) grown at College of Agriculture, University of Sargodha, Pakistan in 2012–2013 growth season (Unpublished data) where *GD* days to germination, *L1–L8* day to 1st leaf to 8th leaf stages and *R1–R9* 1st to 9th reproductive stage. (Source: Saeed Rauf unpublished)

16.2 Germplasm Resources

Sunflower belongs to the genus *Helianthus* which is indigenous to North America. There are about 52 species and 19 subspecies which are widely distributed in central Mexico, the USA and southern Canada. These species have annual (18) and perennial (34) growth habits. The somatic chromosome number of diploid species is 2n = 2x = 34 (Tahara 1915). However, ploidy levels such as tetraploid (2n = 4x = 68) and hexaploid (2n = 6x = 102) have also been observed in various species of *Helianthus*. Among them, all annuals are diploid while polyploidy species are perennials. However, annual diploid species also exist within the genus. Some species occurs in dual ploidy levels such as *H. ciliaris* DC which has both tetraploids and hexaploid, whereas *H. decapetalus* L. has diploid and tetraploid forms.

16.2.1 Extent of Related Species Geographically

Crop wild relatives (CWR) are characterized on the basis of the extent their hybridization with related species. The primary germplasm is comprised of cultivated and wild species of *Helianthus annuus* and winter sunflower, *H. winterii* J.C. Stebbins, while secondary germplasm includes species such as *H. anomalus* S.F. Blake, *H. paradoxus* Heiser, *H. petiolaris* Nutt. and *H. deserticola* Heiser. Secondary species have undergone some degree of genetic differentiation from primary germplasm. Tertiary germplasm varies due to a high degree of genetic and cytological differentiation. The species considered tertiary germplasm are *H. hirsutus* Raf., *H. tuberosus* L. and *H. divericatus* L. These interspecific hybrids required specialized techniques such as embryo rescue for their recovery (Warburton et al. 2017). Differentiation can be accessed through molecular, cytological and morphological bases to characterize species.

The extent of wild species utilization depends on several factors such as ploidy level, growth habit and reproductive barrier. The reproductive barrier occurs through the evolution of any form of reproductive isolation such as prezygotic and postzygotic barriers. In plants, prezygotic barriers are more common than postzygotic, in contrast to animals where postzygotic barriers are more common and hybrid forms have selective disadvantages (Maheshwari and Barbash 2011). Prezygotic barriers include failure of pollen to germinate over the stigma, failure of the pollen tube to grow through the stylar tissue or failure of fertilization. These prezygotic barriers occur due to the divergence between species and adaptability to the particular habitat. Postzygotic barriers include embryo abortion after fertilization, hybrid unviability or sterility. Hybrid sterility is included due to negative interactions between loci. The speed of gene transfer from wild species depends on the ploidy level, the speediest transfer occurs at the diploid level followed by the tetraploid and hexaploid (Alix et al. 2017). The slow transfer of genes occurs due to time-consuming removal of extra chromosome through backcrosses (Jan et al. 2014). The speedy restoration of chromosome to 2n = 34 can also occur through the use of polyploid species as a male parent. The characterization of the wild species is poorly understood and many sister species are not well characterized (Vanzela et al. 2002).

Many *Helianthus* species originated from multiple homoploid hybrid speciation. Multiple homoploid events led to the origin of many species from common ancestral species due to chromosome sorting and rearrangement (Lai et al. 2005). Hybridization between *H. annuus* and *H. petiolaris* followed by the doubling of genome due to unreduced gametes, led to the evolution of three different diploid hybrid species (*H. anomalus*, *H. paradoxus*, *H. deserticola*) (Gross et al. 2003, Fig. 16.6). These species have undergone significant differentiation and adaptation to particular ecological conditions due to transgressive segregation of the chromosomes (Rosenthal et al. 2002). Transgressive segregation led to the appearance of extreme traits which led to the adaptation under extreme environment. For instance, *H. anomalus*, *H. paradoxus* and *H. deserticola* are adapted to sand dunes, salt marshes and high deserts, respectively (Rosenthal et al. 2002). It was noted that

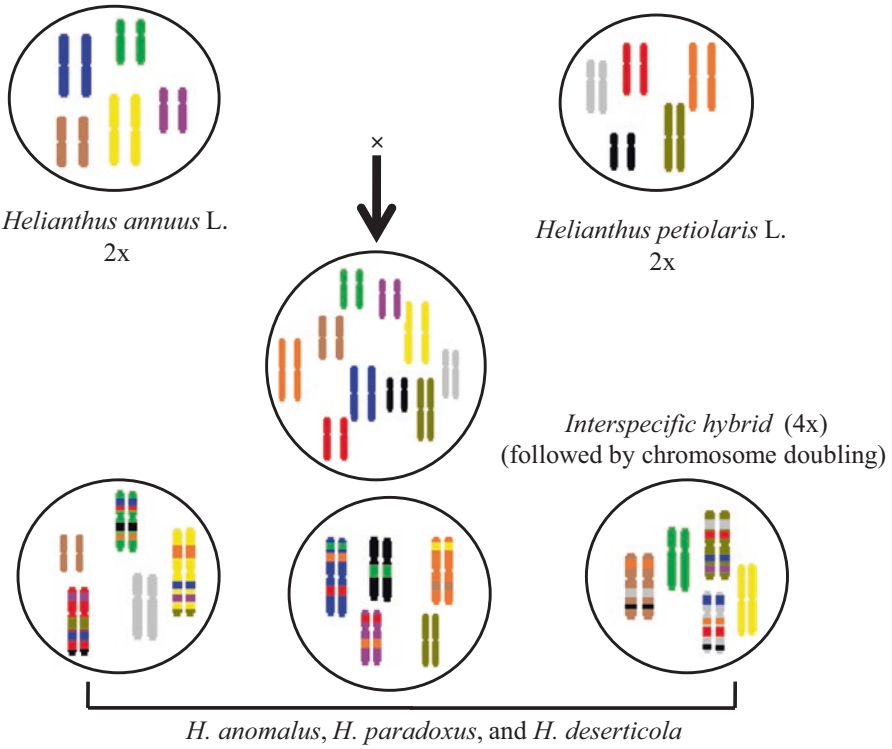


Fig. 16.6 Origin of three homoploid species i.e. *H. anomalus*, *H. paradoxus* and *H. deserticola* due to chromosome sorting and rearrangement. (Figure prepared Saeed Rauf)

these hybrid species were divergent in karyotype and gene order. Gene order differences were observed at 9 to 11 linkage groups due to chromosome sorting and de novo breakage and fusion cycle (Lai et al. 2005). The schematic origin of three species is shown in Fig. 16.6.

Sunflower species grow in a diverse range of habitats i.e. plains, deserts and salt marshes (Fig. 16.7). As a result of their adaptation to assorted ecological conditions, the wild species can be regarded as sanctuaries of new alleles to achieve diverse breeding goals (Kantar et al. 2015). The potential utilization of related species in breeding programs to provide various alleles is well documented (Seiler 1992, 2007a, b) and could be exploited for disease resistance breeding and diversified cytoplasmic sources, as well as drought, heat and salinity resistance. Moreover, these species could also be exploited for the modification of fatty acids and other industrial products. The monetary benefits to the sunflower industry due to wild crop relative contributions of economically-relevant traits have been estimated to be worth than USD 1 billion (Seiler and Marek 2011). The trait of greatest value was cytoplasmic male sterility (PET1) exploited by the sunflower hybrid seed industry from wild species of *Helianthus petiolaris*. Other important traits are disease and insect resistance genes which provide resilience and sustainability to the achene yield (Feng et al. 2009).

Fig. 16.7 Diversity in canopy and foliage color and adaptations among various species of sunflower. (**a**) *Helianthus petiolaris* (ex situ: Sargodha, Pakistan, Saeed Rauf), (**b**) *H. argophyllus* Torr. & A. Gray (ex situ: Sargodha, Pakistan, Saeed Rauf), (**c**) *H. anomolous* (San Juan County, Utah, SW of Cal Black Airport, Gerald Seiler, permission granted), (**d**) *H. debilis* Nutt. (in situ: Florida, USDA, Gerald Seiler, permission granted), (**e**) *H. paradoxus*(in situ: New Mexico, USDA, Gerald Seiler, permission granted), (**f**) *H. niveus* (Benth.) Brandeg. (in situ: California, USDA, Gerald Seiler, permission granted)

16.2.2 Germplasm Collections

Germplasm collections safeguard the genetic diversity within species in addition to providing rare alleles for crop improvement. Several germplasm collections have been created, for instance, the USDA-ARS collection of elite sunflower germplasm at the National Plant Germplasm System, North Central Regional Plant Introduction Station, Ames, Iowa in1948. On the other hand a sunflower wild related species collection was established at the USDA-ARS Bushland, Texas station in 1976. There were over 30 expeditions (Canada, USA, Australia), covering 175,000 km, carried out to collect wild sunflower diversity, which is considered as the most updated collections in the world. The USDA collection contains 4087 accessions: 1886 cultivated *H. annuus* and 2201 wild, of which 1359 are annual and 842 are perennial species (Seiler and Marek 2011). The collection may help to improve economically-relevant traits related to the yield and quality and also supply biotic and abiotic

Table 16.1 Worldwide examples of sunflower germplasm resources collections

Institutes	Collection
USDA collection	4087 accessions: 1886 cultivated, 2201 wild accessions
French National Institute for Agricultural Research Toulouse, France	5576 cultivated accessions, >500 wild ecotypes
The Indian Institute of Oilseeds Research, India	3273 accessions: 1200 exotic lines, 97 genetic stocks, 360 inbred lines, 42 wild species, and 154 wild species derivatives, Dudhe and Sujatha (2016)
The Vavilov Research Institute of Plant Industry, Russia	2230 cultivated sunflowers and 550 wild accessions, Gavrilova et al.(2014)
The Oil Crop Research Institute of the Chinese Academy of Agriculture Science Wuhan, China	2813 accessions, predominantly cultivated, Gao et al. (2001)
The Institute of Field and Vegetable Crops Novi Sad, Serbia	Several thousand lines (S. Terzic, personal communication 2016), wild sunflower collection of just over 1000 accessions of 47 wild species, Atlagić and Terzić(2015)

resistance genes. Moreover, the elite germplasm may help to encourage creation of sunflower breeding programs in many countries. Germplasm collections are given in Table 16.1.

16.2.3 Diversity Present in Primary and Secondary Gene Pools

The genetic diversity in the primary gene pool (*Helianthus annuus*) has been characterized among 433 accessions collected from North America and Europe, along with the 24 wild populations (Mandel et al. 2011). The diversity index was 0.47, compared to the wild of 0.7, showing that cultivated germplasm retained two-thirds of the diversity. A core set of 288 accessions was sufficient to capture 90% of the allelic diversity, while only 12 accessions retained 50% of the allelic diversity showing a narrow base in the primary gene pool (Mandel et al. 2011). *Helianthus argophyllus*, *H. annuus*, *H. petiolaris* and *H. debilis* were crossed with cultivated sunflower to increase the diversity of the cultivated sunflower (Sujatha et al. 2008). Chromosomal pairing between the wild and cultivated sunflower led to the structural rearrangements in lines derived from *H. petiolaris*. The genetic distance of 40 diverse lines and 2 controls was measured using 118 simple sequence repeat (SSR) markers of known map location. A total of 204 alleles were identified and number of alleles per locus was 2–5. A total of 46 distinctive alleles were identified and number of distinctive alleles was highest in *H. petiolaris* derived lines, and the observed PIC value was 0.05–0.575. Pair-wise comparison value was 0.143–0.486 based on the dissimilarity estimate using molecular markers. Results suggest that the wild diploid species are a source of introgression of novel traits, especially from *H. petiolaris*.

Diploid annual and perennial species of sunflower do not cross easily due to the presence of different genomes in the species. Similarly, the genome of wild species is different from the cultivated species. However, annual species are mutually crossable and also with cultivated sunflower. However, sometimes techniques such as in vitro fertilization and embryo rescue are exploited to obtain viable seedlings of hybrids from perennial parental lines. It has been noted that annual wild species have shown karyotypic differences due to translocations and inversions. The diploid annuals have been shown to be susceptible to diseases and thus they are less exploited by the breeders for introgression in cultivated germplasm. However, they could be exploited for introgression of adaptability to abiotic stresses. On the other hand wild perennials have been exploited to introduce disease resistance genes (*Helianthus maximiliani* Schrad., *H. giganteus* L.), Liu et al. (2010) and morphological traits such as oil content (*H. salicifolius* A. Dietr.), Jovanka (2004) modification in cultivated sunflower. However, crossability between the species was poor and requires embryo rescue technique for the recovery of the hybrids.

Wild crop relative collections have contributed to the sunflower industry in several ways. The wild crop relatives have been extensively exploited in breeding programs as a source of resistance to major sunflower diseases i.e. rust, downy mildew, *Verticillium* wilt, powdery mildew, *Phomopsis* stem canker, *Sclerotinia* wilt, charcoal rot, *Phoma* black stem and the parasitic weed broomrape (Seiler 2010). Both horizontal and vertical resistance is known to exist in crop wild relatives. The resistance to all multiple races of rust was high in wild annuals while resistance for all races of powdery mildew was only present in two populations of *Helianthus argophyllus* and *H.debilis* (Jan and Chandler 1985). *Helianthus tuberosus* was useful for resistance to stem infecting disease i.e. *Phomopsis* stem canker, *Phoma* black stem and charcoal rot, while perennial species showed resistance to broomrape.

Germplasm evaluation showed that broomrape resistance and immunity was identified in 7 annuals and 32 perennials, providing breeders a broad genetic base for resistance to new races. (Christov 2004; Petcu and and Pâcureanu 2011; Seiler and Jan 2014). Perennial species of genus *Helianthus* were resistant, but *H. divaricatus, H. maximiliani* and *H. pauciflorus* Nutt. showed susceptibility to the diseases. Annual wild species *H. anomalus* and *H. agrestis* Pollard were resistant, while *H. debilis* ssp. *cucumerifolius* and *H. exilis* A. Gray showed heterozygosity for the resistance (Fernandez-Martinez et al. 2000a, b).

Among the species, *Helianthus argophyllus* has developed specific phenotypes which help them to adapt under drought; is also known as silver sunflower due to it intense hairiness and thick leaves. The presence of high pubescence and smaller leaf area could help to reflect light and to protect the leaves from the transpiration losses. *Helianthus argophyllus* was the best source of stress resistance genes and used in interspecific hybridization. *Helianthus paradoxus* was utilized as a genetic source of salinity resistance (Škorić et al. 2008).

Development of perennial sunflower could benefit sustainable agriculture and remedy agriculture soil degradation. Perennial traits could be transferred to the cultivated type through introgression between the cultivated species *Helianthus tuberosus* and *H. annuus* L. (Kantar et al. 2014). The resulting selected transgres-

sive segregants could have tuber and have sustainable seed yield traits. Tuber traits are positively related with head diameter and seed traits.

16.3 Sunflower Breeding History

Sunflower was domesticated in North America about 4000–5000 years ago (Smith 2006). It was domesticated by Native Americans for its multiple uses such as food, body painting and folk medicine such as treating warts and snake bites, expelling worms and improving eyesight.

The sunflower plant has great aesthetic value and is famous for its peculiar heliotrope movements to track maximum radiation. Wild sunflower is characterized by multiple branches tipped by numerous flowers containing small achenes. Spanish travelers brought this plant to Europe during the fifteenth century from where it was introduced to Russia. Russian academician V.S. Pustovoitat, of the VNIIMK Research Institute, made tremendous improvement in sunflower to make it one of the leading oilseed crops of the world during the first half of the nineteenth century. Sunflower oil content was improved through a modified recurrent selection method called *seed reserve* (Fig. 16.8). This method involves progeny testing and subsequent cross-pollination of selected superior progenies; as a result of this selection sunflower oil content increased from 33 to nearly 43% over three decades of selections (1913–1943). Cultivar Peredovick was released in 1958 with an oil content greater than 50% which was adopted by the rest of the world and became the source of global cultivation. The source of cytoplasmic male sterility in *Helianthus petiolaris* was discovered by P. Leclercq (1969) at INRA in France and a fertility restorer system was identified by Kinman at the USDA (Kinman 1970) which paved the way for exploitation of commercial heterosis which significantly improved the achene yield without sacrificing the seed oil content.

The first attempt to develop a hybrid was carried out by Putt (Vera-Ruiz 2006) at Morden Manitoba, Canada, without the presence of effective male sterility system, by crossing line S37.388RR with commercial cv. Sunrise to develop hybrid cv. Advent which was later discovered as a source of powdery mildew resistance by Vranceanu and Stoenescu (1969) at ICCPT, Romania. Canadian lines 953.88 and 953.102 (progenies from natural crosses with wild sunflower) were supplied to M. Kinman and he subsequently used them with French CMS line selected from the Russian population Cernianka (PI343765), which led to discovery of the restorer gene *Rf1* (Kinman 1970). The first USDA restorer RHA line was obtained by crossing (Peredovik × 953.102) and another line HA-61 came from the cross of (953.88 × Armavir3497). This line was a source of recessive branching and thus suppressed the branching in the sunflower hybrid. The Canadian sunflower team led by Putt also selected a line called CM-303 from Russian open pollinated cultivar which was further selected by USDA sunflower breeders and called HA-89.

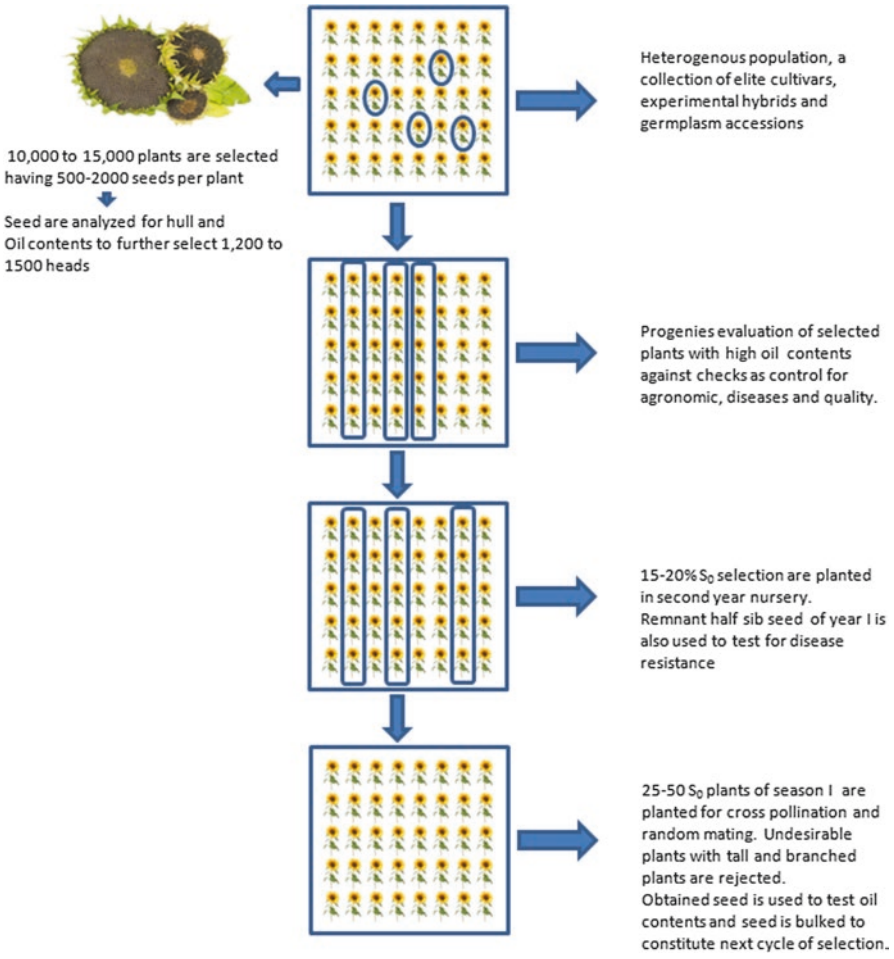


Fig. 16.8 Procedure for Pustovoit's famous *method of reserve* to develop high oil contents progenies. (Photo by Saeed Rauf)

Initially sunflower hybrids had little yield advantage over the open-pollinated cultivars, but hybrids were more uniform in maturity and harvesting. Commercial hybrids had a 30–40% yield advantage and were uniform in maturity. Today, sunflower is the second major field crop after maize being cultivated through hybrid seed. The cultivated hybrid sunflower is non-branching, a large leaf area with a single head (capitulum), which ceases to show heliotropic movement after the initiation of reproductive growth cycle.

16.4 CLEARFIELD Technology to Control Weeds

The sunflower plant is poor competitor with weeds during its early growth stages and broad leaf weeds are a major yield limiting factor due to their competition for light, soil nutrition and moisture. Moreover, weeds act as alternative hosts for the spread of insects and diseases (Pfenning et al. 2008). CLEARFIELD technology has been introduced to introgress imidazolinone herbicide resistance in elite sunflower hybrids. Imidazolinone is a post emergence herbicide for broadleaf weeds. It inhibits the enzyme acetohydroxyl acid synthase (AHAS). Conventional hybrids are sensitive to this herbicide while CLEARFIELD hybrids are known to carry a mutant form of AHAS gene which reduces their sensitivity to the broad leaf herbicide. The source of the resistance is a naturally-occurring mutation in the wild sunflower which was transferred to elite inbred lines by plant breeding methods (Pfenning et al. 2008). CLEARFIELD plus has been introduced to improve the effectiveness of the herbicide-resistant imidazolinone gene in sunflower. The gene was modified through mutation of *Ahas1* and was designated as CLHA-plus or *AhasII-3*. The beneficial effects of the genes were observed such as improved oil content, stability and reliability of herbicide tolerance due to better weed management (Weston et al. 2012).

16.5 Breeding Objectives

16.5.1 Hybrid Breeding

More than 90% of sunflowers are cultivated by hybrid seed. Hybrid crops were found to be superior to open-pollinated and synthetic cultivars due to higher yield potential and uniformity in maturity. The hybrid vigor and heterozygous genetic base of the hybrids allow them to show better yield than synthetic cultivars even under stress condition. The superior performance of hybrids is due to manifestation of heterosis, defined as the superiority of F_1 over the mid parent value or better performance of F_1 over the superior parent or best commercial cultivar. Heterosis is manifested due to genetic divergence of the parents and superior combining ability of both parents, causing the combination of diverse alleles in a single genotype. There is a positive relationship between genetic distance and best parent heterosis (Hladni et al. 2018). In contrast to open-pollinated varieties (OPVs), which only exploited additive gene action for the improvement of plant traits, the performance of a hybrid depends on both additive and non-additive gene action. Some plant traits such as oil content, day to maturity, biotic or abiotic are fixed during inbred line development through pedigree selection and may be controlled through additive alleles. Allelic or nonallelic interaction may be broken to release additive alleles through recurrent selection. However, grain yield potential of a hybrid is based on

over dominance, a genetic phenomenon, in which deviation from mid parents value greatly exceeds both parents.

A single cross hybrid has been developed in sunflower using a cytoplasmic male sterility system in A lines, maintaining it through B lines, and R fertility restorer lines are developed as male lines carrying fertility restorer genes. Morphological traits such as fertility of female lines, synchronization between A and R lines are vital for the development of hybrids. Hybrid seed production in sunflower is done by using cytoplasmic a male sterile female line and a fertility restorer male line at a ratio of 4:2; pollination is facilitated through honey bee hives (2 hives ha^{-1}), Greenleaf and Kremen (2006).

16.5.2 Diversification of Cytoplasmic Male Sterility Source

Sunflower belongs to a genus of highly diverse species and thus cytoplasmic male sterility sources could be diversified using these species as a maternal parent (Table 16.1). Cytoplasmic male sterility is alloplasmic, meaning that it originates from a combination of interspecific and intergeneric crosses. The first sources of cytoplasmic male sterility were *Helianthus petiolaris* species which was transferred into sunflower lines through interspecific crossing. CMS-PET-1 has been commercially exploited for hybrid breeding. However, single use of a male sterility source could increase the vulnerability to diseases and insect pest due to a decrease in genetic diversity. Therefore, expansion of male sterility sources is one of the prime objectives of sunflower hybrid breeding. More than 70 male sterility sources have so far been exploited; however, very few of the sources have the potential to achieve commercial success. This may partly be due to the absence of male fertility restoration genes to completely overcome the cytoplasmic male sterility in hybrid breeding. New sources have been developed through mutagenesis, crossing with wild sunflower or its closely-related species and discovered spontaneously in sunflower fields (Christov 1999). Molecular characterization of 22 cytoplasmic male sterility sources of sunflower showed considerable similarity among the sources and could be differentiated in 10 mitochondrial types based on RFLP marker analyses (Horn 2002). Comparison of PET-1 and PET-2 showed variability and rearrangement in the mitochondrial sequences. PET-1 was characterized by the presence of atpA and orfH522 and 16KDA CMS specific proteins whereas PET-2 had two new open reading frames, orf288 and orf231. The orf encode protein of 11.1 KDa and 7.9 KDa, respectively (Horn et al. 2016) (Table 16.2).

Table 16.2 List of cytoplasmic sources for sunflower

Cytoplasm code	Species/Sources	References
CMS-PET-1, CMS-PET2, CMS-PET-4	*Helianthus petiolaris*	Leclercq (1969), Havekes et al. (1991), and Christov (1994)
CMS-Arg-1, Arg-2, Arg-3, Arg-4	*H. argophyllus*	Christov (1990) and Christov (1999)
CMS-GIG1/141,GIG1/477 GIG1/647, CMS-GIG2	*H. giganteus*	Jiuhuan et al. (2015)
ANN-1, ANN-2, ANN-3, ANN-5, ANN-10, ANN-11, ANN-12, ANN-13, ANN-14	Wild sunflower	Marinković and Miller (1995) and Christov (1999)
DEB1	*H. debilis*	Christov (1999)
ANT1/479, ANT1/ 645	*H. texanus*(= *H. annuus* ssp. *texanus*	Chepurnaya et al. (2003)
PRR1, PRH1	*H. praecox* Engelm. & A. Gray	Christov (1999)
PEF1	*H. fallax* (=*H. petiolaris* Nutt. ssp. *fallax* Heiser)	Miller (1996)
cmsMUT7, cmsMUT8, cmsMUT9, cmsMUT10, cmsMUT11, cmsMUT12	Mutagen, gamma rays 70 to 250 Gy	Christov (1999)
CMS 514A	*H. tuberosus*	Liu et al. (2013)
RIG1, RIG-2, RIG-L	*H. rigidus* (Cass.) Desf. (=*H. pauciflorus*)	Christov (1999) and Chepurnaya et al. (2003)
STR-1	*H. strumosus*L. M – 056	Christov (1999)
NEG-1	*H. neglectus* Heiser	Christov (1999)
EXI-2	*H. exilis*	Christov (1999)

16.5.3 Oil Content

Improvement in oil content is a major objective of sunflower breeding. Oil contents are quantitatively inherited traits and genetic variation is affected by additive genes. Oil content ranges from 30 to more than 50% and have shown tremendous improvement due to selection by plant breeders. Historically oil contents were subjected to improvement during the earlier part of nineteenth century through a method of seed reserve proposed by Pustovoit (Rauf et al. 2008) which led to the development of high oil contents lines. Seed morphological traits such as embryo size and testa thickness were important traits affecting oil content. Smaller seed size, with a larger embryo and a thin testa, give higher oil recovery (Rauf et al. 2017). Improvement in oil content occurred due to higher kernel to achene ratio (Pereira et al. 2000). Oil content accumulation is not constant during the grain filling period; the accumulation rate is slower after anthesis, but increases over the time and becomes constant at physiological maturity (Rondanini et al. 2003). Oil concentration is also dependent on the size and concentration of oil bodies. However, high oil content lines have a higher concentration of oil bodies rather than larger oil bodies. Oil bodies are the storehouse of triglycerides containing a single layer of phospholipid which is

kept intact through oleosin and caleosin proteins (Murphy 1990). The range of oil body diameter is 0.65–2.0 μm in various crop species including sunflower. The accumulation rate of oil bodies is negatively affected by protein content. However, oil content is significantly affected by the environment and medium heritability (0.57) of the trait was estimated over multiple environments (Mokrani et al. 2002). Several QTL have been identified on various linkage groups for seed oil content. These QTL have additive to dominant affects and are closely related to domesticated-related traits in sunflower (Burke et al. 2005; Leon et al. 2001).

16.5.4 Broomrape

Orobanche cumanais is an obligatory non-photosynthetic parasitic plant of sunflower. Yield losses occurring in Asia and Europe where its growth is unchecked can reach 80%. Infested plants have stunted plant height and reduced head diameter. Eight races of *O. cumana* (A to H) have been identified with races F, G and H more prevalent in various countries. Race F was identified in the middle 1990s and is prevalent in countries like Turkey, Spain, Romania and Bulgaria. Races G and H were identified in countries around the Black Sea (Martín-Sanz et al. 2016). Genetic factors such as recombination, mutations and high diversity in wild and parasitic populations led to the evolution of new races of broomrape. Resistance against broomrape infestation is the most efficient method to control this parasitic plant. Resistance is simply inherited with a single dominant gene. Wild species have been known to carry resistance genes which could be transferred through interspecific crossing. *Helianthus debilis* carries a dominant resistant gene for G race (Höniges et al. 2008). Dominant genes designated as *O1*, *O2*, *O3*, *O4* and *Or5* have been identified which confer resistance to each of the races, A to E, respectively (Louarn et al. 2016).

16.5.5 Rust Resistance

Rust is a disease caused by *Puccinia helianthi* Schwein and one of the major factors affecting yield and quality of sunflower seed. There are about four species of rust. It is found in the fields of Canada and the USA and can be identified on plants as cinnamon red pustules on the leaves and other parts of the plant. It causes reduction of green leaf area, reducing the overall assimilation of photosynthates and translocation. It also causes reduction of seed weight, size and oil content. Rust thrives in warm and humid conditions and fungicides can be used to control the diseases, but chemical control is expensive and difficult. Therefore incorporating rust resistance in inbred lines is a preferred method to control diseases. Several rust resistance genes (*R1*, *R2*, *R4u*, *R5*, *R12*, *R13a*, *RHAR6*) were identified in various breeding lines, cultivars and restorers. A single source of resistance is not sufficient due to

emergence of virulent races which may defeat single-source resistance in sunflower. Molecular markers have been developed to stake these genes in single genotypes which increases the durability and spectrum of resistance (Paniego et al. 2012). Two genes have been recently identified in HA-R6 and RHA-397 which provide resistance against all virulent races of rust. Pedigree and marker-assisted selection was carried out to develop two inbred lines (HA-R12, HA-R13) which contained multiple rust resistant genes. HA-R12 contained the rust resistance (*R*) genes, *R2* from MC29 (AUS) and *R13a* from HA-R6. HA-R13 contained *R5* from HA-R2 and *R13a* from HA-R6. Application of rust specific markers confirmed that HA-R12 and HA-R13 contained two rust resistance genes in the homozygous condition, with both lines showing high levels of resistance to rust races 336 and 777, which are the most predominant and virulent races (Ma et al. 2016).

16.5.6 Powdery Mildew

Powdery mildew, *Golovinomyces cichoracearum*, disease causes chlorosis, curling and death of leaves. Affected leaves have a lustrous white powdery growth on the upper surface. The disease can cause complete loss of sunflower yield in the absence of resistance or chemical control due to severe infestation. Powdery mildew occurs in the spring season and the infection increases under dry conditions. Intraspecific variability has been known to occur for resistance to this disease under artificial screening. Artificial infestation is generally carried out by spraying a suspension of inoculum along with 1% sucrose solution. A screening trial containing 120 accessions showed that only 2 restorer lines were resistant and 48 were medium resistant (Kulkarni et al. 2015). The screened accessions may be used to transfer resistant genes into the elite breeding lines with superior combining ability or may be directly exploited in hybrid breeding.

16.5.7 Downy Mildew

Downy mildew is one of the major diseases of sunflower caused by *Plasmopara halstedi* and found on all the habitable continents except Australia. A total of 36 pathotypes of downy mildew have been isolated in various studies, whereas more than 20 major genes (Pl_1–Pl_{21}) and (*PlArg*, Pl_{PMI}) have been identified as source of resistance. Thirteen genes (Pl_1, Pl_2, Pl_5 – Pl_8, Pl_{17}–$Pl_{21}$and Pl_{Arg}) have been mapped on various linkage groups (LG1, 2, 4, 8, 13) (Mirzahosein-Tabrizi 2017). Most of resistant genes were identified in wild sunflower, whereas *Pl5* was identified in *Helianthus tuberosus* and Pl_7in *H. praecox*. Resistant genes Pl_1 and Pl_2 have been extensively used in resistance breeding programs against races 100 and 300 of the

pathogen and are typical examples of vertical resistance. Resistant genes may not be effective due to the evolution of new races of pathogens; therefore, diversification of resistant sources is one of the major objectives of disease-resistant breeding. An example of genes Pl_6 and Pl_7 which were extensively used in French breeding lines, failed to provide resistance against a new race of pathogen (304) and disease infestation thereby increased to 88% in 2002, which had been less than 1% in 1989 (Mestries et al. 2004).The Pl_{Arg} and Pl_8 genes originate from *H. argophyllus* which is resistant against four tested races (Dussle et al. 2004). The Pl_{13} gene was found resistant to 13 races of downy mildew (Mulpuri et al. 2009). Gene pyramiding could be done to incorporate multiple resistant genes from various sources. A study showed that monoculture of a single hybrid with single resistant genes led to the loss of efficient resistance after 3 years and increased the vulnerability of the sunflower crop to downy mildew where a combination of different resistant genes and genes alternation provided long-term solution for the management and control of the downy mildew (De Labrouhe et al. 2010).

16.5.8 Drought

Drought is a major production constraint of sunflower, causing significant yield losses around the world. Drought is the phenomenon that occur sat the highest magnitude among all types of stresses (Rauf et al. 2016). Drought stress adversely effects the photosynthate assimilation and mobilization within plants due to closure of stomata, and a reduced photosynthesis process. Genetic variation among the breeding lines has been observed within sunflower germplasm (Khalil et al. 2016; Rauf 2008; Rauf and Sadaqat 2008; Rauf et al. 2009). Wild species are known to carry drought-resistant related traits. For instance, *Helianthu sargophyllus* has traits such as cuticular wax, intense leaf hairiness and small leaf area (Hussain et al. 2016, 2018). These traits increase the chances of survival of plants under drought stress and have higher water use efficiency due to lower water losses during transpiration. Moreover, intense hairiness is related to higher radiation reflection and repels sucking pests, thus plants have a higher stay-green trait. Cuticular wax has medium heritability and thus selection was effective in F_2 generation to develop drought resistant F_3 plant progenies. Silver canopy color marker was used to select plant with high cuticular wax and intense hairiness. Canopy temperature depression (CTD) was also effective in selecting plant progenies having higher transpiration under drought stress. Canopy temperature depression was an index of transpiration cooling and plants with higher ability for CTD with reference to air temperature had longer root length to explore water from deeper in the soil profile and such genotypes had better production under drought stress. CTD has practical utilization in a plant breeding program, and good heritability for selection in segregating generations for establishment of breeding lines (Rauf et al. 2016).

16.5.9 Heat Stress

Global warming due to emission of greenhouse gases into the atmosphere has caused a rapid rise in air temperatures (Kalyar et al. 2014). Higher temperatures causes increased leaf senescence, early maturity or premature phenological development due to higher accumulation of heat units in plants (Kalyar et al. 2013a, b). It causes higher gematophytic sterility and reduces grain filling. It also accelerates the impact of other stresses such as water and salinity. Sunflower showed the highest growth at 27 °C and temperatures higher than 30 °C was not known to induce temperature stress (Kalyar et al. 2014). Plant phenological traits such as downward head position and erect leaves reduced pollen sterility and lower canopy temperature. Both traits are selectable in segregating population and have medium realized heritability (Kalyar et al. 2013a, b).

16.5.10 Oil Quality Traits

16.5.10.1 Tocopherols and Sterols

Tocopherols are an important component of sunflower seed and human health as they impart antioxidant activity. They have a range of 314.5–1024.5 mg/kg in seed and 562.8–1872.8 mg/kg in sunflower oil (Velasco et al. 2004). Tocopherols have four derivatives, alpha-tocopherol being 90% of the four types (Fernández-Martínez et al. 2007). A variant of genes such as tph_1 (50% α- and 50% β-tocopherol), tph_2 (0%–5% α- and 95%–100% γ-tocopherol), and tph_1tph_2 (8%–40% α-, 0%–25% β-, 25%–84% γ- and 8%–50% δ-tocopherol) produces variable quantity of four derivative of tocopherols (Škorić et al. 2008). Tocopehrol content of sunflower lines was modified through mutation breeding; for example IAST-1 and IAST-540 had 95% of gamma tocopherol (95%). Genetic recombination and transgressive segregation produced breeding lines such as LG-15 (high 30–40% beta tocopherol) and LG-17 (> 90% high gamma tocopherol) (Velasco and Fernández-Martínez 2003).

Phytosterols are known for their antioxidant properties and role in reduction of low density lipids and thus their higher concentration is desirable for human health (Roche et al. 2010). Sunflower elite breeding material contains a high concentration of phytosterol content, which are concentrated in the embryo (72%) (Roche et al. 2010). Sunflower oil contains about 2100–4540 μg g^{-1} of phytosterol (Vlahakis and Hazebroek 2000). Wild germplasm contains1017–4308 mg per kg, while campesterol (5.1–16.3%), stigmasterol (3.1–23.9%), beta-sitosterol (35.1–72.3%), delta-5-avenasterol (1.9–20.5%), delta-7-stigmastenol (1.1–20.3%), and delta-7-avenasterol (0.3–10.6%) (Fernández-Cuesta et al. 2014). Selection for higher phytosterol contents led to the development of breeding line IASP-18 having a two-fold higher concentration than parental lines (Velasco et al. 2014). Both tocopherols and phytosterols have a polygenic mood of inheritance and are significantly affected by the environment (Merah et al. 2012).

16.5.10.2 High to Mid Oleic Acid Sunflower

Traditionally sunflower edible oil is rich in two major fatty acids: linoleic 18:2 andoleic acid 18:1). The concentration of linoleic acid ranges from 55–69% in traditional non-oleic acid types. Linoleic is a major polyunsaturated omega 6- fatty acid which is known to have health benefits by lowering serum cholesterol levels. However, sunflower oil rich in linoleic acid degrades under high temperature and produces toxic oxidants or radicals. Mutation breeding was used to produce mid to high oleic acid content sunflower genotypes. The oleic acid content in mutants range is 60–85%. The most useful source of high oleic acid content developed is through exposure to the chemical mutagen Pervenent (Soldatov 1976). However, a commercial cultivar named NUSUN was released in USA with mid oleic acid contents. Pervenent has been extensively used as a parent in backcross programs for development of high oleic acid content cultivars (León 2013a, b). However, high oleic acid cultivars have low yield potential and only express under a warm environment (Smith et al. 2007). High oleic acid content genotypes were dominant over low oleic acid content and are controlled by the genotype of the embryo (Fernandez-Martinez et al. 1989).

16.5.10.3 High Stearic Acid Sunflower

High stearic acid content is desirable for the production of margarine and vegetable fat for deep frying. Stearic acid (18:0) has neutral affects over the accumulation of low density lipids in comparison to palmitic acid which is known to induce cardiovascular diseases. Therefore, increasing stearic acid content at the expense of palmitic or linoleic acid is desirable for the production of high quality industrial oil. Mutagens such as X-ray have been used to modify the fatty acid profile of sunflower oil. Mutant line CAS-12 has been selected with 55% stearic acid contents and 5% linoleic acid contents (Fernández-Martínez et al. 1997). Similarly, lines such as CAS-29 and CAS-30 had 24.9% and 17.4% stearic acid, respectfully (Fernández-Moya et al. 2005). Two genes (*es1es1* and *es2es2*) were collectively known to produce high stearic acid content in sunflower (Fernández-Moya et al. 2005); medium stearic acid lines had a single recessive gene (Perez-Vich et al. 2004).

16.5.10.4 Sunflower Meal Quality

Sunflower seed is crushed to obtain oil; seed meal is a by-product which can be fed to animals, birds/poultry or may be directly consumed by humans in confectionary and baking products. The comparison of conventional sunflower hybrids between various species for seed meal shows that sunflower meal (20% crude protein) contained lower proportion of protein percentage when compared with other species such as cotton (42%) and soybean (50%). Therefore, there is a need to improve the

protein content at the expense of polysaccharides in sunflower seed, which may increase the value of hull contents. Ease of hulling by reducing the fiber contents not only enhances the protein content but may also have positive impact over oil crushing.

Studies have shown variation in protein and oil contents of sunflower elite germplasm. The maximum whole seed protein content identified in sunflower was 35–50% (Warburton et al. 2017). The variation in protein content was due to hull content. Thus protein content may be improved at the expense of reduction in fiber content and improvement of hull content. Moreover, variation within sunflower germplasm was observed for anti-nutritional components such as chlarogenic acid. High protein content was dependent over kernel-to-hull ratio and reduced fiber content. A decrease in fiber content increases the digestibility of the hull. Ease of hull removal is also an important criterion in the evaluation of confectionary sunflower. Both traits were known to be controlled by high heritability.

Improving the sunflower meal quality is one of the major breeding objectives, meal quality depends on reducing the fiber content, antinutritional factors such as chlorogenic acid, phytic acid and improving the protein content. Phytic acid is a chelating agent that binds with metallic ions and reduces the availability of the Ca++, Mg++ and Zn ions for animals. The total concentration of phytic acid in sunflower meal is 4.5%; it may be decreased to improve the meal quality. Phytic acid also makes complexes with amino acid making them unavailable to the non-ruminant animals. Improving the nutritional value of sunflower meal by enhancing the protein content is also an important breeding objective of sunflower. Sunflower meal carries all the essential amino acid contents except lysine, which may be improved in sunflower meal by exploiting the initial variation within elite or breeding lines. Phenolic compounds such as chlorogenic and caffeic acids interact with amino acids and denature the proteins and inhibit the functioning of enzymes in animals and their concentration needs to be reduced in sunflower.

16.6 Breeding Methods

16.6.1 Conventional Breeding Procedures

Sunflower is a cross-pollinated species and all breeding methods of cross-pollinated species such as recurrent selection methods are applicable for population improvement programs. However, the pure-line selection method is applicable for the development of inbred lines with an additional step of combining ability analysis of the inbred lines. The backcross method is used to introgress disease resistant or monogenic traits.

16.6.2 Inbred Line Development

Superior inbred lines are created by crossing appropriate breeding lines such as disease resistant or drought tolerant inbred lines with highly fertile, high oil content and early maturing lines. Disease resistant or drought tolerant lines are generally developed from interspecific crosses which have poor agronomic characteristics such as high plant height, low oil contents and late maturing (Hussain et al. 2016; Shehbaz et al. 2018). Therefore, they could not be directly exploited in hybrid breeding programs. Resistant genes may be transferred to elite lines through backcross methods or segregating populations may be developed for the pedigree selection in subsequent generations (Shehbaz et al. 2018). Pedigree selection is generally carried out for traits having high additive genetic variance (Kalyar et al. 2013a). Heritability in a narrow sense or realized heritability are indicators of selection response. Oil quality traits such as tocopherol and oleic acid contents have been transferred in elite germplasm through backcross schemes (Jonic et al. 2000). Traits such as oil content may be subjected to the pedigree selection for the development of high oil contents inbred lines. Generally, five to six rounds of selection (F2–F6) are sufficient for improvement and fixation of characteristics in inbred lines. Traits related to heat resistance such as downward head orientation at the time of anthesis have been used as a selection marker for the development of heat-resistant inbred lines (Kalyar et al. 2013a). These selections led to the development of some superior heat-resistant hybrids (Khan et al. 2017). Morphological traits such as reduced leaf area and cuticular wax have been used as marker traits for the selection of drought tolerant inbred lines in segregating generations (Hussain et al. 2016, 2018).

16.6.3 Combining Ability Analysis

Combining ability analysis is an important step in the evaluation of sunflower breeding lines for their performance in hybrid breeding. Combining ability is the ability of a breeding line to produce superior progeny upon crossing with testers. A tester may be low performing, if the aim is to uncover deleterious or recessive alleles carried by the female lines (Kalyar et al. 2013b). However, superior lines may be crossed to determine the best specific combiners, or cross combination, which can be used for the development of high-performing hybrids (Khan et al. 2018). Plants selected within F_2 and F_3 generations were selected on the basis of canopy temperature depression, canopy orientation and were crossed with randomly-selected plants to test their general combining ability (Kalyar et al. 2013a, b). A general combining ability test was also useful to uncover recessive lethal alleles within selected plants during early segregating generation (Kalyar et al. 2013a).Line × tester or diallel mating designs have been used to determine the general and specific combining ability of the inbred lines (Turkec and Goksoy 2006). Crossing of elite CMS lines with restorers led to the development of single cross hybrids and to the identification of superior combiners (Khan et al. 2018; Turkec and Goksoy 2006).

16.6.4 In Vitro Techniques

Sunflower inbred line development requires 6 cycles of self-pollination, and 3–4 years to achieve homozygosity. It takes an additional year to test combining ability of the developed homozygous lines. In vitro (anther or ovular culture) or in vivo (doubled haploid inducer lines) methods of haploid line development can reduce the time required to achieve homozygosity. Once the protocol for the development of haploid line is optimized, the required homozygosity can be achieved in a single year. The anthers collected between diad and tetrad stages were found more responsive for the haploid induction in sunflower. Anthers pretreated with high temperature treatment (35 °C) for 12 days were found more successful. Generally half-strength MS medium supplemented with Morel and Wetmore Vitamins with B-12 and a mixture of amino acids, 120 g L^{-1}glucose, pH 5.9 plus 0.5 g L^{-1} naphthalene acetic acid (NAA) and benzylaminopurine(BAP) were considered for haploid plant regeneration from anther culture (Mezzarobba and Jonard 1986).

A sunflower embryo rescue technique has been used to excise interspecific embryos and to culture them over the growth media after excision from the developing embryo. Seven-day-old embryos (*Helianthus annuus* × *H. mollis* Lam.) were excised from mother plants and inoculated over the Murashige and skoog (MS) media (Faure et al. 2002). In vitro multiplication through nodal culture of interspecific hybrid (*H. annuus* × *H. simulans* E. Watson) was done on MS media supplemented with 0.5 mg L^{-1}benzyladenine (Prabakaran and Sujatha 2004). Immature embryo culture was done to reduce the life cycle and subsequent generation advancement of the crosses. Embryos were excised after 10 days post pollination and inoculated over simple MS media. A majority of the cultured embryo were regenerated into vigorous seedlings with 3–6 leaves (Dagustu et al. 2010). In vitro screening for drought tolerance was carried out on one-half strength MS media supplemented with osmotica 5% polyethylene glycol (PEG-8000) to reduce the osmotic potential of the media (Khalil et al. 2016). Amphidiploid between cultivated and perennial wild sunflower was regenerated through somatic embryogenesis (Fu et al. 2017).

Somatic embryogenesis was optimized using various formulations and concentrations of media and growth regulators along with immature embryo size (Sujatha and Prabakaran 2001). The highest induction of somatic embryogenesis occurred at Gamborg basal salt media(120–210 g L^{-1}) sucrose, 0.8–1.0% agar, smaller-sized embryos (0.5–2 mm) and at an incubation temperature of 28–32 °C. Growth regulators such as 2,4-D promoted direct embryogenesis, BA+NAA facilitated formation of single/multiple shoots while there was no response on 2,4-D + kinetin supplemented medium (Sujatha and Prabakaran 2001).

16.6.5 Somatic Hybridization

Protoplast fusion is a novel technique of fusing genetically incompatible species to produced somatic hybrids. Species that differ in their ploidy levels, growth habit (perennial) and nonsynchronous due to photoperiod sensitivity can be combined through protoplast fusion. However, this technique is itself very delicate and requires a very high degree of expertise, technology and optimization of the protocols for the isolation, fusion of protoplast and finally regeneration of complete plantlets. There are two kinds of fusion (symmetrical, asymmetrical). Asymmetrical fusion induces few chromosomes from the donor species and alien addition lines are constituted as a result of protoplast fusion while symmetrical fusion induces complete sets of chromosomes from donor species (Binsfeld et al. 2000). Generally, callus is induced by invitro culturing over growth media. The obtained callus is plasmolysed and then cultured for enzymatic action to remove the cell wall. The obtained protoplast is filtered and centrifuged and then treated with a growth retardant to inhibit further cell division. The protoplast is fused by passing high voltage current or using polyethylene glycol. Fused protoplast is selected by markers and cultured on suitable media for regeneration.

There are several reports of the sunflower protoplast fusion between cultivated and wild species, and procedures may be followed in other species (Binsfeld et al. 2000; Krasnyanski and Menczel 1995). Protoplast of *Helianthus annuus* and *H. giganteus* was fused by using polyethylene glycol and treated with iodoacetic acid to inhibit the protoplast division before fusion (Krasnyanski and Menczel 1995). The fused protoplast was cultured over V-KM medium containing BAP and NAA acid and embryogenic calli was cultured over the MS media. Regenerated plants were intermediate between the two species and annual growth habit was dominant in hybrids (Krasnyanski and Menczel 1995). Asymmetric hybrids were obtained between cultivated and perennial species of sunflower through polyethylene glycol treatment (Binsfeld et al. 2000). Herbicides amiprophos-methyl or oryzalin were used to induce micronuclei of the perennial sunflower before fusion. The sub-diploid microplast was isolated by centrifugation and filtration over nylon (Binsfeld et al. 2000). Molecular markers were used to identify hybrids and confirmed by chromosome counting. The asymmetric hybrids had 2–8 extra chromosome. Protoplast fusion of *H. annuus* × *H. maximiliani* was carried out to transfer *Sclerotinia sclerotiorum* resistance from the wild species due to poor crossability of the two species in the field. The fused protoplast was embedded in the agrose droplet, developed microcalli and was released from agrose and cultured over shoot regeneration media supplemented with 2.2 mg l^{-1} BAP and 0.01 mg l^{-1} NAA (Taski-Ajdukovic et al. 2006). Protoplast divisions i.e. total division or symmetric division was under genetic control and showed heritability of about 0.87 and 0.89, respectively. QTL analysis showed that important genes encoding traits such as somatic embryogenesis and protoplast divisions were located on linkage group I, XV and XVII (Berrios et al. 2000).

16.6.6 Mutation Breeding

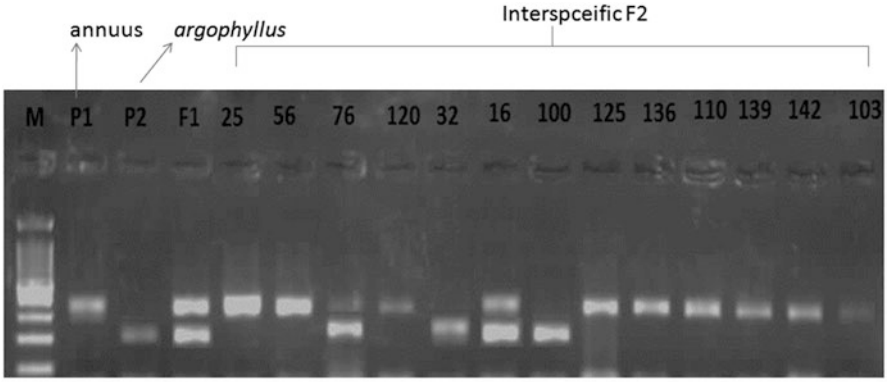
Induced mutation breeding has been used to improve sunflower for several economically-relevant traits such as oil content, nonbranching habit, altered fatty acids, dwarf growth habit, days to flowering, cytoplasmic male sterility, disease resistance and herbicide resistance (Cvejić et al. 2011; Dimitrijevic and Horn 2018). Herbicide tolerant sunflower CLEARFIELD PLUS was developed by selecting plant resistant to imidazolodine (post emergence herbicide) in the M2 population developed through the exposure to ethyl methane sulfonate (EMS). EMS was also used to produce high oleic acid plant genotype Pervenant. Similarly, high stearic acid and mid oleic acid lines were also developed by exposure to the chemical mutagen (Rauf et al. 2017).

Several cyctoplasmic male sterility sources were developed by gamma ray treatment doses 70–225 Gy (Christov 1999). Plants were irradiated with gamma rays (150–165 Gy) and EMS (0.015 mol dm^{-3}) to select resistant plant against *Alternaria* leaf spot (de Oliveira et al. 2004). Inbred lines were treated with physical and chemical mutagens and selection was carried out in M2 and M3 generations. Several mutant lines, e.g. M6, were developed for valuable traits such as high oil content, dwarf and nonbranching breeding lines (Cvejić et al. 2011). Change in ray petal color and dwarf plant size has been obtained when the plants were subjected to mutagens (Vasko and Kyrychenko 2016). A mutant line R 12003 having high oil contents, and resistant to *Orabanche*,was obtained by subjecting immature zygotic embryo to ultrasound treatment (Encheva et al. 2012). Mutation breeding was augmented by advanced molecular techniques such as (TILLING) targeted local lesion in the genome to identify key gene related to fatty acid biosynthesis (Sabetta et al. 2011). It was also used to identify single nucleotide polymorphism in genes such as *Fat4* and *SAD* (Kumar et al. 2013).

16.6.7 Marker-Assisted Selection

Marker-assisted selection (MAS) has been done to incorporate valuable traits in sunflower breeding such as disease resistance, herbicide resistance, oleic acid and male fertility restorer genes. Marker-assisted selection was used to reduce linkage drags and gene pyramiding of various resistant genes. Markers also provide a cost-effective way of selection in highly-laborious traits such as fatty acids i.e. oleic acid. Marker NI-3F/N2-IR was used to amplify the *A-12* gene (oleatedestaure) which has been validated under various genetic backgrounds (Nagarathna et al. 2011; Tilak et al. 2018). High oleic acid was dominant over high linoleic acid and was efficiently detected by the marker F4-R1 (Dimitrijević et al. 2017). Disease resistant genes of downy mildew and rust resistance have been assigned to their respective linkage groups. The marker related to resistant breeding and oleic acid

content have been validated in various genetic backgrounds (Imerovski et al. 2013; Mirzahosein-Tabrizi 2017; Mulpuri et al. 2009; Qi et al. 2011). The presence of closely-linked markers could help to increase the selection efficiency and development of the sunflower genotype with high oil content with betteroil quality (García-Moreno et al. 2006). Molecular markers have also been exploited to confirm interspecific crosses and to reduce linkage drags in segregating generations (Rauf 2008; Fig. 16.9).ORS-728 amplified two bands (250 and 350 bp in parents (P.I. 1806, *Helianthus argophyllus* species and B-124, *H. annuus*), respectively (Fig. 16.10). The band segregating ratios in F_2 plants showed that bands were present in 13 plants and 13 plants had B_{350} band while 24 plants showed heterozygous band A_{250}/B_{350}. The plants showing homozygous genotypes as depicted by marker ORS-728 were determined for oil and oleic acid contents. Multiple regression equations showed significant ($P \leq 0.05$) dependence of phenotypic (oil and oleic acid) and marker data. On the basis of marker ORS-728, plants were grouped into two types. The plants in one group were genotyped as A_{250} while the other group was genotyped as B_{350}. The oleic acid and oil contents of both group was averaged and is shown in Fig. 16.10. The grouping showed significant differences for oleic acid and oil content. The plants carrying marker A_{250} showed oleic acid (39.07%) and oil content (32.05%). The plants in group A showed about 18 and 13% increase in the oleic acid and oil content than plant in group B (Fig. 16.10). These results showed that marker ORS-728 was effective in selection for high oleic acid and oil content.



Primers	Repeat Motif	Forward	Reverse	Size
ORS 728	(AG)7	CTCCATAGCAACCACCTG AAA	CCAAACTCTGAATGAT ACTTGTGAC	317

Fig. 16.9 Polymorphism revealed by the marker ORS 728 in an interspecific population generated by crossing *Helianuthus argophyllus* and *H. annuus* . P1 = *H. argophyllus*P2 = *H. annuus*. (Source: Saeed Rauf (2008) unpublished data)

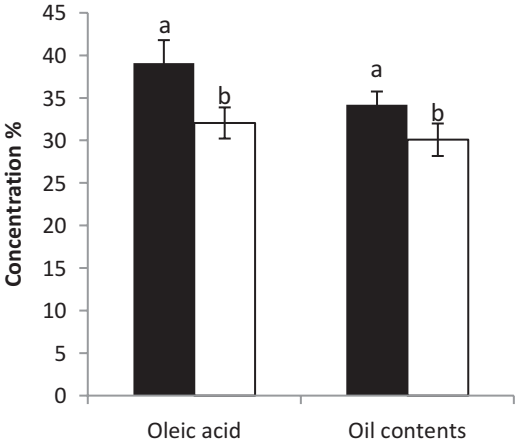


Fig. 16.10 Response of oleic acid and oil contents (%) selected on the basis of polymorphism generated by primer ORS-728, where bars showed two groups of the plants selected on the basis markers generated by primer ORS-728. The black bars indicated significant increase in oleic acid and oil contents due to selection through molecular markers. (Source: Saeed Rauf (2008) unpublished data)

16.6.8 Transgenic Sunflower

Improvement of sunflower through conventional methods is slow, while recombinant DNA technology offers novel and rapid ways to address crop issues such as herbicide tolerance, insect and disease resistance. However, sunflower has been known to be a highly recalcitrant species and difficult to regenerate after delivery of genes through *Agrobacetrium*-mediated transformation (Radonic et al. 2008). A protocol for the transformation of sunflower using microprojectile bombardment, in combination with *Agrobacterium tumeficians*, was optimized which regenerated 7% transgenic plants (Knittel et al. 1994).Insect resistance mediated by*Cry* genes has been experimentally introduced in sunflower (Cantamutto and Poverene 2007). Antifungal genes have been used to transform sunflower to induce disease resistance (Radonic et al. 2008). These genes encode cell wall degradation enzymes (glucanase, chitinase), osmotin and ribosome inhibitor proteins. The wheat oxalate oxidase gene was integrated in sunflower inbred lines and hybrids and is known to enhance *Sclerotinia* head rot resistance (Scelonge et al. 2000). A patent has been granted to genetically transform sunflower for latex production. The gene encoding cis-prenyltransferase was used to transform sunflower for production of latex (Hallahan and Keiper-Hrynko 2007). There is also great potential to induce long chain fatty acids such as decosahexaenoic acid (DHA) and ecosapentaenoic acid (EPA) in edible oil of sunflower, which may increase its medicinal and industrial value (Rauf et al. 2017).

The release of transgenic sunflowers, especially for weedy traits such as resistance to herbicides, diseases and insects has ecological consequences. This is of concern particularly where wild populations grow in close proximity to cultivated fields, as natural gene flow occurs rapidly between both types of sunflower (Cantamutto and Poverene 2007; Gutierrez et al. 2010; Presotto et al. 2012).

16.7 Conclusions and Prospects

Sunflower is an important oilseed crop which is one of the diverse species of the genus *Helianthus*. Oil content was appreciably increased as a result of selection by plant breeders during first half of the previous century, which made it one of the popular oilseed crops for consumers. Modification of the fatty acids resulted in the development of mid to high oleic acid which is better suited for the deep frying. Moreover, development of high stearic sunflower lines has provided new opportunities for making margarine and to produce saturated vegetable fat without harmful industrial processing, such as transesterification. Biotic and abiotic stresses are major yield-limiting factors of sunflower. Genetic variation existing among elite and wild germplasm and introgression of resistant genes was successfully carried out in elite germplasm. Hybrid breeding is used to manipulate heterosis and to increase grain yield. Development of elite breeding lines with superior combining ability is one of the prime breeding objectives of sunflower. In order to expand genetic diversity, cytoplasmic male sterility sources have been expanded which could be used to develop hybrids from novel sources of cytoplasmic male sterility and fertility restorer lines. Mutation breeding has been extensively used to achieve breeding objectives such as dwarf breeding lines, herbicide resistance, oleic acid content, stearic acid content, tocopherols and phytosterols. Marker-assisted selection has been carried out for disease resistance, *Orobanche* resistance and oleic acid content and markers for these traits have been validated in various backgrounds. Transgenic development in sunflower is ecologically complicated by concerns of potential gene escape into closely-related wild sunflower species. Sunflower breeding will be greatly facilitated by new molecular techniques such as whole genome association mapping and genome editing through clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9 (CRISPR/Cas9) technology. Whole genome association mapping could help to tag key genomic regions of breeding interest in wild and cultivated sunflower germplasm and their possible transfer in elite breeding lines with minimum linkage drags. Moreover, CRISPR/Cas9 would help in knocking down the undesirable genes such as disease susceptibility and late crop maturity. Seed and oil quality could be enhanced by knocking down the linoeleic acid and high fiber encoding genes.

Appendices

Appendix I: Research Institutes Relevant to Sunflower

Institution	Specialization and research activities	Website
All Russian Research Institute of Oil Crops, VNIIMK, Russia	Seed production, varietal development and testing, equipment for oil quality analyses, crop husbandry techniques	http://en.vniimk.ru/about/
Trakya Agricultural Research Institute, Turkey	Development of herbicide resistant hybrids	https://ttae.academia.edu/Departments/Sunflower/Documents
INRA Toulouse, France	Genetic resource of wild and cultivated sunflower, disease resistant breeding, genetic resistant against the broomrape	http://www.toulouse.inra.fr/en/contents/list/2519/inra_all/(word)/sunflower/(iLimit)/5/(type)/inra_actualite
Oilseed Research Institute, Faisalabad	Sunflower hybrid development for high yield and oil quality	https://aari.punjab.gov.pk/faqs_ori
Institute of Field and Vegetable Crops, Serbia	Development of sunflower hybrids for high yield potential, high oleic acid, herbicide resistance and confectionary purpose	http://www.nsseme.com/en/products/?opt=oilcrops&cat=products
National Agriculture Technology Institute	Genetic resource and wild species, resistance against biotic stress and herbicide	https://inta.gob.ar/documentos/argentina-national-institute-of-agricultural-technology-inta
Sunflower and Plant Biology Research, Fargo, North Dakota, USA	Genetic enhancement of yield and tolerance to biotic stress, novel weed management solutions	https://www.ars.usda.gov/plains-area/fargo-nd/rrvarc/sun/
Indian Institute of oilseed Research India	Seed production, varietal development and testing, crop husbandry techniques	http://www.icar-iior.org.in/index.php/aicrp-centres/sunflower#
Institute for sustainable agriculture	Development of high quality sunflower genotype, mutation breeding	http://www.ias.csic.es/en/
Directorate of oilseed Research India	Germplasm resources, tissue culture, molecular genetics, disease resistant	http://icar-iior.org.in/index.php/component/content/frontpage
Seed and Plant Improvement Institute, Iran	Genetic resources, molecular markers and disease resistance	https://www.gfar.net/organizations/seed-and-plant-improvement-institute
Oil crop research institute	Germplasm resource maintenance, breeding sunflower for various objectives	http://en.oilcrops.com.cn/

Appendix II: Sunflower Genetic Resources

Cultivar	Important traits	Cultivation location
Mas 88.OL Mas 83. R	High oleic acid Broom rape E tolerant	Maïsadour Semences SA, France – Europe
Parsun-3	High yield and stress tolerance	NARC, Islamabad, Pakistan
DRSH-1 (PCSH 243)	High yield	India
PHB 65A70	High yield, early maturity and resistant to disease	DUPONT, Pioneer, South Africa
7111	CLEARFIELD, Herbicide resistance	Syngenta, World wide
3080	NUSUN Mid oleic acid (55-75)	USA
Camaro II	NUSUN CLEARFIELD Mid oleic acid and herbicide resistance	USA
432E	DuPont ExpressSun (Herbicide resistant)	USA
E76437	High oleic acid, CLEARFIELD	USA
6946 DMR	Downey mildew resistant	Canada
Jaguar DMR	CLEARFIELD and Downey mildew resistant	Canada
PARAISO 1000	CLEARFIELD PLUS and disease resistant	Germany
VELEKA	Orobanche resistant hybrid	Germany
VNIIMK 6540 (k-1872), VNIIMK 8883 (k-1961), VNIIMK 8931 (k-1942), Armavirskii 1813 (k-1588), Armavirskii 3497 (k-1960)	High oil contents (47–51%)	Russia

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Chapter 17
Saffron (*Crocus sativus* L.) Breeding: Opportunities and Challenges

Majid Shokrpour

Abstract Saffron (*Crocus sativus* L.) is an autumnal herbaceous flowering plant belonging to the Iridaceae family. It is considered the most expensive spice in the world and a valuable medicinal herb. The origin of saffron is unclear. The probable center of origin of the plant is Asia Minor (Greece) and/or the Middle East (Iran). From the historical point of view, use of saffron for medical treatment, perfume, food and dye dates back 4000 years. Saffron stigmas contain three important secondary metabolites, crocin, picrocrocin and safranal that are responsible for the saffron color, taste and aroma, respectively. Saffron's adaptation to hot and dry climates has led to widespread cultivation in arid regions, notably Iran where it is a primary income source for many people. The triploid genome of saffron causes the production of abnormal pollen triggering self-sterility. With respect to the clonal nature of saffron, it is believed that there is only one cultivar worldwide. Lack of genetic variation restricts the use of traditional plant breeding based on selection. Probable wild relatives could be an excellent source of genes to alter saffron traits by cross-pollination. In addition, an induced mutation approach with various mutagen agent treatments is an alternative to produce genetic variations. Recent advances in sequencing methods and next-generation sequencing (NGS), provide efficient approaches such as transcriptome sequencing along with proteome and metabolome information, which would help to exploit functional genomics toward genetic engineering of the economic traits of saffron.

Keywords Breeding · Crocus · Clonal selection · Mutation · Saffron · Stigma

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17.1 Introduction

Saffron (*Crocus sativus* L. Iridaceae) is a geophytic, herbaceous, fall-flowering perennial plant, whose stigmas have been mainly used since ancient times as a spice in food, as a dye, in perfume and cosmetic preparations, and for medicinal purposes (Basker and Negbi 1983). Taxonomically, saffron belongs to the genus *Crocus*, subfamily Crocideae, family Iridaceae, and order Asparagales (Fig. 17.1). The genus *Crocus* includes some 90 species native to Central and Southern Europe, North Africa, the Middle East, Mediterranean areas and Western China. They occur in woodland, scrub and meadows at different elevations. Mathew (1982) classified the *Crocus* species using mainly three characters including the corm tunic, prophyll (basal spathe) and the aspect of the style. Molecular analysis has provided complementary data that demonstrate the need to review Mathew's classification. For example, molecular DNA data revealed that *C. banaticus* is a unique species and there are no grounds for isolating it in the subgenus *Crociris*. The species has a prophyll at the base of the pedicel so it can be assigned to section *Crocus*. Despite some inconsistencies among Mathew's classification and other studies, in particular DNA analyses, assigning the species to particular sections and series is well accepted. According to this classification, the genus *Crocus* is divided into two sections: *Crocus* (with a basal prophyll) and *Nudiscapus* (without a basal prophyll); each section is again classified into series based mainly on the corm tunic. *Crocus* species are presented in Appendix II.

Fig. 17.1 Saffron (*Crocus sativus*) plants at flowering stage

17.1.1 Origin and Distribution

The origin of saffron is not clear. It is believed that saffron originated in Asia Minor or the Southwest Greek Islands, while Vavilov (1951) proposed the Middle East (Minor Asia, Turkestan and Iran) as its probable center of origin. Some studies suggested the Mediterranean, notably Crete, as the first place for saffron domestication, during the late Bronze Age. Different wild forms of *Crocus cartwrightianus* have been proposed as the closest wild relative of saffron. From the historical point of view, the oldest evidence of saffron is found in the Papyrus Ebers (1550 years B.C.), which documented using saffron for medical treatments; saffron is depicted in frescoes in the Minoic Palace of Knossos, showing young girls gathering Crocus flowers (Fig. 17.2). Other uses of saffron are for the preparation of perfume, food and dye, as reported by many historical writers including Theophrastus, Hippocrates, Plinius and Celsus (Gresta et al. 2008a).

17.1.2 Economic Importance

One of the most expensive spices in the world, the stigma of *Crocus sativus* is popularly known as saffron (Fig. 17.3). The metabolites crocin, picrocrocin and safranal are responsible for saffron's color, taste and aroma, respectively. These properties

Fig. 17.2 Saffron gatherers appear in Minoan frescos on the island of Santorini in the Aegean Sea. (Source: Anonymous 2012)

Fig. 17.3 The stigmas of *Crocus sativus*, saffron

Table 17.1 Saffron production and cultivation area in Iran during 2010–2016

Year	Production (mt)	Cultivated area (ha 1000)
2010	239	70
2011	254	102.908
2012	261.5	79.39
2013	311	84.738
2014	280	87.93
2015	351.6	95.118
2016	336	105.269

Source: Iran Ministry of Agriculture website 2018

along with other factors especially low input cultivation that is due to minimum irrigation (or no irrigation at some locations), no chemical fertilizer and no chemical weed control, make saffron an appealing organically-grown crop for many farmers and researchers. Indeed, saffron is the main income source for many people in certain countries, particularly Iran. Low water requirement, temperature (hot and cold) resistance and a unique life cycle distinguishes saffron from other crops and provides good opportunities to exploit marginal lands.

The vegetative growth and flowering of saffron begins in the fall just as other crops are harvested or go dormant. The shoots turn yellow and dry during spring and pass the summer in a dormant stage as new underground, *daughter* corms develop. Adaptation to hot and dry climates has led to extensive distribution of saffron cultivation in dry regions of the world. However, significant labor and mechanization difficulties have recently led to decreased cultivation area in countries such as Spain, Greece and Morocco, which were the main world producers. Increased urbanization and agricultural mechanization in those countries have also attributed to this circumstance (Vurdu 2004). Inversely, the noted factors are considered as advantages in some countries like Iran, with extensive dryland areas and large numbers of farm workers (Table 17.1). In Iran, about 90% of the saffron is grown in the North

Table 17.2 Saffron world trade in 2016

Country	Value exported (USD 1000)	Trade balance (USD 1000)	Quantity exported (mt)	Share in world exports (%)
Iran	286,046	285,933	203	70.7
Spain	65,811	12,778	77	16.3
Portugal	11,243	7283	21	2.8
France	6584	−6735	18	1.6
Hong Kong	5208	−3211	19	1.3
Netherlands	4205	1838	35	1
Afghanistan	3869	3869	2	1
Greece	3187	3146	2	0.8
China	3151	−375	173	0.8
UAE	2143	−14,522	14	0.5
Germany	2033	−2469	1	0.5
Switzerland	2005	−3322	1	0.5
India	1386	−13,526	33	0.3
Italy	930	−16,516	13	0.2
World	404,659	177,080	1875	100

Source: International Trade Center website (2018)

Khorasan, South Khorasan and Razavi Khorasan provinces. The cultivation area of 105,269 ha and production of 336 mt, in 2016, made Iran the leading saffron producer in the world. More than 90% of the world's saffron is produced in Iran, which is mainly exported; thereby, Iran dominates quantitatively the export market. Nevertheless, the share of Iran in the world trade in 2016 is just 71% (203 mt), which is related to re-export of saffron by countries such as China, India, United Arab Emirates, Italy, France and Germany (Table 17.2). In terms of exports, following Iran are Greece, Morocco, India (Jammu and Kashmir region), Azerbaijan, Spain and Italy, which are saffron producers (Fig. 17.4). Afghanistan has also increased cultivation area in recent years. Germany, Italy, the USA, Switzerland, the UK and France are the major saffron importers. However, Spain is also known as a major saffron-importing country, especially from Iran, but it re-exports most of it.

Laborious manual harvesting of minute stigmas in saffron is one of the main reasons for the high price of saffron. To produce 1 kg of dry saffron, 110–150 thousands flowers are required. Another difficulty arises in the simultaneous and short flowering period, around 2 weeks, which entails intensive labor. Also, the highest quality stigmas are obtained in the early morning harvest, which requires many workers in the saffron fields. In 2016, the value of the world saffron exports reached USD 404 million; Iran's share was USD 286 million (71%) (Table 17.2).

Fig. 17.4 The world map of the saffron exporting countries, 2016. (Source: International Trade Center website 2018)

17.1.3 Domestication, Selection and Early Improvements

Details surrounding the domestication of saffron are not clear. Although the island of Crete has been documented as the first cultivation and domestication site, Eurasia and Iran, in particular, have ancient cultivation histories going back about 2000 years that may be very important in the saffron domestication process. *Crocus sativus* is a triploid (× = 8; 2n = 3× = 24) and sterile plant. Irregular triploid meiosis results in the production of abnormal pollen and ovules; thus, saffron cannot produce seed and reproduction is done vegetatively by its corms.

There is yet no definite evidence about the genetic origin of saffron. It is assumed that autotriploidy occurred from wild *Crocus* as a diploid unreduced egg cell fertilized by a haploid microspore cell, via a haploid egg cell by two haploid microspores (Chichiriccò 1984; Grilli Caiola 2004, 2005), or by allopolyploidy through the hybridization of *C. cartwrightianus* and *C. hadriaticus* (Castillo et al. 2005). Although there are different views about saffron ancestors, many studies have proposed *C. cartwrightianus* as the most probable (Brandizzi and Grilli Caiola 1998; Brighton 1977; Grilli Caiola et al. 2004; Mathew 1999).

Natural selection is a prerequisite for plant domestication. Regarding clonal reproduction, mutation can be postulated as the only origin of saffron variation. Although phenotypic variations have been found among saffron accessions, there is no strong molecular evidence at the DNA level to confirm this variation.

17.2 Cultivation and Limitations

17.2.1 Current Cultivation Practices

Saffron is propagated by corms. The critical indices used to select corms for planting are a size of more than 2.5 cm in diameter, weight of 5–10 gr (Khorramdel et al. 2015), and lack of defects such as virus, rot or parasites. In this regard, there may also be an unconscious selection to keep or improve qualitative and quantitative yield in saffron. Generally, crop management activities in saffron cultivation are manual in all producing countries. Manual production is the main reason for the high cost of saffron in the market. Due to the intensive labor of cultivation, a perennial cropping system is usually adopted. There are variable durations in perennial cultivation ranging from 3 to 8 years. Douglas et al. (2014) stated that the highest flower and fresh stigma production is in a 3-year cycle. In Iran, a 4-year cropping system is recommended to farmers by the Ministry of Agriculture. The flower numbers and therefore stigma yields decrease after 4–5 years, mainly due to nutrient and water competition, disease infection by the saffron virus (saffron latent virus, SaLV) that was first reported in Iran (Parizad et al. 2017) and annual upward creeping of the daughter corms to the soil surface, usually after 5 years.

Before planting corms, the soil should be prepared by plowing to a depth of 25–30 cm and completely cleared of weeds. Corm sowing is done by hand, at a depth of 10–20 cm and at a planting density of 50–250 corms per square meter (10–15 and 20–25 cm within and between rows, respectively). The sowing time ranges from May to September according to the climatic condition of the cultivation area and local agricultural practices (Gresta et al. 2008b). Some reports claim significant effects of chemical fertilizer to increase saffron stigma yield (Behzad et al. 1992; Goliaris 1999; Hosseini et al. 2004; Sadeghi 1980), while (Behnia et al. 1999) reported less promising results Generally, the most common fertilizer application is the addition of 20–30 mt of organic manure per ha (Koocheki 2004). Watering saffron is not a necessary practice. In fact, this plant is an appropriate crop for drought-prone areas. In semiarid regions, it may not need any irrigation. The most crucial time for irrigation is at the end of summer when the corms are reviving but this usually coincides with the fall rains so it may not be necessary.

Azizi-Zohan et al. (2008) provided information about water requirements and irrigation of saffron by measuring the crop coefficient (Kc), which is the ratio of crop potential evapotranspiration (ETcp) to reference crop evapotranspiration (ETo). They stated that values of Kc varied through the growing season from 0.22–0.24 to 0.94–1.05, and 0.68–0.78 at the beginning, middle and end of the crop cycle, respectively. Flower harvest is manual and requires careful and intensive labor. Gathering the flowers should be done in the early morning, when the corolla is still closed, to keep the maximum quality of stigmas. After harvest, stigma separation from the petals and stamen is made as soon as possible by opening the corolla and cutting the stigmas below the branching where the style changes color from red to yellow (Gresta et al. 2008b).

17.2.2 Current Agricultural Challenges

Mechanization of saffron is difficult due to the delicacy of flowers and corms that need to be handled carefully, so almost all management practices are done manually. Weed control is also a major problem during saffron growing. Saffron is low growing and unable to compete with surrounding weeds. Weed competition begins at the time of flowering in the fall and especially in spring when the leaves are still green. Weeding in spring is not recommended as it may damage the flowers and leaves. Accordingly, after withering of the leaves at the end of spring is the best time to remove weeds. Another challenge recently reported in saffron is the virus infection, SaLV (Parizad et al. 2017). Although there is no present virus damage, it may become of concern in producing high quantitative and qualitative yield of saffron in the future.

Because mechanization of saffron production is difficult, this favors cultivation in countries with low manual labor costs like Iran, India and Afghanistan, where the absence of mechanization is not considered a limiting factor. Best agricultural practices guidelines for efficient exploitation of all inputs to gain the highest production provide many means to overcome current saffron-production problems, including weed control, by increasing plant density and implementing appropriate crop rotation. Use of virus-free corms and a short cropping period (<5 years) in the perennial system could reduce the risk of infections.

17.3 Traditional Breeding Methodologies

17.3.1 Clonal Selection

Vegetative propagation of saffron by corms suggests that it has undergone few changes over time and that saffron all over the world may be represented by a single cultivar. If that is the case, clonal selection does not appear to be a promising approach to genetically improve saffron. The ancient history of saffron cultivation substantiates the effect of some factors that may cause different forms of mutation. In spite of its clonal nature, genetic changes could occur by chromosomal recombination, deletions, inversions, translocations, polyploidy, incomplete segregation or segregation distortion (Mir et al. 2015). Plants genetically changed by natural mutations represent new clones that grow together in a mixed population. However, they can never mix genetically due to their sterility.

Some attempts have been made to assess phenotypic and genotypic variation of saffron among samples collected from various growing areas (Grilli Caiola et al. 2001; Izadpanah et al. 2015; Kalantari et al. 2012). The research found phenotypic differences in terms of flower and leaf attributes such as pistil weight, petal shape, spathe number and leaf length. Over four consecutive years, under identical field condition, 65 Iranian saffron accessions in the collection of the Horticulture

Department, University of Tehran, Karaj, Iran were assessed for quantitative and qualitative attributes by measuring flower and leaf features along with the three main metabolites crocin, picrocrocin and safranal (Ghalamkari et al. 2015; Izadpanah et al. 2014; Kalantari et al. 2012). These studies reported significant differences among the accessions from different Iranian growing areas for most of the measured traits. Nehvi et al. (2007a, b) also found phenotypic variation among Indian saffron genotypes and introduced ten superior genotypes for stigma yield.

Despite the sterility of saffron and the belief of many researchers that the lack of genotypic variation between and within saffron clones, as well as findings in some studies about the existence of variation, some endeavors have been made to select superior clones. An elite subpopulation developed from the progenies of selected corms from extensive saffron belts was identified by Munshi and Zargar (1991) in Kashmir. Agayev et al. (2009) made a clonal selection in saffron samples obtained from five cultivation regions of Iran. They divided the corms into distinct classes by weight and planted then separately in rows. Comparing the clones, each growing from one corm, there appeared to be separate classes of the clones based on the two important attributes of flower number and corm weight. Such results suggest that this approach could be applied in saffron breeding programs to develop new high-yielding cultivars.

17.3.2 Hybridization

Hybridization is a usual process in plant breeding to create segregation within populations and new combinations of genotypes. Typically, hybrid variety development is accomplished by a crossing between lines which are the result of selfing. This is not possible in saffron because of its sterility. Irregular meiosis, due to the triploid genome, apparently causes anomalies in sporogenesis and gametogenesis, producing abnormal pollen, which leads to self-sterility (Chichiriccò 1999; Grilli Caiola 2004). Although in vitro cross-pollination of the saffron (*Crocus sativus*) ovary with pollen of wild species of *C. cartwrightianus* (Grilli Caiola 1999, 2005), *C. tomasii* (Chichiriccò 1999) and *C. hadriaticus* (Grilli Caiola et al. 2001) as probable parents of saffron, produced capsules, and viable seeds, the reciprocal crosses did not succeed and did not form seed (Grilli Caiola 2005). Other possible parents such as *C. mathewii, C. pallasi, C. haussknechtii, C. almehensis* and *C. michelsoni* (Alavi-Kia et al. 2008) also need to be assessed for a successful cross-pollination with *C. sativus*. The potential of the interspecific crosses in saffron affords many opportunities in breeding because of the change of ploidy level and also the transfer of traits from wild species to saffron by hybridization will be possible.

17.3.3 Mutation Breeding

Induced mutation is intended to alter special traits in plants can occur at either the chromosome or gene level. Using mutation in plant breeding, particularly in vegetatively-propagated crops like saffron with no sexual reproduction, can be a viable alternative tool to gain genetic variability. Also, the detection, selection and conservation of M1-generation mutants are feasible due to their clonal nature (Mir et al. 2015). In saffron, traits such as high content of valuable metabolites in the stigma, flower number, high corm multiplication, stigma number and dry weight can be changed by mutation breeding. Khan (2004) applied five doses of gamma rays to induce mutation in saffron corms to create high-yielding mutants. Mutants with five-branched stigmas, higher dry weight and length were considered for this purpose. Another endeavor to increase daughter corms production per mother corm was made using physical (gamma ray) and chemical (EMS, colchicine and ethidium bromide) mutagenic agents (Khan et al. 2011). Among the treatments, gamma radiation of 0.2 KR had a positive effect on improving these characters. The authors suggested that standardization of such a technique could make a considerable increase in the number of saffron corms for areal expansion. Mutagenesis may also help to overcome sterility barriers by duplication of chromosome number and increasing ploidy level, which are often made by colchicine treatment (Zaffar et al. 2004).

17.4 Germplasm Diversity and Conservation

The study of genetic diversity and characterization of germplasm provides necessary data to use in plant breeding programs and also to make appropriate decisions in terms of conservation management of germplasm. Germplasm consists of various types of collections including cultivated varieties, landraces, mutants and wild species.

17.4.1 Germplasm Diversity

Vegetative propagation by means of corms, along with the sterility of saffron due to its triploid genome, creates limitations in the use of traditional breeding approaches for genetic improvement to create new recombinations, crossing and selection. Nevertheless, some studies have reported variation among different accessions. Morphological markers including vegetative attributes (leaf number and length, leaf area, spathe number, fresh and dry weights of the shoot) and flower attributes (style weight and length, petal, calyx dry weight) have been measured to assess phenotypic variation (Izadpanah et al. 2015). In this regard, a significant phenotypic variation among Iranian saffron accessions for all the measured traits has been reported. Biochemical variability has also been found in saffron germplasm originating from different growing regions of Iran (Shokrpour, Kalantari, and Ghalamkari; unpublished data) and different cultivation areas in Greece, Italy and Spain (Maggi et al. 2011). Given the differences found among accessions of saffron in terms of the

apocarotenoids (safranal, picrocrocin, crocin), referred to in Sect. 17.3.1, Iranian saffron accessions could be qualitatively graded for the three metabolites. Maggi et al. (2011) characterized the accessions by 16 key quality parameters by UV-Vis, HPLC and GC analyses, and found the proper assignment of the saffron samples to their respective producing countries.

The most convenient method to assess genetic diversity is the utilization of molecular markers, which present variability at the DNA level. Rubio-Moraga et al. (2009) studied the genetic diversity among 43 saffron isolates from 11 different countries using RAPD, ISSR and microsatellite markers. They stated that all accessions were identical clones and suggested that *Crocus sativus* is a monomorphic species. Contrary to this study, results of research by Izadpanah et al. (2014), Keify and Beiki (2012), Abedi (2013), and Shokrpour et al. (2016) revealed some significant molecular variation among accessions from different Iranian cultivation areas (Fig. 17.5) using RAPD (Fig. 17.6), ISSR (Fig. 17.7) and SRAP (Fig. 17.8) mark-

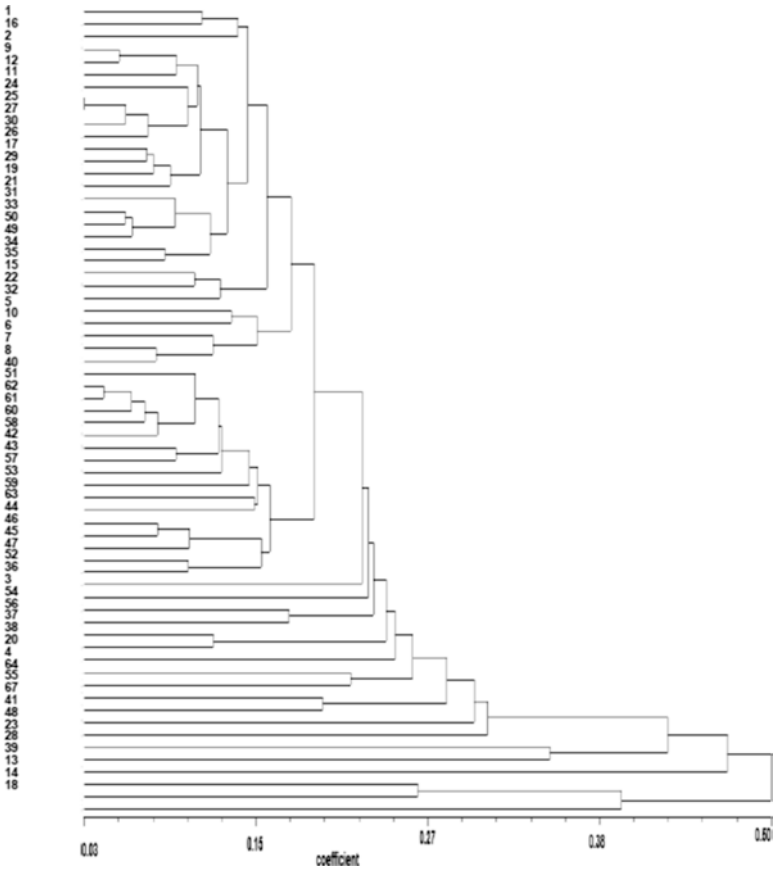

Fig. 17.5 UPGMA clustering the different saffron accessions from Iran using molecular data Numbers 1–12: Torbat; 13–27: Gonabad; 28–32: Mahvelat; 33–48: Ghaen; 49–65: Ferdows. (Source: Shokrpour et al. 2016)

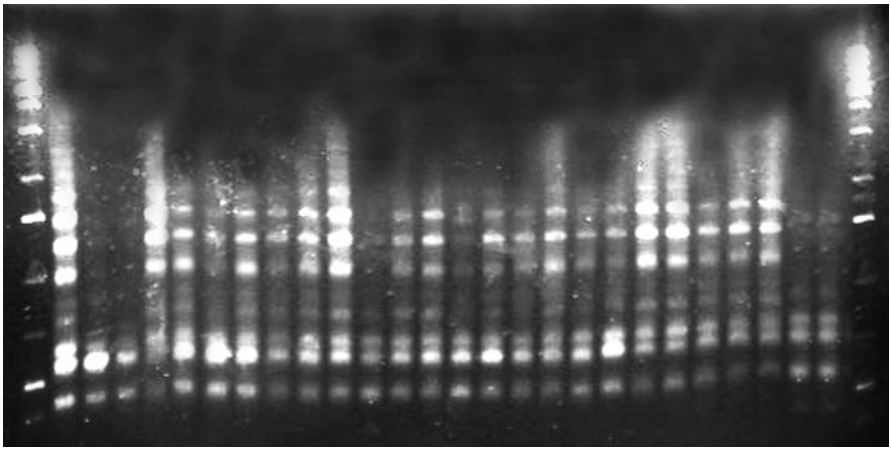

Fig. 17.6 Banding pattern of RAPD analysis (primer sequence: CAGCCGA) in different saffron accessions from Iran. The first and last lanes are 1-kb ladder and the others represent saffron accessions. (Source: Abedi 2013)

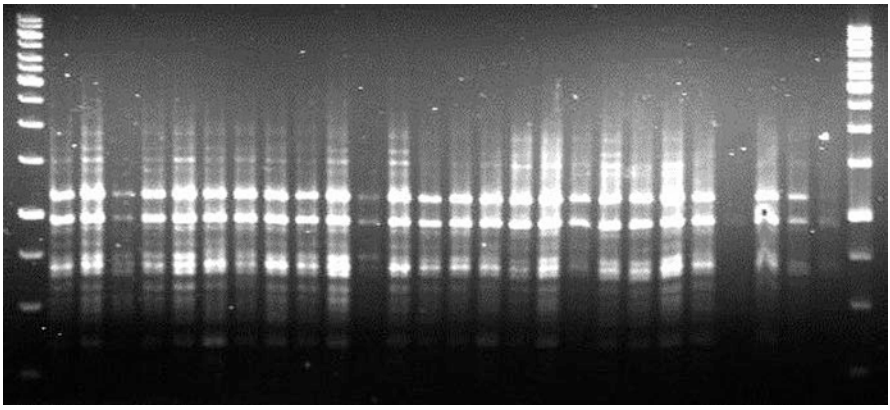

Fig. 17.7 Banding pattern of ISSR analysis (primer sequence: (CACACACACACACACARG) in different saffron accessions from Iran. The first and last lanes are 1-kb ladder and the others represent saffron accessions. (Source: Abedi 2013)

ers. Regarding the challenges surrounding genetic diversity in saffron germplasm, finding efficient and reliable molecular markers, using genome sequencing, mainly by next-generation sequencing (NGS) methods, is inevitable.

Molecular markers have also been applied to the phylogenetic study of the *Crocus* genus (Alavi-Kia et al. 2008; Beiki et al. 2010; Izadpanah et al. 2014). Alavi-Kia et al. (2008) studied phylogenetic relationships of *Crocus* in Iran includ-

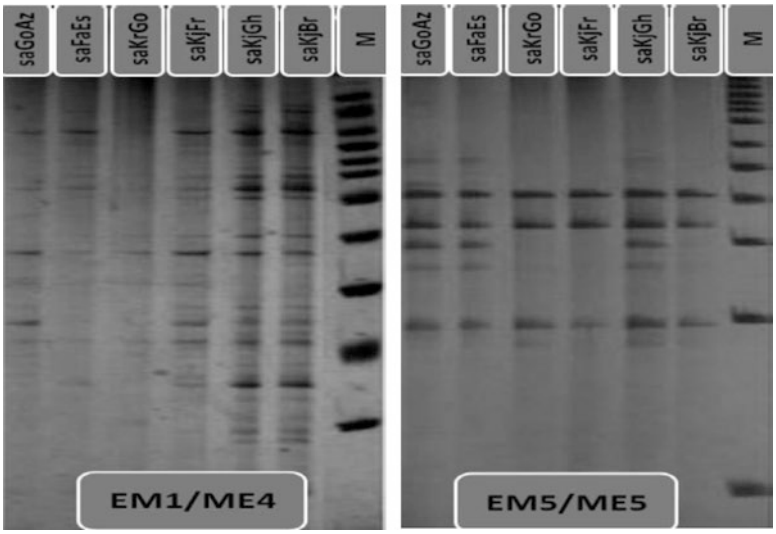

Fig. 17.8 Amplification profile of SRAP with primers of EM1/ME4 and EM5/ME5. Lane M is 1-kb ladder and other lanes represent different saffron genotypes from Iran. (Source: Keify and Beiki 2012)

ing *C. sativus* and eight wild species (Fig. 17.9) using inter-retrotransposon amplified polymorphism (IRAP) markers. Their findings showed a close relationship between saffron and three wild species *C. almehensis*, *C. michelosnii* and *C. cancellatus*. A phylogenetic tree separated all species except *C. caspius* and *C. gilanicus*, based on the Neighbor-Joining algorithm (Fig. 17.10). The authors suggested *C. almehensis* and *C. michelosnii* as the closest relatives of saffron and probably wild ancestors of this cultivated species. However, it has been suggested that saffron is a probable progeny of *C. cartwrightianus*, which contributes to two of the three genomes, while the other parental lineages remain unclear (Fernández 2004). For a complete understanding of the phylogenetic relationships, a study must include all *Crocus* species and use molecular markers with appropriate genomic coverage, e.g., GBS (genotyping by sequencing).

17.4.2 Genetic Resources Conservation Approaches

The low level of variation in saffron genetic resources makes it subject to genetic erosion (Fernandez 2004, 2007). Therefore, conservation of saffron germplasm is of great importance to stop or decrease the threat of disappearing intra- and inter-specific variations. Climatic changes such as long-term drought and rapid industrial

Fig. 17.9 Geographical locations of wild and cultivated *Crocus* species in Iran. (*Crocus* sp. marked in red)

growth are some important contributors to genetic erosion. A typical example in this regard is the Iranian endemic species *Crocus gilanicus*, recently rare in habitat in Guilan province, as attempts to find the plant were unsuccessful during field missions in 2016 and 2017. Saffron genetic conservation should consist of in situ, ex situ and in vitro DNA banks, and include vegetative propagation materials and seeds. Wild crocus species and saffron can be conserved in situ in their original habitats in natural and cultivation areas, respectively. Ex situ conservation in botanic gardens, research centers and gene banks is also an important component of genetic resource conservation. In Iran, the saffron collection established in the Horticulture Research Station, University of Tehran, Karaj, contains more than 70 saffron accessions from different growing areas and 8 wild *Crocus* species.

The first attempts to create an international saffron gene bank were made in 2003 at the First International Symposium on Saffron Biology and Biotechnology, held in Albacete, Spain. A joint consortium of partners from nine countries (CROCUS BANK) was formed with two primary goals (Fernandez 2007): first, the collection and reproduction of saffron bulbs from all countries that cultivate saffron and, sec-

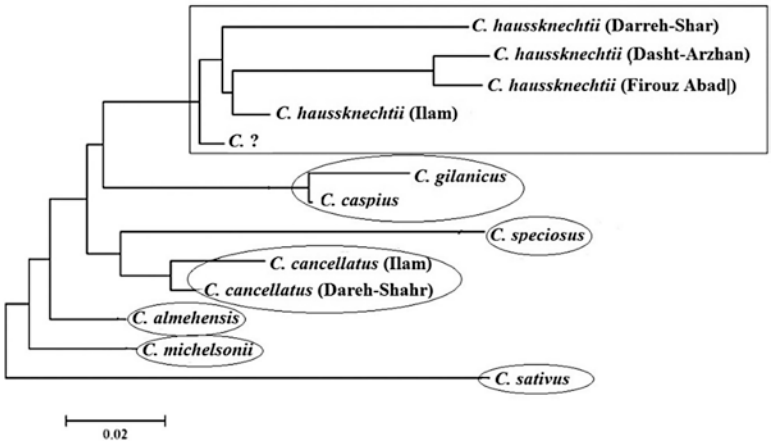

Fig. 17.10 Neighbor-Joining clustering of nine *Crocus* species based on IRAP data. (Source: Alavi-Kia et al. 2008)

ond, the collection of saffron allies for research into the taxonomy, evolution, genetics, physiology, ecology and agronomy of the genus (Husaini et al. 2009).

Since wild *Crocus* species produce seeds, they can be stored in low temperatures for long-term conservation. In vitro conservation is another way to maintain saffron genotypes and is beneficial, due to the lack of seed production in saffron. Different parts of the plant including leaf, flower and corm can be used for explant tissue culture. Callus initiation and plant regeneration are two main steps that should be understood before using in vitro conservation. The techniques of cryopreservation and slow growth are usually applied in this regard: storing in liquid nitrogen at −196 °C and culture under particular condition which retard growth. Somatic embryos represent good plant material to conserve under in vitro conditions because of its more convenient regeneration than undifferentiated callus. Ebrahimzadeh et al. (2000) proposed a protocol to initiate somatic embryos from shoot meristem on LS medium containing BAP (2×10^{-5} M) + NAA (2×10^{-5} M). Mature embryos could be germinated on half-strength MS medium containing GA3 (25 mg/l) to produce matured embryos. Then, transferring the embryos to half-strength MS medium supplemented with BAP (5×10^{-6} M) combined with NAA (5×10^{-6} M) produced plantlets with well-developed root systems and corm formation. Genomic DNA of a plant provides a proper means for the long-term preservation of germplasm. Plant genomic DNA extraction can easily be done by different methods such as cetyltrimethylammonium bromide (CTAB). Although the regeneration of a complete plant from its DNA has not yet been achieved, the advantages of DNA conservation such as long-term preservation, the need for only a small storage space and cost-effectiveness are reasons for the increasing trend toward using this method. In addition, it is expected that a regeneration method for saffron will be developed in the near future.

Table 17.3 Ploidy level and C-value of some *Crocus* species

Species	Ploidy level	1C (pg)
Crocus pallasii		2.98
C. etruscus	2	3.63
C. chrysanthus		3.90
C. cartwrightianus	2	3.97
C. biflorus	2	4.34
C. thomasii	2	4.35
C. sativus	3	5.90
C. angustifolius x *flavus*		6.55
C. vernus	2	11.55
Mean		5.24

Source: C-value database of Royal Botanic Garden Kew

17.4.3 Cytogenetics

Crocus sativus is a triploid with a chromosome number of 2n = 3× = 24, although the chromosome numbers within the genus can be 2n = 6, 8, 10, 11, 12, 14, 16, 18, and up to 64 or 70 (Brighton 1977; Uslu et al. 2012). According to Uslu et al. (2012), *C. abantensis*, *C. speciosus* and *C. biflorus* have 2n = 8 chromosomes, while *C. ancyrensis* and *C. olivieri* have 2n = 6 chromosomes. C-value is the DNA content, in pictograms (pg), of a haploid nucleus (e.g. a gamete) or one-half the amount in a diploid somatic cell of a eukaryotic organism. The Royal Botanic Garden Kew provides a C-value database of many plants, including *Crocus* (Table 17.3). The C-value of nine *Crocus* species varied from 2.98 pg in *C. pallasii* to 11.55 pg in *C. vernus*, with an average of 5.24 pg, which is near that of *C. sativus* (5.90 pg). These wild species of *Crocus* have a ploidy level of 2×.

17.5 Biotechnology and Molecular Breeding

Advances in biotechnology have provided effective tools for improving the qualitative and quantitative yield of saffron. Several studies have been conducted on genetic variation among different saffron accessions by molecular markers such as RAPD, ISSR, SRAP, SSR and AFLP (Izadpanah et al. 2015; Kefy and Beiki 2012; Rubio-Moraga et al. 2009; Shokrpour et al. 2016; Siracusa et al. 2013). A phylogenetic study of different species of *Crocus* was conducted using molecular markers (Alavi-Kia et al. 2008; Beiki et al. 2010; Izadpanah et al. 2014). Regarding the importance of biosynthetic pathways of apocarotenoids in saffron, some contributions clarify the pathways, particularly at the transcriptome level (Ahrazem et al. 2015; Ashraf et al. 2015; Baba et al. 2015b; Iqbal-Mzr et al. 2013; Jain et al. 2016).

17.5.1 Functional Genomics

The primary motivation of saffron functional genomics research is to compile a limited list of candidate genes involved in the production of the main metabolites of the saffron stigma. Enhancing their expression using a transgenic technique leads to improved stigma quality (Husaini et al. 2009). The large and complex saffron genome (c.a. 10 Gb) (Busconi et al. 2015) poses a challenge for its whole genome sequencing. Accordingly, functional genomics in saffron has mainly been studied using transcriptome sequencing. Ashraf et al. (2015) identified, cloned and characterized an ultrapetala transcription factor called CsULT1 and suggested it as a novel regulator of *Crocus* apocarotenoid biosynthesis. A novel dioxygenase, carotenoid cleavage dioxygenase 2 (CCD2), was identified by deep transcriptome sequencing, expressed early during stigma development (Frusciante et al. 2014). It catalyzes the first dedicated step in saffron crocin biosynthesis from *Crocus*. Baba et al. (2015b) used transcriptome sequencing of stigma and other flower tissue to discover and express genes involved in apocarotenoid biosynthesis (Fig. 17.11). Jain et al. (2016) also applied de novo transcriptome sequencing at different tissue and developmental stages and obtained a total of 105,269 unique transcripts from 206 million

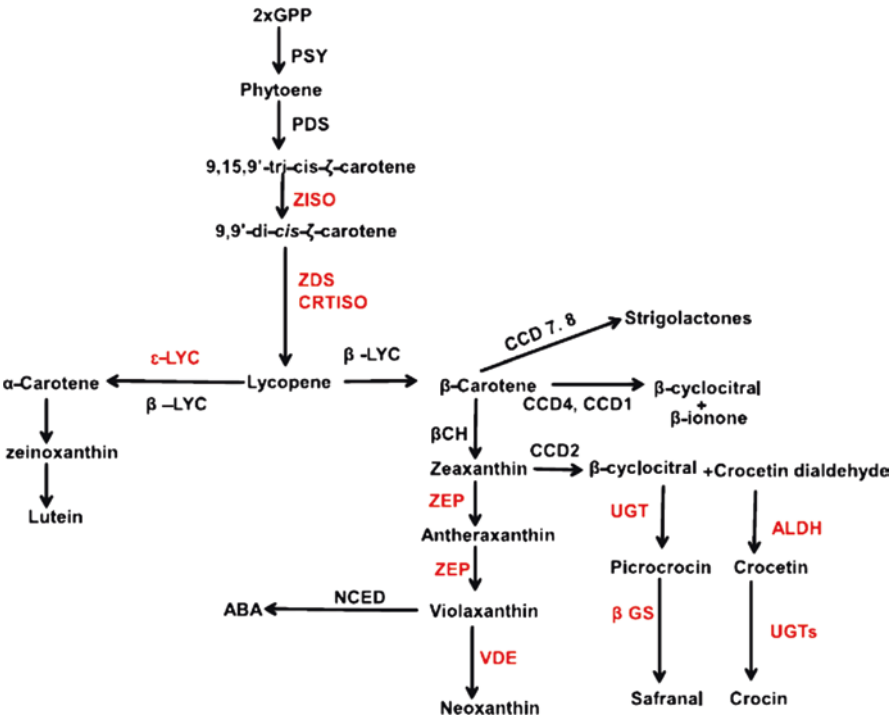

Fig. 17.11 Apocarotenoid pathway showing different enzymes and intermediates involved in the pathway; genes marked in red. (Source: Baba et al. 2015b)

high-quality, paired-end reads. They reported the differential expression of transcripts encoding for transcription factors (MYB, MYB related, WRKY, C2C2-YABBY and bHLH) involved in secondary metabolism.

17.5.2 Bioinformatics

Bioinformatics plays an essential role in contemporary plant science. Currently, in addition to *Arabidopsis thaliana*, information on rice and other crop genome sequences have built a rich and diverse repository of information about plant DNA sequences. *Crocus* bank provides a valuable source of such genomic data on saffron. In order to support and strengthen collaborative research on using omics approaches to characterize the saffron genome, DNA fingerprinting to protect the quality and improve the genetic, chemical fingerprinting, proteomics, transcriptomics, and metabolomics, an initiative of the European Cooperation in Science and Technology (COST) called *saffronomics* (http://www.saffronomics.org) is available. D'Agustino et al. (2007) produced a set of 6603 high-quality ESTs from a saffron stigma cDNA library (available at http://www.saffrongenes.org). To the present, there are 6768 saffron ESTs available at the gene bank of the National Center for Biotechnology Information (NCBI).

17.5.3 In Vitro Culture

Regarding clonal propagation of saffron, the development of efficient in vitro techniques to produce pathogen-free corms will be necessary. In vitro techniques applied in saffron include micropropagation with adventitious corms and roots (Zeybek et al. 2012), cormlet production (Parray et al. 2012) and somatic embryogenesis (Sheibani et al. 2007). In addition, somaclonal variation induced in cell and tissue cultures would be helpful to select variant mutants for biotic and abiotic stresses (Mir et al. 2015).

Induction of somatic embryogenesis and the various embryogenic stages in saffron were found in which somatic embryo development proceeded through well recognized sequences (globular to embryoid) with clearly discernible bipolar regions (Ebrahimzadeh et al. 2000) (Fig. 17.12). Germination of mature embryos facilitated the proliferation of plantlets from the somatic embryos and proved to be a useful alternative method for micropropagation. However, micropropagation of saffron holds promise only if flowering induction is feasible on a commercial scale. The first report on saffron flowering from in vitro raised cormlets under greenhouse conditions was described by Parray et al. (2012). MS half-strength medium supplemented with thidiazuron (TDZ) (20 μM), indoleacetic acid (IAA) (10 μM) and sucrose (40 g/l) regenerated the highest number of cormlets (70 ± 30) per corm slice

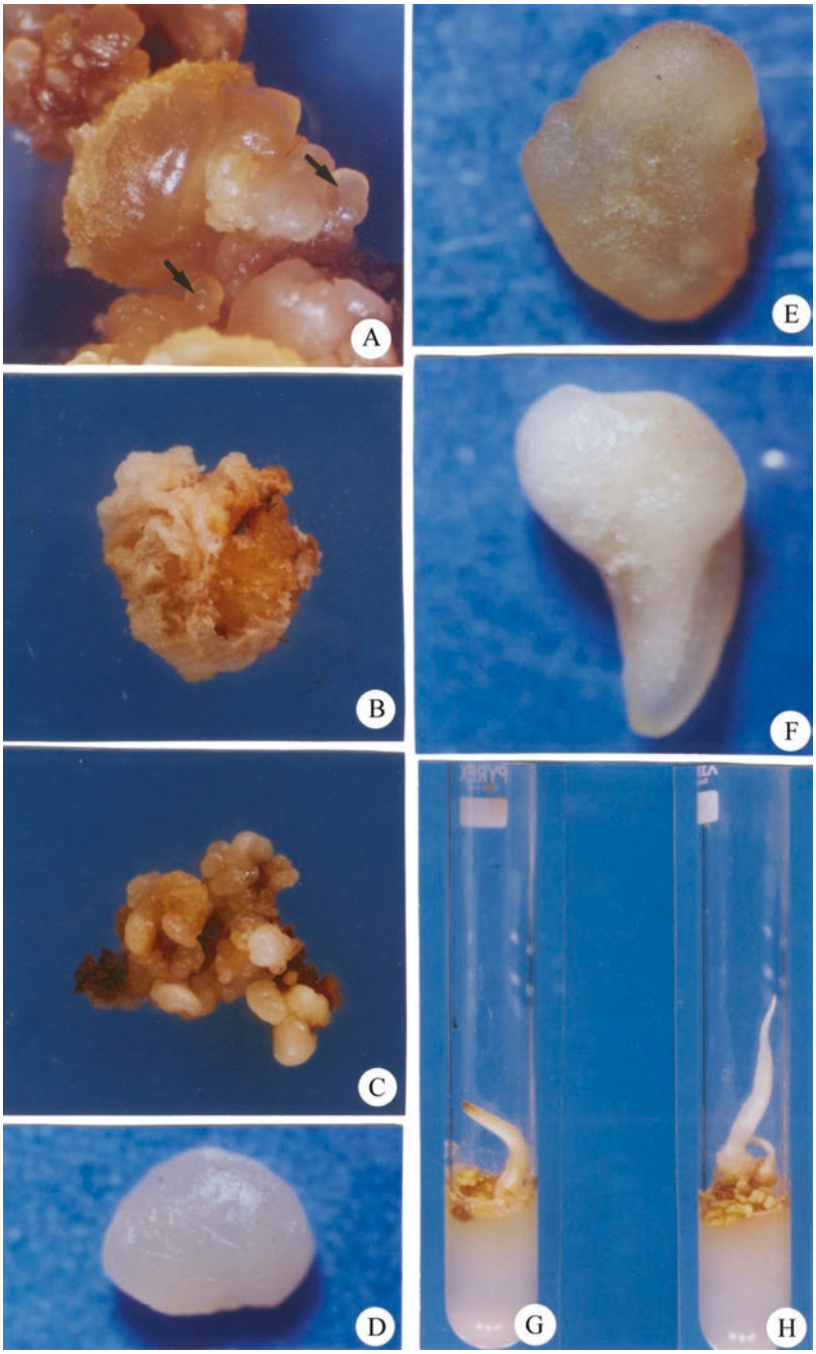

Fig. 17.12 Plantlet regeneration via somatic embryogenesis in *Crocus sativus*. (**a**) Appearance of nonembryogenic, embryogenic and clusters of embryoids, (**b**) Nonembryogenic, friable calli, (**c**) Embryogenic calli, (**d**) Globular embryoids ×20, (**e**) Heart-shaped somatic embryo ×20, (**f**) Torpedo somatic embryo showing bipolarity ×20, (**g**) Plantlet regeneration from somatic embryo, (**h**) Plantlet with corm. (Source: Ebrahimzadeh et al. 2000)

(explant). Also the in vitro developed cormlets with a mean weight of 2.5 g showed maximum flowering (25%) as well as vegetative growth (55%).

17.5.4 Genetic Engineering Potential

The saffron triploid genome and its sterility restrict genetic variation and therefore limits selection of superior individuals for stigma quality and quantity. Genetic engineering provides a great opportunity to develop new cultivars or clones harboring economic and other useful features. Recent findings on the functional genomics of saffron, as noted above, are expected to pave the way to engineering apocarotenoid biosynthesis pathways to elevate or modify stigma quality and to develop high-yielding stigma in the future. Baba et al. (2015a) successfully overexpressed *Crocus* carotenoid cleavage dioxygenase, CsCCD4b in *Arabidopsis*, which imparts tolerance to dehydration, salt and oxidative stresses by modulating reactive oxygen species (ROS) mechanisms.

17.6 Conclusion and Prospects

17.6.1 Current Status Overview

Saffron is regarded as one of the world's most highly-valued spice and medicinal plants. It is particularly important in Iran which provides more than 90% of world production. The annual world export of saffron reaches more than USD 400 million, attesting to its economic significance. Accordingly, researchers are motivated to study saffron to enhance the quality and quantity of its stigmas. Considering the lack of or low genetic variation in saffron due to its sterility, traditional plant breeding approaches are impractical. Nevertheless, one can expect to find the occurrence of natural plant mutations over its long history of cultivation, especially in major growing areas like Iran. Clonal selection may result in developing new high-yielding plants. The use of mutation breeding, genetic engineering, in vitro culture and interspecific crosses could be helpful in creating new genetic variations. Many efforts have been made to create genetic variability and find superior genotypes. Research on genetic improvement of saffron is in its early stages; no improved cultivars of saffron have yet been developed. Given that apparently there is only one variety of saffron in the world, further studies must be undertaken.

17.6.2 Current Research Initiatives to Combat Global Climate Change

Climate changes in recent years, particularly in terms of temperature increases (global warming) and rainfall reduction or greater irregularity in distribution, have led to increasing soil depletion and diminishing areas of tillable land in certain areas. Plant growth and development are affected by the changing environmental parameters of temperature and humidity. Saffron is mainly influenced by environmental factors, especially temperature, which affects the time of its flower initiation and duration (Molina et al. 2004). Based on the results of research on flowering behavior, under a temperature rise due to global warming, flowering duration can be expected to increase by 32–38 days per 1 °C increase in mean daily temperature (Koocheki et al. 2009). Simulation models of the study predicted a temperature increase of 1.5–2 °C due to climate change, which would result in delay of flower emergence until early to late December in the central saffron production regions of the Khorasan provinces in Iran. The resilience of saffron production to climate change may be significant concerning cultivation methods. A combination of traditional and technological methods can be adapted to climate change and provide optimal results (Bidad 2017). The incidence of abiotic stresses from high temperature and drought are among the consequences of climate change. Hence, developing new varieties that tolerate such stresses is necessary. Biotechnology and genetic engineering have a critical role to play in saffron improvement due to its propagation by clones. Using biotechnology, agronomic practices that increase carbon sequestration can easily be adapted and thus render additional benefits such as increased root biomass, soil organic matter, water and nutrient retention capacity, and, hence, increased saffron productivity (Husaini 2014).

17.6.3 Recommendations for Future Research

Given the low or lack of genetic variability in saffron, selecting superior clones is not an adequate method of improvement and therefore the use of induced genetic variation is crucial. Induced genetic variation can be advanced by different means, such as interspecific crosses, induced mutation and genetic engineering. Furthermore, the development of genomic resources by next-generation sequencing approaches, e.g. RNA sequencing and genotyping by sequencing (GBS), along with proteome and metabolome information, can facilitate the development of new saffron varieties capable of producing larger quantities of superior-quality stigmas.

Appendices

Appendix I: Research Institutes Relevant to Saffron

Institution	Specialization and research activities	Contact information and website
Department of Horticulture, College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran	Germplasm conservation, molecular and traditional breeding, agronomy	Telefax: +98 26 32248721 utcan.ac.ir
The North America Center for Saffron Research And Development, University of Vermont, Burlington, VT 05405, USA	Agronomy, production management	Tel.: +1 802-656-5440 https://www.uvm.edu/%7Esaffron/
Saffron Institute of Torbat-e-Heydarieh, University of Torbat-e- Heydarieh, Iran	Production management, marketing	Tel.: +98 51 52210145 http://si.um.ac.ir/
SaffronGlobnet, University of Ferdowsi, Mashhad, Iran	A platform for scientific exchange between interested individuals and institutions	Email: saffronglobnet@um.ac.ir Tel/Fax: +98-511-8796841 http://saffronglobnet.um.ac.ir/index.php
Departamento de Ciencia y Tecnología Agroforestal y Genética, Facultad de Farmacia, Instituto Botánico, Universidad de Castilla-La Mancha, Albacete, Spain	Phytochemical and molecular studies	Tel.: 967 599 200 Fax: 967 599 238 https://previa.uclm.es/dep/ctagroforestal/index.asp

Appendix II: Crocus *Taxonomy for Section, Series and Species*

Section	Series	Species	Flowering time
Crocus	*Verni* Corms with reticulated fibers	*Crocus etruscus* Parl. *C. ilvensis* Peruzzi & Carta *C. kosaninii* Pulevic *C. longiflorus* Raf.	Spring (except of *longiflorus*)
		C. tommasinianus Herb. *C. vernus* (L.)	
		C. vernus ssp. *albiflorus* (Kit. ex Schult.) Asch. & Graebn.	
		C. vernus ssp. *vernus*	

(continued)

Section	Series	Species	Flowering time
	Baytopi Corms with strongly reticulated fibers, bracts absent; anthers extrorsely dehiscent	*C. baytopiorum* B. Mathew	Spring
	Scardici Leaves have no pale stripe on the upper surface	*C. pelistericus* Pulevic *C. scardicus* Kos.	Spring
	Versicolores Corms with tunics, which for the most part have parallel fibers, flowers with conspicuous exterior striping	*C. cambessedesii* J. Gay *C. versicolor* Ker Gawl. – cloth-of-silver *C.* *C. corsicus* Vanucchi ex Maw *C. imperati* Ten. *C. imperati* ssp. *imperati* *C. imperati* ssp. *suaveolens* (Bertol.) B. Mathew *C. minimus* DC.	Spring
	Longiflori: Yellow anthers, styles much divided	*C. goulimyi* Turrill (see also Constantine Goulimis) *C. ligusticus* M.G. Mariotti (Syn. *C. medius* Balb.) *C. niveus* Bowles *C. nudiflorus* Smith.	Autumn
		C. serotinus Salisb. – late *C.* *C. serotinus* ssp. *clusii* (J. Gay) B. Mathew	
		C. serotinus ssp. *salzmannii* (J. Gay) B. Mathew *C. serotinus* ssp. *serotinus*	
	Kotschyani Anthers white, styles for the most part three-forked	*C. autranii* Albov. *C. gilanicus* B. Matthew *C. karduchorum* Kotschy ex Maw *C. kotschyanus* K. Koch – Kotschy's *C.* (syn. *C. zonatus*)	Autumn
		C. kotschyanus ssp. *cappadocicus* B. Mathew *C. kotschyanus* ssp. *hakkariensis* B. Mathew	
		C. kotschyanus ssp. *kotschyanus* *C. kotschyanus* ssp. *suworowianus* (K. Koch) B. Mathew	
		C. ochroleucus Boiss. & Gaill. *C. scharojanii* Ruprecht *C. scharojanii* ssp. *scharojanii*	

(continued)

Section	Series	Species	Flowering time
		C. scharojanii ssp. *lazicus* (Boiss.) B. Mathew *C. vallicola* Herb	
	Crocus Anthers yellow, style distinctly three-branched	*C. asumaniae* B. Mathew & T. Baytop *C. cartwrightianus* Herb. *C. sativus* L. – saffron *C.* (a sterile triploid mutant or hybrid), presumably derived from *C. cartwrightianus*	Autumn
		C. hadriaticus Herb. *C. hadriaticus* ssp. *hadriaticus* *C. hadriaticus* ssp. *parnassicus* (B. Mathew) B. Mathew *C. hadriaticus* ssp. *parnonicus* B. Mathew	
		C. moabiticus Bornm. & Dinsmore ex Bornm. *C. mathewii* H. Kemdorff & E. Pasche (1994) *C. naqabensis* Al-Eisawi (2001) *C. oreocreticus* B.L. Burtt	
		C. pallasii Goldb. *C. pallasii* ssp. *dispathaceus* (Bowles) B. Mathew *C. pallasii* ssp. *haussknechtii* (Boiss. & Reut. ex Maw) B. Mathew	
		C. pallasii ssp. *pallasii* *C. pallasii* ssp. *turcicus* B. Mathew *C. thomasii* Ten	
	Unknown	*C. malyi* Vis. (formerly in Series *Versicolores*) *C. banaticus* Heuff. (formerly in obsolete subgenus *Crociris*)	Spring
Nudiscapus	*Reticulati* Corm tunic for the most part decidedly covered with reticulated fibers, style three-forked or much divided	*C. abantensis* T. Baytop & B. Mathew *C. ancyrensis* (Herb.) Maw – Ankara *C.* *C. angustifolius* Weston – cloth-of-gold *C.* *C. cancellatus* Herb. *C. cancellatus* ssp. *cancellatus*	Winter or Spring

(continued)

Section	Series	Species	Flowering time
		C. cancellatus ssp. *damascenus* (Herb.) B. Mathew	
		C. cancellatus ssp. *lycius* B. Mathew *C. cancellatus* ssp. *mazziaricus* (Herb.) B. Mathew *C. cancellatus* ssp.	
		pamphylicus B. Mathew *C. cvijicii* Kos. *C. dalmaticus* Vis. *C. gargaricus* Herb. *C. herbertii* B. Mathew (became a *true* species) *C. hermoneus* Kotschy ex Maw *C. jablanicensis* N. Randj. & V. Randj.	
		C. reticulatus Steven ex Adams *C. reticulatus* ssp. *hittiticus* (T. Baytop & B. Mathew) B. Mathew	
		C. reticulatus ssp. *reticulatus* *C. robertianus* C. D. Brickell *C. rujanensis* Randjel. & D. A. Hill (1990)	
		C. sieberi J. Gay – Sieber's *C.*, Cretan *C.*	
		C. sieberi ssp. *atticus* (Boiss. & Orph.) B. Mathew	
		C. sieberi ssp. *nivalis* (Bory & Chaub.) B. Mathew *C. sieberi* ssp. *sieberi* *C. sieberi* ssp. *sublimis* (Herb.) B. Mathew	
		C. sieheanus Barr ex B.L. Burtt *C. veluchensis* Her	
	Biflori	*C. aerius* Herb. *C. almehensis* C.D. Brickell & B. Mathew	Winter or Spring

(continued)

Section	Series	Species	Flowering time
	Tunics of corms split into rings at the base, either entire or with tooth like projections, leathery in texture, style three-forked	*C. biflorus* Mill. – silvery *C.*, Scotch *C.* *C. biflorus* ssp. *biflorus*	
		C. biflorus ssp. *adamii* (J. Gay) K. Richt. *C. biflorus* ssp. *alexandri* (Nicic ex Velen.) B. Mathew	
		C. biflorus ssp. *artvinensis* (J.Philippow) B. Mathew	
		C. biflorus ssp. *atrospermus* Kernd. & Pasche *C. biflorus* ssp. *caelestis*	
		Kernd. & Pasche *C. biflorus* ssp. *caricus* Kernd. & Pasche *C. biflorus* ssp. *crewei* (Hook.f.) B. Mathew	
		C. biflorus ssp. *fibroannulatus* Kernd. & Pasche *C. biflorus* ssp. *ionopharynx* Kernd. & Pasche *C. biflorus* ssp. *isauricus* (Siehe ex Bowles) B. Mathew *C. biflorus* ssp. *leucostylosus*	
		Kernd. & Pasche *C. biflorus* ssp. *melantherus* B. Mathew *C. biflorus* ssp. *nubigena* (Herb.) B. Mathew *C. biflorus* ssp.	
		pseudonubigena B. Mathew *C. biflorus* ssp. *pulchricolor* (Herb.) B. Mathew *C. biflorus* ssp. *punctatus* B. Mathew *C. biflorus* ssp. *stridii* (Papan.	
		& Zacharof) B. Mathew *C. biflorus* ssp. *tauri* (Maw) B. Mathew *C. biflorus* ssp. *weldenii* (Hoppe & Fuernr.) B. Mathew	

(continued)

Section	Series	Species	Flowering time
		C. biflorus ssp. *yataganensis* Kernd. & Pasche *C. chrysanthus* Herb. – Golden *C.*, Snow *C.* *C. chrysanthus* ssp. *chrysanthus* *C. chrysanthus* ssp. *multifolius* Papan. & Zacharof	
		C. cyprius Boiss. & Kotschy *C. danfordiae* Maw *C. danfordiae* ssp. *danfordiae* *C. danfordiae* ssp. *kurdistanicus* Maroofi & Assadi *C. hartmannianus* Holmboe *C. kerndorffiorum* Pasche (1993) *C. leichtlinii* (Dewar) Bowles *C. nerimaniae* Yüzbasioglu &	
		Varol (2004) *C. pestalozzae* Boiss. *C. wattiorum* (B. Mathew, 1995) B. Mathew (2000) *C. demirizianus* O. Erol & L. Can (2012) *C. yakarianus* Yıldırım & O. Erol (2013)	
	Speciosi Corm tunic splits into rings at the base, leathery or membranous, foliage after the flowers, style much divided	*C. pulchellus* Herb. – hairy *C. speciosus* M. Bieb. – Bieberstein's *C.*, large purple *C.* *C. speciosus* ssp. *ilgazensis* B. Mathew *C. speciosus* ssp. *speciosus* *C. speciosus* ssp. *xantholaimos* B. Mathew	Autumn
	Orientales Corm with parallel fibers or lightly reticulated, numerous leaves, style three-forked	*C. alatavicus* Semenova & Reg. *C. caspius* Fischer & Meyer (formerly in *Series* Biflori) *C. korolkowii* Regel ex Maw – celandine *C. michelsonii* B. Fedtsch	Spring

(continued)

Section	Series	Species	Flowering time
	Flavi Tunics of the corms membranous, split into parallel fibers, styles much divided	*C. adanensis* T. Baytop & B. Mathew (formerly in Series *Biflori*) *C. antalyensis* Mathew *C. antalyensis* ssp. *antalyensis* *C. antalyensis* ssp. *striatus* O. Erol & M. Koçyiğit (2010) *C. antalyensis* ssp. *gemicii* L. Sik & O. Erol (2011)	Spring
		C. candidus E.D. Clarke *C. flavus* Weston – Yellow *C. flavus* ssp. *flavus* *C. flavus* ssp. *dissectus* T. Baytop & B. Mathew *C. flavus* ssp. *sarichinarensis* Rukšans	
		C. graveolens Boiss. & Reut. *C. hyemalis* Boiss. *C. olivieri* Gray *C. olivieri* ssp. *olivieri* – Balkan and Turkey *C. olivieri* ssp. *balansae* (J. Gay ex Baker) B. Mathew – endemic round İzmir, West-Turkey	
		C. olivieri ssp. *istanbulensis* B. Mathew, Istanbul, Turkey. *C. paschei* H. Kerndorff *C. vitellinus* Wahl	
	Aleppici Tunics of the corms membranous, with split, parallel fibers, foliage produced at the same time as the flowers	*C. aleppicus* Baker *C. boulosii* Greuter *C. veneris* Tappein ex Poech	*Autumn or Winter*
	Carpetani Undersurface of the leaves rounded with grooves, upper surface channeled, style whitish, obscurely divided	*C. carpetanus* Boiss. & Reut. *C. nevadensis* Amo & Campo	Spring
	Ntertexti Corm tunic fibrous with fibers interwoven, spring-flowering	*C. fleischeri* J. Gay	Spring

(continued)

Section	Series	Species	Flowering time
	Laevigatae Corm tunic membranous or splitting into parallel fibers, sometimes leathery, foliage produced at the same time as flowers, anthers white, style much divided	*C. boryi* J. Gay *C. laevigatus* Bory & Chaub. *C. tournefortii* J. Gay	Autumn

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Chapter 18
Vanilla (*Vanilla* spp.) Breeding

Alan H. Chambers

Abstract *Vanilla planifolia* is the botanical source of vanilla extract, but has not generally benefited from strategic plant breeding. *V. planifolia* was cultivated in pre-Columbian Mesoamerica for its desirable aroma, and spread globally by vegetative cuttings starting in the 1500s. In 1837 a method to artificially pollinate *Vanilla* flowers enabled commercial production outside the native range. Today, Madagascar leads *Vanilla* production with significant contributions from other nations. The mass propagation of a few foundational clones has resulted in a global industry reliant on a very narrow germplasm base with threats from multiple biotic and abiotic stresses. Further, the lack of molecular, genomic and definitive phenotypic characterization inhibits improvement within this genus. The establishment of modern *Vanilla* breeding programs could leverage increasingly accessible technologies including advances in genomics and biotechnology to rapidly improve this species for high priority traits like disease resistance, total bean yield, pod uniformity, vigor, non-splitting pods, flower longevity, extract quality and flowers that are able to self-pollinate without manual intervention. While plant breeding is generally a long-term prospect, the potential benefits are justified by the increasing demand for premium ingredients like natural vanilla extract. In the future, genetic improvement of this species could result in more resilient and higher-quality cultivars that reduce price volatility, support growers, improve sustainability and excite modern consumers.

Keywords Breeding · Genomics · Vanilla extract · *Vanilla planifolia* · *Vanilla pompona* · *Vanilla* x *tahitensis*

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18.1 Introduction

Vanilla is often referred to as the world's favorite flavor, and is the second most valuable spice globally after saffron (Gallage and Møller 2018). Natural vanilla flavor is an extract of cured *Vanilla* beans, and is used in various foods and beverages from ice cream and chocolate to fine perfumes and cosmetics. The *Vanilla* genus is pantropic, but *Vanilla planifolia*, the major commercial species, is native to Central America and has been consumed since ancient times by the Mesoamericans (Bruman 1948; Lubinsky et al. 2008a). *V. planifolia* spread globally starting in the late 1500s as part of the spice trade to many islands and continents including key introductions into Martinique (1697), Guadalupe (1701), Europe (1739), Reunion Island (1793), Madagascar (1842), Tahiti (1848), Puerto Rico (1909) and others towards the end of the 1800s (Bory et al. 2008c; Childers 1948; Fouche and Jouve 1999). The vines were not productive in European botanical gardens due to the absence of natural pollinators, and remained a curiosity until the discovery of a method to artificially pollinate *Vanilla* flowers by Charles Morren in 1837, with optimization of the method by Edmund Albius in 1841 (Berenstein 2016; Bory et al. 2008c). Supply constraints in the 1850s and 1860s in Mexico favored expansion of the commercial industry in other countries that could use Albius's technique to produce crops of *Vanilla* beans. Today, Madagascar produces both the highest volume and probably the highest quality *Vanilla* beans with Indonesia, Uganda, India, Comores, Mexico and other countries contributing to global supply (Divakaran et al. 2010). The USA is the largest importer of *Vanilla* beans (~1500–2000 mt per year) that are further processed into vanilla extract (FAO 2009). Despite its global appeal and high value, there is little evidence that *Vanilla* has benefited from systematic plant breeding, though some breeding has been reported (Bory et al. 2008c; Divakaran et al. 2006; Grisoni and Dijoux 2017; Knudson 1950; Menchaca et al. 2011; Theis and Jimenez 1957). In many ways, *Vanilla* can be considered a cultivated, wild species with great opportunities for rapid improvement through modern plant breeding methods.

Vanilla has been cultivated in the United States since the early 1900s. An original *V. planifolia* introduction from Mexico into Puerto Rico in the early 1900s was followed by another introduction from Florida in 1909 (Childers 1948). The Puerto Rican *Vanilla* industry grew until the 1950s when it quickly declined, though material from the former plantings are still cultivated by growers and hobbyists. *Vanilla* cultivation in Hawaii started with introductions from Mexico, Tahiti, Samoa and Fiji before 1900 (Conter 1903; Correll 1953), and is mostly focused on the tourism industry today. Florida, Puerto Rico and Hawaii all have naturalized populations of *V. planifolia*. Florida and Puerto Rico also have a number of native *Vanilla* species. Commercial production of *V. planifolia* in southern Florida is currently being established as a new, high-value industry. Native *Vanilla* species like *V. phaeantha* and *V. mexicana* set beans in natural areas in the absence of manual pollination (Chambers 2018) (Fig. 18.1). Natural pod set could be facilitated by unknown orchid pollinators, and possibly enhanced by an introduced species of orchid bee,

Fig. 18.1 (**a**) Naturally pollinated beans of *V. phaeantha*, (**b**) *V. mexicana*, (**c**) Manually pollinated *V. planifolia* beans shown for comparison. (Photo: Alan H. Chambers)

Euglossa dilemma (Pemberton and Wheeler 2006; Skov and Wiley 2005) (Fig. 18.2). There might also be variation in floral biology that allows for self-pollination, as has been reported for *V. mexicana* in Guadalupe (Gigant et al. 2016). Observation of *V. mexicana* flowers in Florida revealed that this species lacks a rostellum and should therefore be capable of autogamy without manual interveion (A. Chambers pers. obs.) (Fig. 18.3).

Vanilla is commercially valuable for the flavor extract that comes from its cured seed capsules (commonly called *beans*). Orchid-based vanilla flavor contains ~2% vanillin, but also includes a complex bouquet of other aromas that is considered to

Fig. 18.2 Orchid bees (*Euglossa dilemma*) have become established in Florida and could enhance natural pollination of orchids, including *Vanilla* flowers. This specimen was captured at the University of Florida, Tropical Research and Education Center. (Photo: Daniel Carrillo)

Fig. 18.3 *V. mexicana* flower in Florida. The petals and column have been removed to show the absence of the rostellum, the flap-like organ that prevents self-pollination in most *Vanilla* species. (Photo: Alan H. Chambers)

be richer and more desirable than synthetic alternatives. This premium product comprises approximately 1% of the total vanilla market and is restricted primarily by insufficient supply. Therefore, global demand for vanilla flavor can only be satisfied through alternatives that focus on vanillin, the most characteristic compound in vanilla extract. Chemical synthesis of vanillin can be achieved using multiple substrates with lignin (a byproduct from the pulp and paper mill industry) being one of the most common (Gallage and Møller 2015). The second route to produce vanillin leverages microbial biofermentation. This product can be labeled *natural*; however, this is currently a more expensive and technologically-challenging proposition than other methods. The various routes to produce vanilla flavor provide options for companies striving to meet price points or deliver premium products through improved sensory experiences even when vanilla extract is limiting.

The primary objective of this chapter is to highlight the tools currently available for establishing modern *Vanilla* breeding programs, and to identify capabilities that still need to be developed. Breeding methods and suggested targets for improvement are also discussed. Overall, the challenges facing commercial *Vanilla* production are tractable, especially with the availability of modern molecular and genomics-based tools.

18.1.1 Botanical Classification and Distribution

The genus *Vanilla* belongs to the large orchid family, Orchidaceae. Over 100 recognized *Vanilla* species are native to tropical areas around the world, mostly between latitudes 27°N and 27°S. The primary species for commercial use is *V. planifolia*, but *V.* x *tahitensis* and *V. pompona* are also cultivated on a more limited scale. Vanilla extract has a USA standard of identity, Code of Federal Regulations (CFR 169.3) that is strictly limited to *V. planifolia* and *V.* x *tahitensis* to prevent adulteration of the consumer product. Other *Vanilla* species can be interesting from ornamental or genetics perspectives, but most are poorly characterized and therefore have unknown utility.

V. planifolia is the primary species cultivated for vanilla extract. It originates from North and Central America where it has been used since pre-Columbian times by native populations, including the Aztecs. *V. planifolia* is generally thought to comprise a very narrow gene pool due to the success of vegetative propagation during early dispersal of a few foundational clones. *V. planifolia* seed dispersal can be facilitated by air, water, or perhaps even bats. Yield and extract quality can vary greatly across geographies due to the growing environment and as a result of different curing practices. The impact of genetics on extract quality has been minimally explored and could be a contributing factor to quality differences.

V. x *tahitensis* is closely related to *V. planifolia* and is commonly believed to be a hybrid between *V. planifolia* and *V. odorata* (Lubinsky et al. 2008a). *V.* x *tahitensis*

probably arose as a spontaneous seedling either in a vanillery or in a natural setting and was then propagated by cuttings. The exact origin of *V.* x *tahitensis* is still unknown (Gretzinger and Dean 2018), but most likely spread from southern Mexico or Central America to Hawaii, the Philippines, and then to Tahiti where an industry based on *V.* x *tahitensis* exists today (Bory et al. 2008c). *V.* x *tahitensis* has a unique flavor profile compared to *V. planifolia* including intense anise flavor notes (Brunschwig et al. 2016, 2017). The beans of *V.* x *tahitensis* can also be matured on the vine longer than *V. planifolia* without splitting. This would be a favorable trait to incorporate into *V. planifolia* to reduce postharvest waste.

V. pompona is a hardy species with only limited commercial value in the global marketplace, but the potential of this species as a specialty crop has been proposed (Maruenda et al. 2013). *V. pompona* could also be used as a source of genetic resistance to *Fusarium* root rot that threatens most commercial *V. planifolia* production (Koyyappurath et al. 2015). *V. planifolia* x *V. pompona* hybrids are already in cultivation in some countries including Costa Rica, and have been developed in Madagascar as well (Belanger and Havkin-Frenkel 2011; Delassus 1963; Dequaire 1976). In southern Florida, *V. pompona* is grown by plant enthusiasts, and is frequently misclassified as *V. planifolia*. The vigor and precocious flowering of *V. pompona* make this species attractive from a breeding perspective.

18.1.2 Importance

Vanilla extract from cured *Vanilla* beans is the second most valuable spice globally. Vanilla is often referred to as the world's favorite flavor, and is used in numerous products including ice cream, chocolate, baked goods, beer and cosmetics. *Vanilla* production is concentrated in a few countries (Fig. 18.4), and was grown globally on around 35,000 ha worth approx. USD 130 million in 2017 (FAO, Figs. 18.5, and 18.6).

Fig. 18.4 *Vanilla* production by country. Data shown is an average for 2010–2017. Darker colors indicate higher average tonnage (FAO)

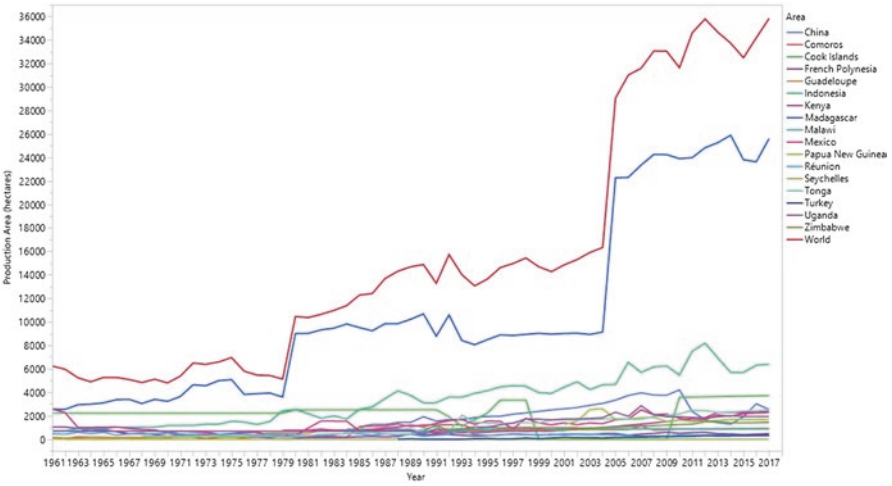

Fig. 18.5 *Vanilla* production area in hectares, 1961–2017, for the top 17 *Vanilla*-growing countries. The figure also includes overall world production (FAO)

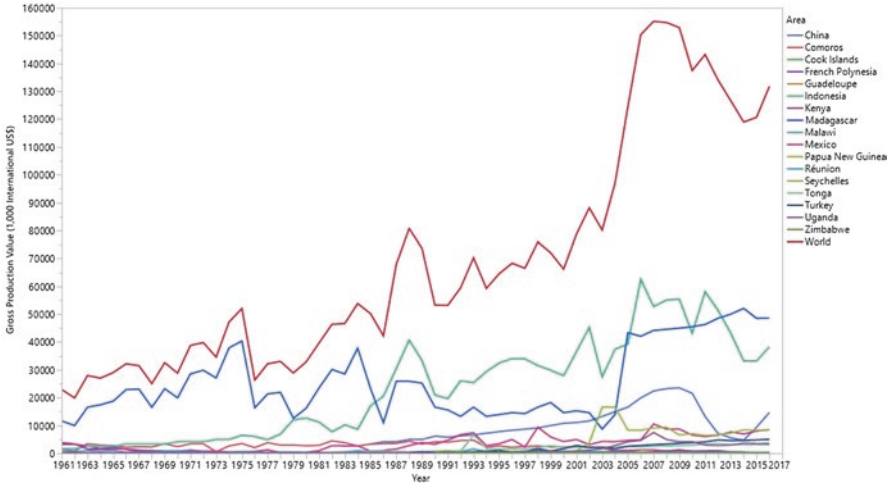

Fig. 18.6 *Vanilla* production value in USD 1000, 1961–2017 for the top 17 *Vanilla*-growing countries. The figure also includes overall world gross production value (FAO)

18.1.3 Domestication, Selection and Early Improvements

Like all plant species, *Vanilla* faces both biotic and abiotic challenges. High-value traits for improvement include resistance to *Fusarium*, non-splitting beans, a shorter juvenile period, increased bean quality including vanillin content and other traits. While *Vanilla* has been cultivated for hundreds of years, evidence for domestication

through selective breeding is lacking. The spread of *Vanilla* germplasm was facilitated by the ease of propagation through vegetative cuttings, but a few challenges could have prevented the genetic improvement of this genus until recently.

The efficient breeding of *Vanilla* requires an understanding of the genetic and phenotypic diversity within compatible species. *Vanilla* diversity at the genomics level has only recently been explored, and provides useful insights into selecting distinct accessions for comprehensive evaluation (Hu et al. 2019). Early *Vanilla* research in Madagascar provides insights into useful phenotypes for plant breeding today (Delassus 1963; Dequaire 1976). Major breeding targets historically included disease resistance, vanillin yield and indehiscence. Some species of early interest as listed in the original documents include *V. planifolia* (*V. fragrans* syn.), *V. pompona*, *V.* x *tahitensis*, *V. phaeantha*, *V. madagascariensis*, *V. humblotii*, *V. phaleanopsis*, *V. dekaryana*, *V. zanzibarica*, *V. moonii*, *V. françoisii* and *V. montagnacii*. The available information from these early breeding efforts provide many useful insights into *Vanilla* breeding, including a method for immature seed germination, attempts at mutagenesis and a description of the challenges arising from the generation of sterile hybrids. Unfortunately, the early breeding program in Madagascar declined and much of the information and material from this pioneering work is not readily available, if it still exists.

Additionally, efficient *Vanilla* seed germination is necessary in order to support domestication through selection. *Vanilla* pods produce tens of thousands of seeds each. The seeds have a thick seed coat that likely requires mycorrhizal associations to facilitate germination in a natural setting. Historically, seed germination has been a limiting factor for creating segregating populations. Today, the in vitro germination of immature *Vanilla* seeds is a viable option for creating segregating populations for the genetic improvement of this species. Many of the above challenges have been (or will soon be) overcome and *Vanilla* improvement could see rapid advances in the coming years, especially through the application of genomics-assisted breeding.

18.2 Cultivation and Traditional Breeding

18.2.1 Current Cultivation Practices

There are two general methods for *Vanilla* cultivation. The first includes the use of tutor trees as trellises and requires lower initial investment. Many different tree species can be used as tutors, but selection of the optimal tutor tree species is important. *Vanilla* requires partial shade for optimal growth. Therefore, tutor trees need to provide filtered light to protect the vines without over shading. The best tutor trees are those that can be easily maintained with minimal pruning and that provide adequate light penetration and air movement to reduce free water and disease. Tutor tree spacing is usually between 2.5–3 mm with 3–3.5 m between rows. *Gliricidia*

sepium, *Jatropha curcas*, *Leucaena leucocephala*, *Erythrina* sp., *Citrus* x *sinensis*, *Casuarina equisetifolia*, *Plumeria alba* and others have been recommended as suitable tutor tree supports for *Vanilla* (Bianchessi 2012; Hernández and Lubinsky 2010). The selection of the best tutor tree depends on the growing environment and availability of inexpensive planting stock.

The alternative cultivation method involves high density planting under artificial shade structures. Shade cloth with 30–50% light filtering is common with increased shade during seasons of intense solar radiation and reduced shade during the rainy season (Hernández and Lubinsky 2010). *Vanilla* vines are maintained in these structures on post and wire trellises with mulch 10–20 cm deep and up to 100 cm wide as a growth substrate. The shade house cultivation method is more intensive with closer plant spacing and generally has higher yields per unit area than the tutor tree method. The spread of disease can be problematic with intensive cultivation as pathogens can more easily spread from plant to plant. Ultimately, the economics of the whole production system needs to be taken into account when establishing a new vanillery.

18.2.2 Vanilla Curing

Another important consideration for *Vanilla* cultivation and production is the curing process. Green *Vanilla* beans have little to no aroma when harvested. The current vanillin biosynthesis model includes the accumulation of gluco-vanillin prior to harvest, and the development of aroma including vanillin during the curing process (Childers 1948; Fock-Bastide et al. 2014; Yang et al. 2017). Traditional curing includes heat killing, sweating, slow drying and conditioning (Bianchessi 2012; Odoux 2010). Pods are first harvested and sorted by size. Then the initial heat killing step arrests further pod maturation and splitting. The heat treatment is usually 63–65°C for 3 min for 16 cm or larger pods with slightly less time required for medium pods. Minimizing splitting is especially important for reducing postharvest culls for *V. planifolia*. *V.* x *tahitensis* is naturally nondehiscent and therefore does not need to be heat killed to prevent pod splitting. Sweating is the process of maintaining the *Vanilla* pods at elevated temperatures for 24–48 h, above 45°C, after the initial heat killing. Gradual drying over 90 (or more) days decreases the pod moisture from 85% to <25%. The final step, conditioning, includes the continued development of flavor after packing into boxes while monitoring for mold growth. Many variations to traditional curing methods have been attempted, as previously reviewed (Odoux 2010).

18.2.3 Current Agricultural Problems and Challenges

Like many other crops, *Vanilla* yield needs to be greatly improved to meet growing demand. The fastest route to improving *Vanilla* yield is through education and extension activities to improve horticultural practices and sustainably increase *Vanilla* production on the same amount of land. The potential for yield increase through genetic improvement of this species can only be estimated at this point as few hybrids have been created, characterized and published. As a mostly wild species, there are multiple traits that could be targeted to improve yield and quality through directed domestication.

All cultivated plant species have disease challenges. *Vanilla* is easily propagated by cuttings and is genetically uniform across production areas. Vegetative propagation facilitates the spread of viruses which can reduce yield and cause vine decline. The lack of genetic diversity in commercial production also enables other successful pathogens, like *Fusarium*, to further threaten the entire industry. *Fusarium* can be a limiting factor in both *V. planifolia* cultivation methods, though resistance is reported in other species including *V. pompona*, *V. phaeantha* and others (Delassus 1963; Knudson 1950; Koyyappurath et al. 2015; Theis and Jimenez 1957).

Vanilla extract quality is a result of plant genetics, growing environment and the processing parameters selected during curing and extraction. Increased vanillin would be a favorable trait in an improved *Vanilla* cultivar. Increasing vanillin would most likely be achieved by increasing the gluco-vanillin substrate. The impact and interaction of genetics, environment and processing on vanilla flavor should be further investigated.

18.2.4 Improvement Strategies

The genetic improvement of *Vanilla* is essential to meet the existing and future needs of the industry. Classical breeding should facilitate the introgression of traits that could be simply inherited, including disease resistance and non-shattering seed pods, as part of pedigree-based breeding and backcrossing strategies, depending on the source and inheritance of the trait. One major challenge to plant breeding within *V. planifolia* is the general lack of phenotypic data available from diversity collections and segregating populations. Either this information has been kept proprietary, or *Vanilla*, like many other tropical species, is poorly characterized in general. There are a few reports of the successful creation or identification of interspecific hybrids in addition to those described earlier in this chapter. These include hybrids between *V. planifolia* and *V. aphylla* (Divakaran et al. 2006; Nissar et al. 2006), *V. planifolia* and *V. pompona* (Belanger and Havkin-Frenkel 2011), and *V. claviculata* and *V. barbellata* (Nielsen 2000). A *V. x tahitensis* x *V. pompona* mapping population of 90 progeny was used to create a genetic linkage map with AFLP markers (Lepers-Andrzejewski et al. 2012). Interspecific hybrids demonstrate that even wide crosses

in *Vanilla* are possible, though the impacts on mature plant performance are still unknown or unpublished.

18.2.5 Traditional Breeding Methodologies and Limitations

Vanilla flowers are formed on an inflorescence with the sequential opening of one or two flowers at any time for a single inflorescence. *V. planifolia* flowers are a cream-green color, though this species can often be mistaken for *V. pompona* that produces yellow flowers. Stigmata are physically separated from the pollinia (orchid arrangement of anthers with pollen) by the rostellum, a flap-like structure that prevents self-pollination (Fig. 18.7). The rostellum efficiently prevents self-pollination and therefore autogamy and bean development. *Vanilla* flowers are self-compatible and will produce viable seeds through manual pollination or pollination facilitated by orchid bees (*Euglossa* spp.) that are attracted to compounds like 1–8-cineol in the *Vanilla* flowers (Soto Arenas and Dressler 2010). In either case, the rostellum must be circumvented to initiate fertilization. This can be done manually as demonstrated in Fig. 18.8.

Traditional breeding of *Vanilla* will be the most efficient method for creating superior cultivars. A single cross can yield tens of thousands of hybrid seeds. *Vanilla* seeds can be germinated at high density in vitro (García 2018) and screened using molecular markers at an early developmental stage. Seedlings with desirable traits can be vegetatively propagated by cuttings enabling the phenotypic analysis of genetically identical material as for other vegetatively propagated crops.

Fig. 18.7 *V. planifolia* flower cut to show pollinia **P** with pollen, rostellum **R** and stigmata **S**. The stigmatic surfaces are shielded directly behind the rostellum. The rostellum must be circumvented in order to deposit pollen onto the stigmata. (Photo: Alan Chambers)

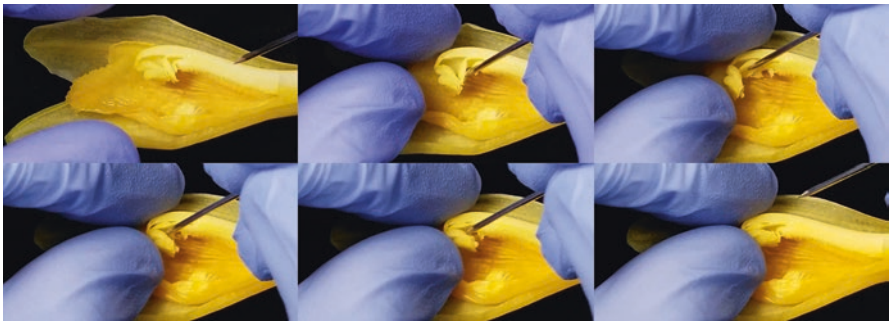

Fig. 18.8 Sequential photographs showing manual pollination of a *Vanilla pompona* flower. Half of the flower was removed to demonstrate the process. The rostellum is pushed towards the pollinia using any narrow object, and then the anthers are gently pushed onto the stigmatic surfaces. (Photo: Alan Chambers)

Vanilla has a number of biological limitations that need to be understood from a breeding perspective. Juvenility is a challenge for many tropical plant breeding programs. The juvenile period of *Vanilla* depends on vine biomass, and is expected to take 3–4 years for a seedling to reach a flowering stage. This will prolong breeding cycles and justifies the investment for developing marker-trait associations to ensure resources are only expended on valuable material.

18.2.6 Role of Biotechnology

Probably the greatest role of biotechnology in support of the *Vanilla* industry is in the production of disease-free planting material. Vegetatively propagated crops tend to accumulate viruses over time, and the tissue culture of clean material can be used to provide disease-free material for new plantings. *Vanilla* propagation by tissue culture is routinely practiced and a number of studies and reviews have reported increased efficiency of in vitro propagation methods (Divakaran et al. 2010; Gantait and Kundu 2017; García 2018). Optimized protocols for micropropagation including initiating aseptic cultures, regeneration, shoot multiplication, rooting, plant hardening and the creation of synthetic seeds have also been reviewed (Gantait and Kundu 2017). Tissue culture companies in many countries could meaningfully contribute to the industry given the ease of in vitro *Vanilla* propagation and the benefits associated with disease-free material.

Other types of biotechnology might be favored for historical reasons. The *Vanilla* standard of identity specifies that only the extract from *V. planifolia* and *V.* x *tahitensis* can be labeled as vanilla bean flavor. This restriction blurs the permissibility of using other species to create hybrids even when using a backcross breeding strategy. This might favor the application of gene editing to create specific allelic variants within *V. planifolia* and *V.* x *tahitensis* accessions that would still clearly fall within the current standard of identity.

18.3 Germplasm Biodiversity and Conservation

Every breeding program is limited by the diversity of germplasm in that program. Diversity includes variation for high-value traits like yield, disease resistance and quality. Obtaining diverse germplasm within *Vanilla* species can be challenging due to restrictions placed on the movement of germplasm across borders, the global predominance of a few foundational clones, the lack of germplasm characterized at the genomics level and the lack of research focused on plant breeding to develop elite material with novel trait combinations. The difficulty of obtaining diverse and well-characterized *Vanilla* germplasm within a single species is somewhat offset by the ease of creating hybrids between species. Interspecific hybridization could be one approach to incorporating novel genetics into the relatively narrow genetic base of *V. planifolia*.

18.3.1 Germplasm Diversity

Vanilla diversity has been analyzed by multiple groups using various molecular tools. Most of this research has focused on diversity analysis using single gene sequences (Bouetard et al. 2010; Cameron 2004; Cameron and Carmen Molina 2006; Soto Arenas and Dressler 2010); RAPDs (Besse et al. 2004; Minoo et al. 2008; Schlüter et al. 2007); AFLPs (Bory et al. 2008d; Lepers-Andrzejewski et al. 2012; Lubinsky et al. 2008a); microsatellites (Bory et al. 2008b; Perez et al. 2016; Ramos-Castellá et al. 2017) and others (Cameron 2009; Divakaran et al. 2006; Lubinsky et al. 2008b; Nielsen and Siegismund 1999; Sreedhar et al. 2007; Verma et al. 2009; Villanueva-Viramontes et al. 2017). This research is useful for diversity analysis, but is not especially suitable for marker-assisted breeding. Marker abundance, cost, reproducibility and flexibility are all important qualities for establishing marker-assisted breeding.

Marker-assisted breeding in *Vanilla* will most likely rely on the development of single nucleotide polymorphism (SNP) markers. SNPs are abundant, relatively inexpensive to develop, highly reproducible, transferrable between labs, information rich and are amenable to multiple genotyping formats. Recently, a draft *V. planifolia* genome and genotyping by sequencing of 112 *Vanilla* accessions was completed (Hu et al. 2019). The accessions included 23 *Vanilla* species with a focus on *V. planifolia* and *V. pompona*. Over 5000 out of 521,732 total SNPs were used to analyze diversity among the accessions (Fig. 18.9). The study revealed previously hidden diversity including the identification of an unexpected number of hybrids. While no phenotypic information was available from this new collection of *Vanilla* accessions, it is anticipated that the marker density made possible with these new genomic tools will enable marker-trait associations in the future.

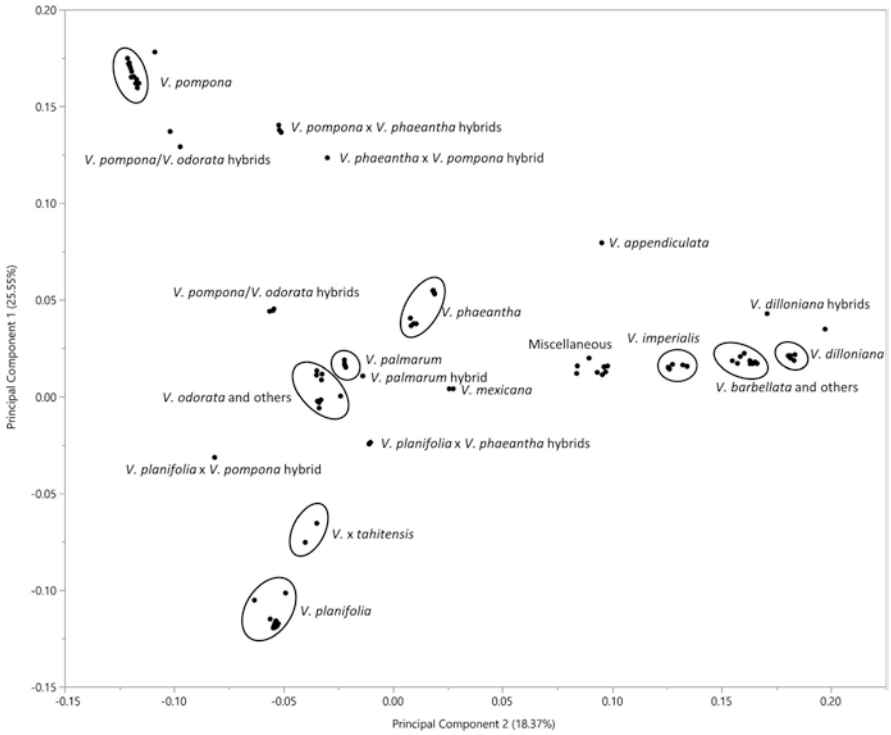

Fig. 18.9 Genomics-based diversity of 112 *Vanilla* accessions from 23 species. A total of 5082 SNPs were used to analyze diversity within this collection. Major groupings of species are bounded by ovals. Hybrids are shown using common designation as female parent followed by male parent where available. (Credit: Figure created by Alan H. Chambers based on data published in Hu et al. 2019)

18.3.2 Cultivar Characterization and Phylogeny

While there are few named *Vanilla* cultivars in total, there are a few distinguishable types defined by morphological features (Appendix II). In Reunion these include cvs. Classique, Mexique (or Bleue), Aiguille, Grosse Vanille, Sterile and Variegata (Bory et al. 2008c). *V. planifolia* types in Veracruz, Mexico include cvs. Mansa/Dura, Variegata/Acamaya/Rayada, Albo-marginata and Oreja de Burro (Soto Arenas and Dressler 2010). Mansa is the most popular type grown in Mexico, and a single clone of this type is probably the founder of the world's *Vanilla* plantings (Bory et al. 2008d; Soto Arenas and Dressler 2010). The Variegata types have yellow striping on the leaves and are popular as ornamental plants. Albo-Marginata has leaves with white margins and is believed to be a mutant form of cv. Mansa from Madagascar (Soto Arenas and Dressler 2010). Oreja de Burro is also similar to Mansa, but is reported to be self-incompatible, leading to 74% premature fruit abortion (Martinez and Engleman 1993). Common to all *V. planifolia* is the inability to self-pollinate without insect or human intervention.

V. x *tahitensis* is commonly held to be a hybrid between *V. planifolia* and *V. odorata* (Lubinsky et al. 2008b), and is also grown commercially. It most likely arose as a chance seedling and is considered to be a specialty type with intense anise flavor notes compared to *V. planifolia* extract. *V.* x *tahitensis* was introduced from the Philippines into Tahiti and French Polynesia in the mid-1800s (Lepers-Andrzejewski et al. 2012). The accumulation of spontaneous mutations or chance germinated seedlings of *V.* x *tahitensis* have resulted in a few types distinguishable by bean size, bean shape, stem shape, leaf shape and leaf color. Some of these types including Tahiti, Parahurahu and Rea rea are considered to be diploid and others like Haapape and Tiarei are tetraploid (Lepers-Andrzejewski et al. 2011). Haapape has been reported to be an autoplolyploid of Tahiti due to their genetic similarity (Duval et al. 2006). The tetraploids are generally larger and this could be advantageous for the industry as larger beans command higher prices, but also creates issues with uniformity when curing mixed types.

18.3.3 Genetic Resources Conservation Approaches

Living collections are most useful for a *Vanilla* breeding program, though herbarium specimens can be useful in some ways. Ultimately, a breeding program is limited by its genetic diversity. Further, *Vanilla* grows in many natural areas that are threatened by encroaching agriculture and development. Therefore, establishing, maintaining and developing novel genetic material is essential for the future conservation and improvement of *Vanilla*.

Ex situ conservation includes the collection, curation and maintenance of living material. Only a few living *Vanilla* collections have been established for conservation and to support breeding efforts. Limited information on these collections is available in the literature based on publications investigating diversity within these accessions (Appendix I). In general, *Vanilla* germplasm is restricted to those institutions that have established the collections and viable genetic material is not readily available for distribution. One collection has been established in the French island of Reunion off the coast of Madagascar supporting research at CIRAD (Roux-Cuvelier and Grisoni 2010). Another collection at Vanilla Establishment of Tahiti has several hundred accessions focusing on *V.* x *tahitensis*. A collection in India has been reported with ~300 accessions (Sasikumar 2010). A new collection with over 200 accessions has been established at the University of Florida Tropical Research and Education Center to support commercialization efforts and breeding research. The UF-TREC collection includes material from botanical gardens, enthusiasts, online vendors and native species. Supporting collections in other countries like Mexico are vital to conserving material from the center of diversity.

18.3.4 Cytogenetics

Cytogenetic analysis of *Vanilla* is challenging due to the phenomenon of partial endoreduplication where several rounds of DNA synthesis occur in the absence of mitosis (Joubes and Chevalier 2000). This process is common for orchids in general and can lead to incorrect flow cytometry-based genome size estimates and higher chromosome counts depending on the tissue sampled (Brown et al. 2017). This has led to various genome size estimates for *V. planifolia* including 2C = 4.59 pg (Brown et al. 2017), 2C = 5.06–10.38 pg (Duval et al. 2006), 2C = 5.22 pg (Lepers-Andrzejewski et al. 2011), 2C = 6.3 pg (Kausch and Horner 1984), 1C = 2.31 pg (Travnicek et al. 2015) and 2C = 5.03 pg (Bory et al. 2008a) with some higher estimates probably from endoreduplicated nuclei populations (Arumuganathan and Earle 1991; Leitch et al. 2009; Zonneveld et al. 2005). Diploid *V.* x *tahitensis* is most likely similar to *V. planifolia* with a 2C = 4.23–5.23 pg (Brown et al. 2017; Lepers-Andrzejewski et al. 2011), and *V. pompona* is larger than *V. planifolia* with a 2C = 7.02 pg in agreement with the thicker stems and larger leaves of this species (Adams and Brown 2007). Chromosome number estimates for *V. planifolia* and *V.* x *tahitensis* range from 2n = 13–32 (Bory et al. 2008a).

18.4 Molecular Breeding

18.4.1 Molecular Marker-Assisted Breeding

Most of the molecular marker research in *Vanilla* has been used for diversity analysis and not for marker-assisted breeding. The exception includes using AFLP markers for genotyping a mapping population between *V.* x *tahitensis* and *V. pompona* hybrid progeny from controlled crosses (Lepers-Andrzejewski et al. 2012). The development of species-specific markers to confirm hybrid progeny will probably be the first application of marker-assisted breeding in *Vanilla* breeding programs. Molecular markers between distinct individuals within the same species will then follow. The development of sufficient numbers of informative molecular markers (e.g. SNPs) will enable marker-trait associations in the future. Additionally, analysis of segregating populations by RNA-seq will be a useful tool for identifying candidate genes for high-value, simply inherited traits.

18.4.2 Functional Genomics and Bioinformatics

A previous study used RNA-seq to investigate seed pod development in *V. planifolia* (Rao et al. 2014). The study was centered on interrogating pathways for vanillin and C-lignin biosynthesis, and generated a transcript database for leaf, stem, root and

various other tissues within the developing *Vanilla* pod (https://sc.noble.org/vanilla/blast/blast.php). Other publicly available genomics databases can be found at sequence repositories like NCBI (https://www.ncbi.nlm.nih.gov/). Though limited, these can be useful for initiating diversity studies and generating hypotheses. The available datasets primarily include chloroplast DNA sequences or tissue-specific RNA-seq datasets for *V. planifolia*, *V. pompona*, *V. aphylla* and *V. shenzhenica*.

Dedicated bioinformatics support for tropical crops including *Vanilla* can be limited. The development of publicly available bioinformatics platforms like Cyverse (Goff et al. 2011; Merchant et al. 2016) and Galaxy (Afgan et al. 2018) are empowering biologists to analyze their own computationally-intensive datasets. The user-friendly interface and tool offerings are continually making these computing options more attractive for biological scientists and breeders. These tools will be more valuable for *Vanilla* with the completion of a genome to unlock more of these online capabilities.

18.5 Genetic Engineering

18.5.1 Methodologies

An optimized transformation protocol for *V. planifolia* protocorm-like bodies (PLBs) has been reported (Malabadi and Nataraja 2007; Retheesh and Bhat 2011). Production of PLBs using MS-based medium with 0.45 μM thidiazuron had the highest proliferation per shoot tip. PLBs were separated and co-inoculated with *Agrobacterium* for 30 min with 100 μM acetosyringone, blotted dry and incubated for 3 days in the dark on regeneration medium (0.3% sucrose, 4.43 μM BA, 2.68 μM NAA). Plants were noticeably regenerating after 60–75 days. Transformed plants were confirmed by PCR, GUS-staining, Southern hybridization and Northern hybridization.

18.5.2 Transgenic Cultivars

There is currently no evidence for commercial cultivation of any transgenic *Vanilla* cultivar. It is likely, though, that new technologies like gene editing will be invaluable in the genetic improvement of this species. This is especially likely given the propensity to cultivate clonally propagated *V. planifolia*, the restrictions established by the standard of identity and the challenges associated with funding a long-term *Vanilla* breeding program.

18.6 Mutation Breeding

18.6.1 Conventional Mutagenesis of Seeds

Attempts at conventional mutagenesis of *Vanilla* seeds have been reported (Dequaire 1976). This was part of research conducted by JP Tonnier in Madagascar starting in 1954. Soaking seeds in silver nitrate (1%) for 30–120 min, or irradiation using Cobalt 60 from 0–3 kilorads was attempted. Plants regenerated from these experiments were transferred to field plantings in 1971, but the status of these plants is currently unknown. Mutagenesis by ethyl methanesulfonate (EMS) inhibited seed germination at 0.1% EMS, but the dose response was not shown (Jose 2005).

EMS can be a useful tool for inducing point mutations in various crops, but there are at least two primary limitations for EMS-based mutagenesis in *Vanilla*. The first is obtaining a sufficient number of germinating seeds for treatment. This is not as routine as for cereals, for example, where tens of thousands of seeds can be harvested, treated and planted in high density. EMS-treated *Vanilla* seeds would most likely require maintenance in tissue culture, and this would increase the complexity of creating the initial population. The second barrier includes the space and time necessary to self the first generation of seedlings in order to obtain a sufficient population of individuals with homozygous, mutated alleles for phenotyping. This would also be more challenging than in cereals where this technique is more commonly applied. Still, EMS combined with high-throughput screening that targets specific genes could be a useful strategy to increase resource efficiency and develop accessions with novel phenotypes without regulatory hurdles.

18.6.2 In Vitro Mutagenesis and Selection

Creating genetic variation through somaclonal variants in *Vanilla* has been reported (Ramirez-Mosqueda and Iglesias-Andreu 2015). These authors reported high rates of survival, morphological variation and confirmation of genetic variability through the use of inter-simple sequence repeats (ISSR) molecular markers. These techniques could easily be expanded to include mutagenesis treatments to generate genetic variability in *V. planifolia*.

A recent study demonstrated the utility of in vitro selection for *Vanilla* somaclonal variants to find resistance to the soil fungus *Fusarium oxysporum* f. sp. *vanillae* (Ramírez-Mosqueda et al. 2019). Callus cultures were induced from selfed, immature seeds of a “Mansa” morphotype accession, and plants were regenerated from the resultant friable callus. *Fusarium* cell filtrate above the median lethal dose (30% v/v) was used for two rounds of in vitro screening. Resistant shoots were then propagated and challenged with a *Fusarium* mycelial suspension. The authors reported that ~26% of 8-week-old plants continued to show resistance to *Fusarium*. This research is encouraging pending field-based evaluations. It is also interesting

to note that the use of selfed seeds as a starting material resulted in plants showing increased resistance to *Fusarium* similar to the method of development for cv. 'Handa' that is also reportedly resistant to *Fusarium*. Understanding the heritability of the resistance mechanism either from *de novo* somaclonal variation, or, perhaps, the segregation of a recessive susceptibility allele, would be valuable follow up research.

18.6.3 Colchicine-Induced Polyploidy

Natural polyploidy in *Vanilla* represents genetic barriers for plant breeding. Sterile *V. planifolia* types have been reported, and these could be the result of hybrids from parents with different ploidy levels. Colchicine is a popular and effective method to induce polyploidy to overcome these barriers. Colchicine treatment around 0.2% was sufficient to reduce survival of *Vanilla* seeds by half, and 0.4% resulted in the highest rate of chromosome doubling (Jose 2005). The induced polyploids were morphologically distinct from their diploid controls, including the presence of thicker leaves.

18.7 Hybridization

18.7.1 Conventional Hybridization

Hybridizing *Vanilla* is a fairly straightforward approach to creating progeny with novel genetics, but some technical expertise is required. *V. planifolia* flowers are short-lived and must be pollinated in the morning (~6 am to noon) of the day that they open. This is also the case with other species like *V. pompona*, but some *Vanilla* species have flowers that stay open and fresh for multiple days like *V. aphylla* (Nissar et al. 2006) and *V. imperialis* (Alan Chambers pers. obs.).

V. planifolia flower anatomy is designed to prevent autogamy, though the flowers are self-compatible. The rostellum is a flap-like organ that efficiently prevents pollen from fertilizing the stigmatic surfaces of the flower unless circumvented. This can be a useful feature in a breeding program because it eliminates the need for emasculation prior to hybridization. The best strategy to ensure successful pollination is to collect fresh pollen from the male parent to fertilize a fresh flower as the female parent. Successful pollinations are apparent one week after hybridization as evidenced by the flower column greening and remaining on the vine. The column will then rapidly begin to grow. The flower and column will wither and drop if not successfully fertilized. Each manually pollinated flower should be labeled with the date of pollination to streamline seed germination after ~45 days. Otherwise, the *Vanilla* seed capsule will mature over the following 9 months.

18.7.2 Seed Germination

Vanilla seed germination is not as simple as many cultivated species, because the seeds are small with very little endosperm and heavily protected by a thick seed coat making them difficult to germinate. Seed germination in the wild is thought to benefit from mycorrhizal symbionts as with other orchid species, and could be aided by scarification through animal dispersion via bats or rodents (García 2018). Mature seeds can be aseptically incubated on artificial medium in vitro, but this often yields only a few germinated seedlings (Divakaran et al. 2006; Grisoni and Dijoux 2017). A few pioneering methods for germinating mature *Vanilla* seeds have been published with subsequent optimizations (Bouriquet and Boiteau 1947; Dequaire 1976; García 2018; Knudson 1950). Historical research focused on nutritional supplementation, and the incubation environment including germinating seeds in the dark and at elevated temperatures (~32 °C), to improve germination rates.

An alternative strategy relies on the in vitro germination of immature seeds, and can generate segregating population sizes suitable for genetic research (García 2018; Menchaca et al. 2011). The critical component of this method relies on aseptically-harvesting seeds prior to full seed coat development ~40–45 days post pollination. Harvested seed capsules should be washed with soap and water, and then surface sterilized with 10% bleach for 30 min followed by multiple rinses with sterile water. The seed capsules are ready for aseptic dissection after surface sterilization. Seeds are gently scraped from the seed capsule and spread across MS-based medium supplemented with 400 mg/l glutamine and 80 mg/l adenine sulfate for germination. Seeds will begin to swell noticeably after ~30 days. Germination rates of 50–80% have been reported 200 days after initial incubation (García 2018).

The use of tissue culture to germinate seeds is designed to grow plants with enough vigor to survive hardening. Germinated seeds are individually plated on secondary MS plates under lights and allowed to grow until shoots and roots develop. Individual test tubes can be used to grow plants until they are ~12–15 cm long at which point they can be removed from their aseptic conditions and hardened in soilless mix under humidity domes with supplemental lighting for 2–3 months. Hardened seedlings can then be transplanted to a shade house. The general process of seed germination is shown in Fig. 18.10.

18.7.3 Somatic Cell Hybridization

Somatic cell hybridization, or protoplast fusion, would be useful in *Vanilla* when attempting to combine the complete genomes of multiple species without undergoing meiosis. This would be advantageous for combining the genetics of two *Vanilla* accessions when hybridization by crossing is not possible or is inefficient. In this way, high-value traits like disease resistance could be incorporated into a *V. planifolia* background.

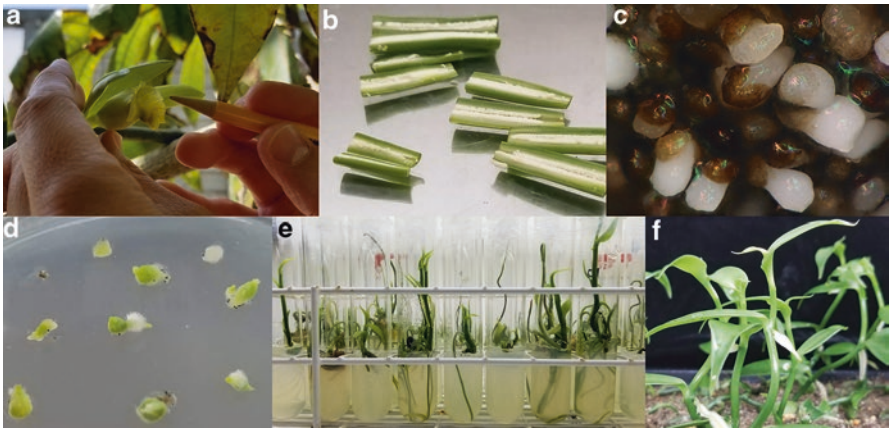

Fig. 18.10 In vitro germination of *Vanilla* seeds. (**a**) Making a controlled cross by manual pollination, (**b**) Aseptic seed harvesting ~45 days after pollination for germination on growth medium, (**c**) Germinating seeds ~30 days after plating on growth medium, (**d**) Germinated seeds are individually plated on growth medium under artificial lighting until roots and shoots develop, (**e**) Seedling growth in individual tubes until true leaves develop, (**f**) Hardening of seedlings under artificial lighting and under humidity domes. (Photo: Alan Chambers)

A few studies have been published on protoplast isolation and fusion in *Vanilla*. One study isolated protoplasts from *V. planifolia* and *V. wightiana* tissue using cellulose, pectinase, hemicelluase and macerozyme enzymes (Mathew et al. 2012). These authors noted the presence of calcium oxalate crystals, and eliminated post digestion centrifugation in order to avoid rupturing protoplasts. PEG-mediated protoplast fusion using macerozyme R-10 and cellulase R-10 was optimized for *V. planifolia* and *V. andamanica* Rolfe (Divakaran et al. 2008, 2010). Fused protoplasts were identified by arrangement of chlorophyll in potentially fused protoplasts. Another study found that 1% cellulose, 0.5% pectolyase and 1% hemicellulose resulted in up to 81 and 79% viability for *V. planifolia* and *V. pompona*, respectively (Montero-Carmona and Jiménez 2015). Plating efficiency was 9.4% when observing microcalli regenerating on MS-based medium.

There are a few significant concerns relative to *Vanilla* protoplast isolation, fusion and regeneration. First, these preliminary studies are very useful for optimizing protoplast isolation, but more research is needed to demonstrate successful regeneration into viable plants. Second, the successful fusion of protoplasts must be confirmed in viable plants using molecular markers to confirm true fusion events. Finally, and most challenging, is the need to demonstrate that using protoplasts derived from leaf tissue is even suitable for a species with extensive partial endoreduplication. It is probable that the populations of endoreduplicated leaf nuclei are unable to re-establish a base genome composition. This would eventually be necessary in order to successfully complete meiosis supporting seed development and pod maturation. While protoplast-related work in *Vanilla* is indeed promising, there is still much research that needs to be done.

18.7.4 Hybrid Cultivars

Documentation of hybrid *Vanilla* cultivars is extremely limited. A breeding program in Reunion released a *Vanilla* cv. Handa as specified in a plant patent application (Grisoni and Dijoux 2017). Limited technical information on the development of cv. Handa is found in the patent application. An undisclosed *V. planifolia* parent was selfed and the seeds were germinated in vitro. Eleven seeds germinated and these were maintained over years until the selection of one seedling that was named cv. Handa. Handa cv. is reported to have resistance to *Fusarium* and has reduced bean splitting. Another type called Vaitsy was possibly developed in Madagascar as a cultivar with resistance to *Fusarium* (Belanger and Havkin-Frenkel 2011; Quirós 2010). Vaitsy cv. is most likely a hybrid between *V. planifolia* and *V. pompona*, and could be similar to, or the source of, the *Vanilla* widely grown in Costa Rica. A recent genomics-based sequencing project included a commercial *Vanilla* accession from Costa Rica and found evidence that it was most likely a *V. planifolia* x *V. pompona* hybrid backcrossed to *V. planifolia* (Hu et al. 2019). Additionally, there is a report of a hybrid (55-295) between *V. planifolia* and *V.* x *tahitensis* Haapape that is indehiscent with a higher percentage of vanillin than common *V. planifolia* (Dequaire 1976). The development of superior plant material like cvs. Handa, Vaitsy and 55-295 is certainly an indicator of the possibilities for *Vanilla* improvement, but there is still much research to be done. The genomic characterization and phenotypic interrogation of these accessions across multiple environments is necessary in order to demonstrate their utility.

18.8 Conclusions and Prospects

Vanilla is poised to benefit from genomics-enabled plant breeding. Some of the major technological hurdles have already been overcome including the ability to hybridize selected parents and generate large segregating populations. Many high-value traits have been identified as targets that would benefit growers, the extract industry and consumers. The advances in molecular biology and genomics including studies leveraging RNA-seq and genomics-based diversity analyses are expanding the possibilities for *Vanilla* improvement. Multiple announcements of groups developing *Vanilla* genomes suggest that this species may finally enter the genomics era soon. Overall, there seems to be greater interest in advancing *Vanilla* science in recent years, and this will most likely lead to great discoveries in the near future.

Some anticipated initiatives to increase *Vanilla* supply include the expansion of areas under cultivation especially within its native distribution. This could help alleviate supply restrictions and generate economic revenue for diverse growers. Additional benefits include producing greater volumes of *Vanilla* beans in closer proximity to the major importers, thus improving sustainability. Some of these

growers will be able to focus on niche and specialty *Vanilla* to meet the needs of an evolving consumer base that values novelty. The expansion of agritourism is another possibility for a species like *Vanilla* that resonates with consumers.

The development of superior *Vanilla* cultivars is necessary in order to alleviate current production challenges. Marker-trait associations will improve the efficiency of *Vanilla* breeding as research advances for this species. The development of higher yielding cultivars with higher quality beans that require less labor and fewer inputs should be a priority. Overall, the allure of natural vanilla flavor is expected to increase, and it is our opportunity to ensure a sustainable path forward leveraging advanced genetics, breeding and horticultural solutions.

Appendices

Appendix I: Research Institutes and Online Resources Relevant to Vanilla

Institute name	Specialization Research Activities	Contact information
University of Florida Tropical Research and Education Center	Genetics, genomics, and plant breeding program. ~200 *Vanilla* accessions from multiple species	Alan Chambers, ac@ufl.edu
VATEL Biological Resources Center	~400 *Vanilla* accessions from multiple species including hybrids	Michel Grisoni, michel.grisoni@cirad.fr
Genetic Resources Center Vanille de Tahiti	~140 *V.* x *tahitensis* accessions for breeding and research	Sandra Lepers-Andrzejewski, sandra.lepers@vanilledetahiti.pf
National Laboratory for Genomics of Biodiversity	Mesoamerican crops and their wild relatives including 140 accessions of cultivated and wild *Vanilla* species	Angélica Cibrian Jaramillo, angelica.cibrian@cinvestav.mx
Unidad de manejo para la conservación de la Vida Silvestre	*Vanilla* germplasm management, maintenance, in vitro cultivation and producer training. 70 accessions	David Moreno Martínez, dmoreno29@hotmail.com
Orquidario de la Universidad Veracruzana	In vitro germplasm coordinator and orchid research and conservation. 14 accessions	Rebeca Menchaca García, rebecamenchaca@hotmail.com
Indian Institute of Spices Research	~300 *Vanilla* accessions	Bhas Sasikumar, bhaskaransasikumar@yahoo.com

Appendix II: Vanilla *Genetic Resources*

Cultivar or Morphotype	Important traits	Cultivation location
Handa	Selfed *V. planifolia* with resistance to *Fusarium*	Madagascar
Vaitsy	Possible *V. planifolia* x *V. pompona* hybrid	Madagascar
Albomarginata	*V. planifolia* Mansa'type with white leaf margins	Madagascar
Classique	Light green, flat leaves	Madagascar
Mexique	Darker, blue-colored leaves	Madagascar
Aiguille	Similar to Classique with more slender leaves and pods	Madagascar
Sterile	Similar to Classique yet with self-incompatibility	Madagascar
Mansa	Common commercial type of *V. planifolia*	Mexico
Oreja de Burro	Similar to Mansa but probably self-incompatible	Mexico
Variegata	Variegated *V. planifolia*	Mexico
Tahiti	Diploid *V.* x *tahitensis*	Tahiti
Parahurahu	Diploid *V.* x *tahitensis*	Tahiti
Rea rea	Diploid *V.* x *tahitensis*	Tahiti
Haapape	Probable autopolyploid of Tahiti	Tahiti
Tiarei	Tetraploid *V.* x *tahitensis*	Tahiti

The morphotype accessions as described in the literature are most likely not exclusively distinct as those in different geographies may call the same genotype by different names, and genotypes that are genetically distinct may be called by the same name

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