HANDBOOK OF PLANT BREEDING

Johann Vollmann
Istvan Rajcan
Editors

Oil Crops

Springer

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Foreword

When one is privileged to participate long enough in a professional capacity, certain trends may be observed in the dynamics of how challenges are met or how problems are solved. Agricultural research is no exception in view of how the plant sciences have moved forward in the past 30 years. For example, the once grand but now nearly forgotten art of whole plant physiology has given way almost completely to the more sophisticated realm of molecular biology. What once was the *American Society of Plant Physiologists'* is now the *American Society of Plant Molecular Biology*; a democratic decision to indemnify efforts to go beyond the limits of the classical science and actually begin to understand the underlying biological basis for genetic regulation of metabolic mechanisms in plants. Yet, as new technologies open windows of light on the inner workings of biological processes, one might reminisce with faint nostalgia on days long past when the artisans of plant physiology, biochemistry, analytical chemistry and other scientific disciplines ebbed and waned in prominence.

No intentional reference is made here regarding Darwinism; the plant sciences always have been extremely competitive. Technology is pivotal. Those who develop and/or implement innovative concepts typically are regarded as leaders in their respective fields. Each positive incremental step helps bring recognition and the impetus to push a scientific discipline forward with timely approaches to address relevant opportunities.

So, it might be interesting to know how those skilled in the art of statistical analysis and the field of classical plant quantitative genetics are coping with the intensifying research emphasis on biotechnology, genomics, proteomics, and the like. After all, high-throughput whole genome sequence analyses and advanced bioinformatic resources for gene discovery will soon render the characterization of haplotypes, in entire germplasm collections and among progeny of segregating breeding populations, a routine event. Will the day come when breeders are told which parents to mate for a particular objective? No doubt an interesting dialog will ensue, but by-in-large taking the mystery out of plant science should be viewed as a good thing for all the constituent professions.

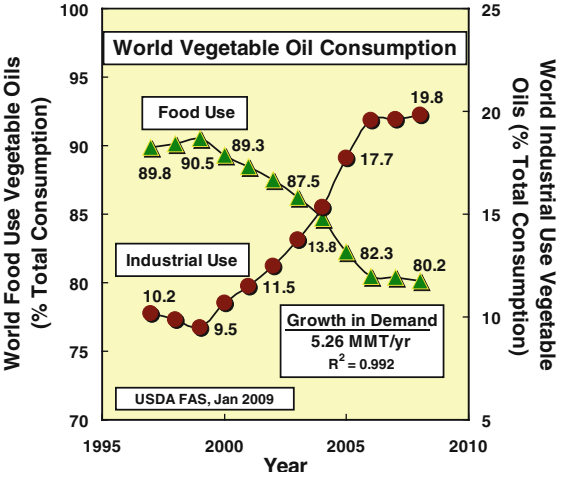
Is a physician's ability impaired by the advent of new diagnostic technologies and a more effective range of pharmaceuticals? Even a NASCAR driver

benefits from all of the computerized signals that monitor every aspect of a race cars performance. So, it is the same for breeding and quantitative genetics. Knowledge and skill are still needed to associate phenotypic traits with a haplotype. Ability is still required to reduce all of these ancillary tools to successful practice. Thus, the renaissance that is underway will position plant quantitative genetics to emerge with increased capacity to provide solutions to major problems and address the needs of world agriculture in a timely manner.

What are those needs with regard to oilseeds? Based on world production, USDA Foreign Agricultural Service reports show that soybean (56.0%), rapeseed (13.4%), cottonseed (10.1%), peanut (8.1%), sunflower (8.0%), and palm plus palm kernel (2.8%) are the major oilseed crops. These commodities represent essentially the entire commercial source of vegetable protein and oil. Annual world consumption of vegetable oil has averaged about 90.0% of total vegetable oil supply since 1997, leaving on average enough end-of-year stocks for about a 30-day buffer; whereas annual world use of oilseed meal has averaged about 95.7% of total supply, leaving on average a carryover equivalent to about an 11-day cushion of meal. These trends suggest that consumer demand for these products is limited only by availability, and that any natural disaster that may limit oilseed production could severely compromise the global food chain.

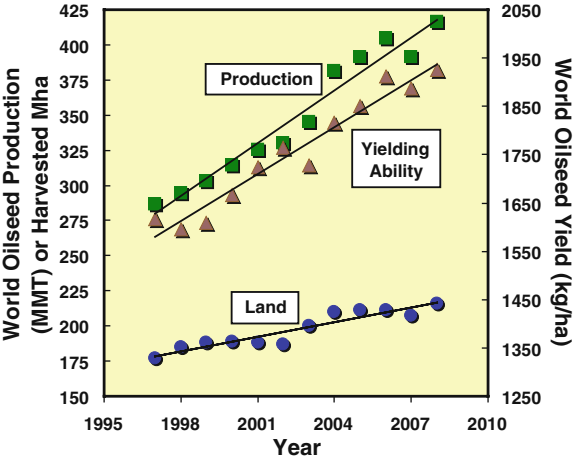
Although crushing capacity has expanded significantly in the US and abroad, the proportion crushed has averaged about 81% of total world oilseed production for decades. Considering the need to service export markets, a significant escalation of oilseed crush levels to increase the supply of meal and oil is unlikely. Hence, the greatest need that oilseed breeders face is simply to ensure a sufficient oilseed supply to meet the elastic demand for protein and oil; which on its own merit is a major contribution to alleviate world hunger.

However in recent years, a number of constraints have emerged that could mitigate efforts to increase global oilseed production for food use. The most prominent factor is renewed interest in vegetable oil as a source of biodiesel fuel. This concern recognizes that annual global vegetable oil resources could barely make a dent in the demand for energy. However, as shown in the adjacent figure, the market forces that direct food and industrial demand for



vegetable oils appear to have established a temporary equilibrium at about 80% (food):20% (industrial). Perhaps this will hold long enough for appropriate adjustments in markets for oilseed products. In addition, breeding efforts to develop varieties for commercial production of industrial oilseeds like lesquerella, cuphea and various non-food biotech innovations should help stabilize this situation.

Achieving greater genetic gain for oilseed productivity may be a lesser priority to some in the oilseed industry who subscribe to the paradigm that farmers will expand harvested area to increase the production of oilseeds. However, in view of escalating costs of oilseed production and competition for land from non-oilseed crops, the flexibility of countries to devote more agricultural resources to oilseeds remains to be seen. At this time, the rate of increase in harvested area since 1997 may be the best estimate of how much more harvested area might be available in future years. Regression analysis of these data in the figure below estimates the rate of increase at +3.45 Mha per year (R^2, 0.88). Assuming continuation of a linear trend, there might be a total of 258 Mha in global oilseed production in the year 2020, an increase of about 41 Mha over the level in 2008. One must wonder if this would be enough to make a significant difference.



Questions about future levels of harvested area place more pressure on the remaining variable in the yield equation for increased production. Regression analysis of these data in the adjacent figure estimates the rate of increase in world oilseed production at +12.5 MMT per year (R^2, 0.96). Assuming continuation of a linear trend, there might be a total of 704 MMT in global oilseed production in the year 2020, an increase of about 196 MMT over the level in 2008. Again, using simple arithmetic and assuming 258 Mha would be available to harvest, the world average oilseed yield in 2020 could be about 2.7 MT per ha (or 3.2 MT per ha if no additional land became available). Reaching that plateau would require a 40% increase in average total oilseed yield (70% without the projected increase in land) given an average global oilseed yield of 1.9 MT per ha in 2008.

In the past decade, average global oilseed yield has increased only 20%. Therefore, it appears that a great deal is riding on the development and application of oilseed biotechnology and genomics in the next decade. These technologies should enable quantum leaps in genetic progress. However, it all

depends upon a renaissance in quantitative genetics and the application of those technologies now and by the next generation of public and private oilseed breeders. Perhaps, it would be wise to redouble the effort to train and deploy that future workforce now.

Raleigh, North Carolina Richard F. Wilson

Preface

Vegetable oils have gained in importance during the past few decades resulting in the doubling of the world oil crop production in the last 25 years. Oil crops have been increasingly used as raw materials for food, livestock feed and non-food industrial applications. Plant breeding has played an essential role in supporting these developments: Breeding for higher yield and oil content allowed for an increase in oil production per unit area, whereas breeding for better oil quality has improved both the human health value of vegetable oils as well as the suitability of particular oils in specific industrial applications. Moreover, newly developed unique oil qualities are opening new opportunities in agricultural production and processing.

Cereals, legumes or forages each represent relatively homogeneous groups of crops belonging to one or a few plant families with similar botanical characteristics in which comparable breeding procedures could be used. In contrast, oil crop species have been developed in various botanical families from both the monocots and dicots. Thus, oil crops are a highly diverse set of species from short season annuals to perennials with a life span of over 2000 years. Consequently, breeding methods used for oil crop improvement include clonal breeding, pure line breeding, improvement of open-pollinated populations as well as hybrid breeding. In particular, the breeding procedures and techniques include almost every activity from simple mass selection and hybridization to specialized biotechnologies such as *in vitro* propagation or genetic engineering. Despite the differences at the species and breeding levels, some major breeding goals are remarkably similar, which justifies treating them in one volume such as: high oil content, altering fatty acid composition to suit the needs for either human consumption or non-food utilization, and a high quality of by-products. In addition, issues such as the biosynthetic pathways of particular fatty acids and their manipulation, QTL analysis for quality characters, genetic diversity, or oil and fatty acid analytics during selection are of common interest to all oil crop breeders. Therefore, this volume was prepared as a state-of-the-art compilation and a major reference text on oil crop breeding, which has been lacking for several decades. While the information accumulated in this volume is of primary interest to plant breeders, valuable insights are also offered to agronomists, molecular biologists, physiologists,

plant pathologists, food scientists and university scholars from the comparative treatment of various oil crop species.

Apart from an introductory chapter on oil crop breeding and a chapter highlighting genetic modification of vegetable oils, this volume presents 17 chapters devoted to breeding of particular oil crop species. Oil crops with world-wide distribution such as soybean, sunflower, oilseed rape and related brassicas are presented side-by-side with tropical and subtropical species such as cotton seed, peanut or castor, the perennials oil palm, coconut and olive, minor oil crops of regional importance such as safflower, poppy, oil pumpkin or maize, and new oil crops such as lesquerella and cuphea. Origin and domestication, varietal groups, genetic resources, major achievements and current breeding goals, breeding methods, techniques and biotechnologies, and seed production are addressed depending on their relevance in a particular crop.

Each crop chapter has been written by outstanding experts in their respective fields. Whenever possible authors from different institutions or countries worked together on particular chapters, which contributed to broadened and well-balanced views on particular species or topics.

The editors acknowledge the excellent contributions of all chapter authors who devoted much time and effort in delivering their part to this high quality volume. The editors extend heartfelt thanks to the staff at Springer, particularly to Hannah Schorr and Jinnie Kim, for their highly professional support during all stages of the publishing process. Moreover, the editors would like to thank Editors-in-chief of the Springer series *Handbook of Plant Breeding*, Professors Jaime Prohens, Fernando Nuez and Marcelo Carena, both for considering a volume exclusively devoted to oil crops and for their helpful input throughout the preparation of this volume.

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Chapter 1
Oil Crop Breeding and Genetics

Johann Vollmann and Istvan Rajcan

1.1 Introduction

Oil crops have considerably gained in importance to world agriculture and associated industries over the past 25 years. The total area of land devoted to oil crop cultivation has seen an increase from 160 million hectares in 1980 to 247 million hectares in 2005 (Fig. 1.1), whereas the world-wide acreage of cereals has dropped from 717 to about 670 million hectares over the same period of time. Annual world oil crop production has risen from 278 million metric tonnes in 1980 to about 711 million metric tonnes in year 2005 (Fig. 1.1). This remarkable expansion of production is due to the process of concentration on major oil crop species and at the same time to yield increases per unit area through refined agronomic practice and plant breeding. As illustrated in Table 1.1, soybean, rapeseed, sunflower and oil palm are the major crops contributing to the increase of the overall oil crop cultivation area, whereas the acreages of cotton seed, linseed, safflower and castor had significant decreases. Increases in yield per unit area from the 1979–1981 period to the 2002–2004 period (Table 1.1) were 82.2 and 69.0% for oil palm and rapeseed, respectively, and were also high for linseed and castor. A more moderate yield increase from 1701 to 2284 kg/ha (i.e. 34.3%) was noticeable for soybean, whereas progress was very slow in sunflower and safflower, and even negative in poppy. Taking the 2005/2006 marketing year and the medium-term prospects assessment for agricultural commodities of FAO and OECD as a basis, world oil crop production and vegetable oil output were estimated to rise by another 25–30% by the year 2015 (Thoenes 2006). Projections for the period 2006–2015 show that production increases will slow down in Europe and North America, while they will notably grow in Brazil, Argentina, Malaysia and Indonesia. Both the oil crop production increases since 1980 and the projected growth until 2015 correspond with a steadily growing demand for

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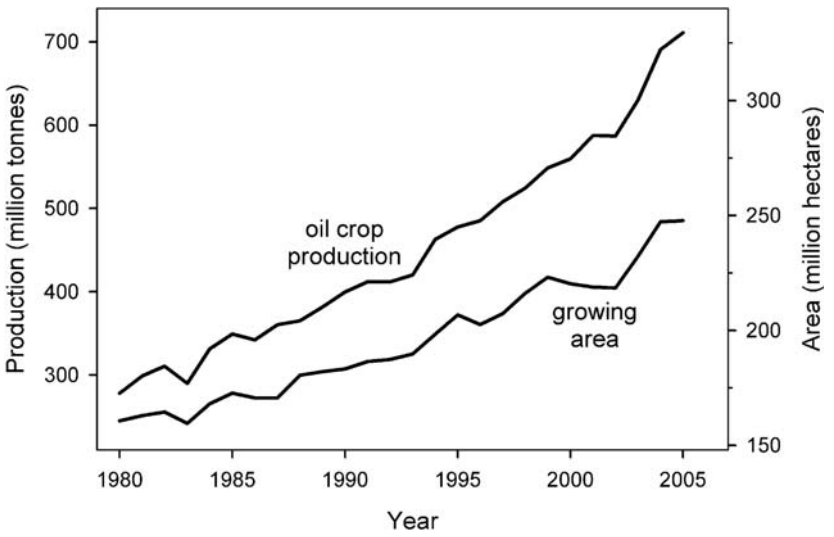

Fig. 1.1 Global oil crop production and acreage from 1980 to 2005 (FAOSTAT 2007)

Table 1.1 Changes in world acreage and average annual seed or fruit yield of major oil crops over a 25 years period of time

	Area in hectares		Change	Average yield in kg/ha		Change
Species	1980	2005	(in %)	1979–1981	2002–2004	(in %)
Castor	1, 540, 418	1, 408, 773	–8.5	572	934	63.1
Coconut	8, 768, 644	10, 685, 108	21.9	3, 717	4, 994	34.4
Cotton seed	34, 523, 000	30, 000, 000	–13.1	1, 246	1, 821	46.1
Groundnut	18, 364, 563	23, 427, 479	27.6	988	1, 452	47.0
Linseed	5, 371, 117	3, 182, 058	–40.8	456	767	68.2
Oil palm	4, 277, 328	12, 395, 528	189.8	7, 052	12, 847	82.2
Olive	5, 130, 401	7, 550, 561	47.2	1, 853	2, 042	10.2
Poppy	58, 573	109, 164	86.4	579	504	–12.9
Rapeseed	10, 992, 015	27, 448, 263	149.7	970	1, 639	69.0
Safflower	1, 322, 348	916, 443	–30.7	694	741	6.7
Sesame	6, 265, 283	7, 279, 469	16.2	303	435	43.7
Soybean	50, 649, 297	92, 369, 299	82.4	1, 701	2, 284	34.3
Sunflower	12, 425, 559	22, 823, 330	83.7	1, 170	1, 225	4.7
Total	160, 618, 770	241, 961, 583	50.6			

Source: FAOSTAT 2007.

and consumption of vegetable oils and fats. From 1980 to 2003, the availability of vegetable oils for food was rising from 19.9 to 26.4 kg per caput per year for North America and from 11.6 to 19.6 kg for Western Europe, whereas it developed from 4.8 to 10.3 kg for Asia and from 7.1 to 8.3 kg for Africa

(FAOSTAT 2007). Subsequently, the projected rise in vegetable oil consumption by 30% during the decade from 2005 to 2015 will be caused by increases of per caput food oil consumption in China, India and Latin American countries, whereas in the European Union and North America it will be driven by the strongly growing demand for bio-fuels (Thoenes 2006).

The largest increases in world average yield were found in oil palm and rapeseed (Table 1.1), which can only partly be attributed to plant breeding, as for both crops the expansion in planting area occurred predominantly in highly productive environments, i.e. Indonesia and Malaysia for oil palm, and Europe for rapeseed. Nevertheless, plant breeding has undoubtedly played a key role in production increases over the past 25 years: In oil palm, the introduction of hybrid cultivars derived from crosses between Deli (thick-shelled dura population) and shell-less pisifera or thin-shelled tenera populations, reciprocal recurrent selection, and the achievement of homogeneous planting populations of a favourable genotype through clonal micro-propagation of hybrids instead of seed propagation are considered driving forces of the huge yield increase (Soh et al. 2003). In oilseed rape, genetic progress is attributable to pure line improvement through enhancement of agronomic features, disease resistance and incorporation of the doubled-haploid technique, whereas high-yielding hybrid cultivars are gaining momentum only recently (Snowdon et al. 2007).

Generally, oil crop breeding is a more complex undertaking than breeding of cereals or legumes, as most oil crops are dual- or multi-purpose crops, which requires the simultaneous manipulation of different quality characters. In soybean, oilseed rape, sunflower and a number of other oil crops, the protein-rich meal is of economic significance beside the oil. However, a highly negative correlation between oil and protein content is a major impediment to breeding progress in these crops. In soybean, average seed oil content is 20% and protein content is 40% with a long-term tendency of slight increases in oil and decreases in protein content; as both constituents are important in international trade, economic models based on oil and protein prices have been proposed as a selection index in breeding of high value soybeans (Leffel 1990). In oilseed rape, selection of strains exhibiting the yellow seed color character, which is associated with a thinner seed coat and lower fibre content than in black-seeded genotypes could be a strategy of simultaneous improvement of both oil and protein content (Badani et al. 2006b). In cotton, fibre yield and fibre quality are the main crop features, whereas cotton seed oil is a by-product and therefore oil content is not the major breeding objective. In linseed or flax, there are two main morphotypes of cultivars for either oil production from seed (linseed) or bast fibre production from stems (fibre flax), whereas dual-purpose cultivars are rare, and production of both high quality seed and fibre from the same crop is difficult agronomically. Only recently, the utilisation of short-fibre linseed straw is discussed for applications in the emerging field of non-woven materials, and selection criteria for breeding of

dual-purpose linseed cultivars have occasionally been suggested (Rennebaum et al. 2002; Foster et al. 2000). Moreover, issues such as the specific requirements of oilseed quality analytics, crop product diversification, the handling of cytoplasmic male sterility in hybrid crops with hermaphroditic flowering, and the introduction of genetically engineered cultivars or traits add to the complexity of oil crop breeding.

Earlier reviews of oil crop breeding have focused on major breeding objectives (Knowles 1983), on breeding methods (Knowles 1989) or on the reproductive systems of oil crop species which determine both the breeding strategy applicable and the resulting type of cultivar (Arthur 1994). More recently, excellent reviews have been published on breeding for specific fatty acid composition (Burton et al. 2004), on the different aspects of improving oil quality (Velasco and Fernández-Martínez 2002), and on genetic engineering the pathways of oil biosynthesis (Dyer and Mullen 2005). This review addresses two key features of present day oil crop breeding, genetic diversity and oil content; the emphasis will be put on annual oilseeds rather than on perennial crops.

1.2 Domestication and Genetic Diversity

Domestication is an evolutionary process of genetic development, in which natural selection is replaced by human selection shaping crop plants for specific needs. Typical changes occurring during the development from a wild plant to a domesticated crop are referred to as the domestication syndrome; they include the loss of seed dormancy, increased rates of self-pollination, adoption of vegetative propagation, increase in yield of seed or other plant organs utilized, compact growth habit, loss of seed dispersal, increase in number and size of seeds and inflorescences, changes in color, taste and texture, and decrease in the content of toxic substances (Gepts 2002). Other important changes include the alteration of photoperiod sensitivity, adaptation to agricultural soils and agronomic treatments, and the adaptation to new environments often far away from the center of origin.

Cereals, legumes and fruits were among the first crop plants utilized by mankind; domestication of cereals dates back some 12 000 years and is considered as the decisive impetus of Neolithic revolution, the transition from a hunting and gathering lifestyle to a sedentary agriculture-based society (Salamini et al. 2002). Oil plants were not among those first crops domesticated, most of them appeared much later in history, as their utilization and handling requires specific knowledge and techniques not available to early agriculturalists. The comparatively late appearance of major oil crops does have consequences on their status of domestication, on the development of genetic diversity, and subsequently on availability of germplasm resources.

1.2.1 Domestication of Oil Crops

The domestication status of oil crops is fairly divergent depending on their agricultural history. While few oil crops are fully domesticated, many others express various wild type characteristics, as illustrated in some prominent examples: Seed dormancy is still a problematic feature of sunflower which disallows rapid germination of lost seed, but instead causes volunteer sunflowers in the following season; pod dehiscence and subsequent seed shattering may cause considerable yield losses in soybean, oilseed rape, sesame and other oilseeds; self-pollination is prohibited in several oilseed brassicas due to self incompatibility; anti-nutritional factors such as protease inhibitors are present in soybean, oleuropein, a bitter phenolic compound is found in olive; and toxic components such as glucosinolates in oilseed brassicas or gossypol in cotton have only been reduced recently. In addition, new oil crops only grown for their unique fatty acid patterns, such as lesquerella, crambe, cuphea, meadowfoam or jojoba exhibit numerous wild type characteristics apart from poor productivity.

Flax or linseed (*Linum usitatissimum* L.) is today considered to be the oldest oilseed in the world having been domesticated in the Near East region 10 000 years ago and serving as a source of both oil and fibre from prehistoric time until present (Allaby et al. 2005). It has been under discussion whether oil or fibre was the primary reason of domestication, and whether domestication took place once or happened several times in independent domestication events in different diversity regions of flax (Diederichsen and Hammer 1995). New evidence from network analysis of genetic diversity in the stearic acid desaturase locus *sad2* suggests a single domestication event of cultivated flax from its wild progenitor *Linum angustifolium* Huds.; moreover, an oilseed type of flax is proposed as the first domesticate, while fibre flax appears as a later descendant from oilseed flax (Allaby et al. 2005).

Sesame (*Sesamum indicum* L.) has often erroneously been described as the oldest oilseed in human use with a probable origin in Africa, as sesame is a historically and culturally important crop plant, and there is a high diversity of *Sesamum* species on the African continent (Bedigian 2003). However, clear evidence from archeology, history as well as from botanical, chemical and genetic data suggests that sesame has been domesticated on the Indian subcontinent during the period from 3050 to 3500 BC, and that *S. malabaricum* Burm., a wild sesame species occurring in India exclusively is the progenitor of cultivated sesame (Bedigian 1998, 2003).

Sunflower (*Helianthus annuus* L.) was domesticated by Native North Americans about 4, 300 years ago from wild *H. annuus* in the now east-central United States (Wills and Burke 2006); in addition, multiple evidence for an indepentent domestication event in Mexico has also been presented (Lentz et al. 2008). Sunflower was then utilized as a multi-purpose crop, but became an oilseed only in the late 18th and early 19th century in Russia, from where it spread over

Europe and was later re-introduced from Russia to North America as an oilseed crop (Putt 1997). From the cross between a cultivated and a wild sunflower genotype with subsequent QTL analysis, Burke et al. (2002) gained insight into the genetics of sunflower domestication: Only a few major QTL were found, the two strongest QTL affected the number of selfed seeds (self-compatibility); moreover, selection for increased achene size was an important feature of sunflower domestication, a high frequency of favourable alleles was present in wild sunflower, and a majority of sunflower domestication traits was non-recessive.

Soybean (*Glycine max* (L.) Merr.) was domesticated from the wild *Glycine soja* Sieb. & Zucc. in the northeast of China (Manchuria) in the period 1500–1100 BC (Hymowitz 2004) probably in multiple domestication events, as suggested by chloroplast DNA diversity between wild and cultivated soybeans (Xu et al. 2002). So, despite the popular myth claiming soybean to be one of the oldest crops utilised by mankind (Hymowitz and Shurtleff 2005), it is a comparatively young crop plant. And much later, during the North Song Dynasty (960–1127) soybean was recognized as a source of vegetable oil (Huan and Bao 1993).

Oilseed rape (*Brassica napus* L.) is known only since the 13th century as an oil crop (Snowdon et al. 2007; Downey and Röbbelen 1989). As an amphidiploid interspecific hybrid and probably with a polyphyletic base (Song et al. 1988) it originated in the Mediterranean region of southwest Europe, where the two diploid parental species *B. oleracea* L. (cabbage) and *B. rapa* L. (turnip) overlap in their natural habitats. Apart from oilseed rape, the species *Brassica napus* is comprised of related forage and vegetable forms (e.g. Soengas et al. 2006), but no true wild forms are known, which also underlines the recent origin of this species.

1.2.2 Oil Crop Germplasm

The availability of germplasm with sufficient genetic diversity is essential for a continuous breeding progress. Jones (1983) emphasized the particular need of preserving oil crop germplasm, as almost all of the major oil crops are now cultivated far away from their primary centers of origin. Therefore, they do have a comparatively narrow genetic base classically made up by relatively few plant introductions who represent the ancestors, from which elite germplasm and further breeding material is developed.

As shown above, most oil crops gained economic importance during the last couple of decades only, and many of them are very young crops in terms of their cultivation and utilisation history as oil plants. These appear to be the main reasons why oil crops are poorly represented in ex situ germplasm collections at present. In Table 1.2, a summary is presented on numbers of accessions for oil crops versus other crops held by the three major genebank associations, which represent the most significant institutions conserving genetic resources. For all three associations, cereals such as *Triticum* sp. (mainly bread and durum

Table 1.2 Accession numbers of crops in general and oil crops in three major genebank associations (ex situ collections)

Genebank association	Crops in general	Accessions	Oil crops	Accessions
CGIAR centers	*Triticum* sp.	114, 721	Soybean[1]	15, 904
(SINGER)	Rice	111, 303	Peanut	14, 694
	Sorghum	36, 805		
	Barley	38, 067		
	Maize	25, 827		
	Chickpea	30, 063		
	Lentil	10, 733		
	CGIAR total	689, 578		
EURISCO	*Triticum* sp.	156, 045	Linseed/flax	17, 226
European Plant	Barley	75, 033	Soybean	11, 408
Genetic	Maize	42, 267	Oilseed rape	4, 879
Resources	Oat	23, 149	Sunflower	4, 444
Search	Rye	10, 254	Poppy	4, 114
Catalogue	Sorghum	6, 234	Peanut	2, 575
	Common bean	30, 845	Cotton	1, 957
	Pea	24, 767	Sesame	1, 661
	Lentil	5, 635	Safflower	728
	Faba bean	5, 600	Olive	421
	EURISCO total	1, 000, 175		
USDA National	*Triticum* sp.	55, 942	Soybean	19, 277
Plant Germplasm	Barley	28, 438	Peanut	6, 831
System	Sorghum	42, 666	Cotton	5, 794
	Corn	25, 468	Linseed/flax	2, 863
	Oat	21, 837	Sunflower	2, 759
	Rice	19, 470	Safflower	2, 373
	Phaseolus sp.	14, 928	Sesame	1, 226
	Chickpea	6, 019	Castor	1, 043
	USDA total	477, 077		

[1]World Vegetable Center (AVRDC, Taiwan, as part of SINGER network).
Sources: CGIAR: http://www.singer.cgiar.org/, 30 April 2007
EURISCO: http://eurisco.ecpgr.org/, 30 April 2007
USDA: http://www.ars-grin.gov/npgs/stats/, 30 April 2007

wheat), rice, barley or sorghum and legumes such as chickpea, pea, lentil or phaseolus beans have been conserved in clearly higher numbers than oil crop species: The genebanks of the international agricultural research centers (CGIAR group, SINGER network) hold a significant peanut collection and a partly vegetable type soybean collection, but generally oil crops are not on their list of mandate crops. The European national germplasm collections, linked together in EURISCO, an internet germplasm search catalogue, hold significant numbers of linseed/flax and soybean accessions; for oilseed rape and sunflower, the two most important European oil crops, accession numbers are much lower and in the same magnitude as for poppy, which is of very regional importance only. The United States National Plant Germplasm

System holds significant collections of soybean, peanut and cotton accessions in their genebanks, which represent the major US oil crop species. In addition to the accessions listed in Table 1.2, important oil crop germplasm is also maintained by institutions in Canada, Argentine, Brazil, China, India, Australia and few other countries.

Generally, the number of accessions per species held in ex situ collections is an indicator of past collection activities and the availability of germplasm, but not a sound measure of genetic diversity. For the accessions of linseed/flax, Diederichsen (2007) reviewed the ex situ collections world-wide: More than 46, 500 accessions of linseed are present in at least 33 public genebanks; however, based on analyses of duplications, the author estimates that only 10–15,000 accessions are unique. In soybean, more than 170,000 accessions are maintained in genebanks, out of which more than two thirds are duplications and about 45,000 are considered unique genotypes (Carter et al. 2004).

Although present in lower number than cereals and legumes, oilseeds such as linseed, soybean and peanut appear to be well represented in ex situ collections, while germplasm availability of minor and new oil crops is very limited (Thompson et al. 1992), and therefore enhancing germplasm collections of these species will be an important activity ensuring future breeding progress.

1.2.3 Genetic Diversity in Oil Crops – Selected Examples

An overview of the genetic diversity present in the primary and further genepools of a given species is of great interest both to plant breeding and conservation management. Technically, estimates of genetic relationship may be obtained from pedigree information, phenotypic data, or molecular polymorphisms on the protein or DNA level, and by applying an appropriate measure of genetic distance (Mohammadi and Prasanna 2003). In oil crops, various conclusions for plant breeding have been drawn from analyses of genetic diversity for particular species and populations, as outlined in selected examples from soybean and oilseed rape.

Soybean genetic diversity has meticulously been investigated from various points of view and was reviewed by Carter et al. (2004). Pedigree analysis and calculation of coefficients of parentage revealed that the genetic base of North American soybean cultivars is narrow as compared to Asian soybeans: While only 26 ancestors contributed 90% of genes to 258 public cultivars in North America (Gizlice et al. 1994), it is more than 339 ancestors which contributed 90% of genes to 651 Chinese soybean cultivars (Cui et al. 2000) and more than 74 ancestors which contributed 90% to 86 modern public Japanese cultivars (Zhou et al. 2000).

Using RAPD markers, Li and Nelson (2001) found a larger genetic diversity in Chinese accessions than in Japanese or South Korean accessions and were able to clearly separate Chinese soybeans and those from Japan or South

Korea, respectively. In a diversity study based on AFLP markers, Ude et al. (2003) suggested to utilize Japanese elite cultivars in order to widen the narrow genetic base of North American soybeans, as they are more distinct from North American cultivars than Chinese ones. In numerous other studies, molecular markers were used to investigate special issues such as variation in vegetable soybeans (Mimura et al. 2007) or diversity between cultivated and wild soybean accessions and their geographical genetic differentiation (Chen and Nelson 2004; Xu and Gai 2003).

The phenotypic diversity determined for 15 traits of over 20,000 soybean accessions from the Chinese national soybean collection is representing a highly valuable information pool for breeding and has further been used to propose a single geographical center of soybean diversity downstream the Yellow River Valley (Dong et al. 2004). Phenotypic data from 25 leaf, stem and seed composition traits of North American and Chinese soybean cultivars have also been utilized to verify the narrow genetic base of North American soybeans, which probably represents a subset of the wider genetic base of Chinese cultivars (Cui et al. 2001); phenotypic distinctness of these two genetic pools is considered to be the result of continuous selection for adaptation to contrasting environmental conditions, which now offers new opportunities for reciprocal broadening the genetic bases by introducing exotic parents.

Marker-assisted introgression of genes from exotic or wild sources through backcrossing is occasionally considered as enhancing the genetic base of soybean (Lee et al. 2007). However, while backcrossing may bring in the beneficial effect of a particular allele into adapted breeding material, it does not enlarge the overall genetic base (Carter et al. 2004); otherwise, backcrossing the genetically engineered tolerance to the herbicide glyphosate into many commercial soybean cultivars also did not reduce the genetic base of North American soybean cultivars (Sneller 2003).

In oilseed rape, genetic diversity is considered to be low because of the short cropping history and the strong breeding focus on seed quality characters, i.e. low erucic acid and low glucosinolate contents which narrowed down the genetic base. Therefore, artificial resynthesis of oilseed rape from its diploid progenitors cabbage and turnip is practised in order to broaden the genetic base of oilseed rape (Becker et al. 1995; Seyis et al. 2003; Basunanda et al. 2007), although resynthesized rapeseed lines exhibit a low yield potential and inferior seed quality. Resynthesis has repeatedly been used for gene introgression into cultivars, e.g. for various disease resistances or yellow seed color (Snowdon et al. 2007). Apart from resynthesis, enriching the genetic base of oilseed rape has been suggested by hybridizing European and Chinese elite oilseed rape lines (Hu et al. 2007), or by utilizing diversity existing in vegetable or fodder crop types of *Brassica napus*, despite their inferior oil and meal quality (Hasan et al. 2006).

Due to the present transition from pure line breeding to hybrid breeding, genetic diversity in oilseed rape is receiving new attention, as heterotic

pools of accessions with sufficiently large genetic distance need to be formed for maximum hybrid performance (Snowdon et al. 2007). Significant relationships between parental genetic distance and hybrid oilseed rape performance have been described (Diers et al. 1996; Riaz et al. 2001; Shen et al. 2006), but were considered not sufficient for prediction of heterosis. For improvement of hybrid performance, Quijada et al. (2004) suggested the introgression of European winter oilseed rape genomic segments into Canadian spring canola, as superior hybrid performance was found in testcrosses between these two genepools. A different strategy for increasing hybrid performance of oilseed rape has been proposed by Li et al. (2006a), who found considerable heterosis in crosses between natural *Brassica napus* parents and a new type of *Brassica napus* containing the A subgenome of *B. rapa* and the C subgenome of *B. carinata* thus realizing intersubgenomic heterosis.

1.3 Recent Milestones in Oil Crop Breeding

Over the past few decades, breeding research in oil crops has seen a number of crucial results which had significant impacts on the subsequent development of world-wide oil crop production (Table 1.3). Improvement of both oil and meal in oilseed rape by reducing erucic acid content of oil (canola quality) and glucosinolate content of meal are two most prominent milestones contributing to the expansion of world oilseed rape acreage from less than 10 million hectares in the early 1970s to more than 27 million hectares in 2005 (FAOSTAT 2007). Moreover, high oleic (Schierholt et al. 2001) and low linolenic (Rücker and Röbbelen 1996) oilseed rape represent further improvements of nutritional value and oxidative stability. Relevant changes in fatty acid composition have also been achieved in sunflower, soybean and linseed (Table 1.3). Additional examples of alterations in fatty acid composition for particular crops have been summarized by Velasco and Fernández-Martínez (2002). In sunflower and oilseed rape, cytoplasmic male sterility (cms) allowed for the development of hybrid cultivars (Table 1.3), whereas in oil palm hybrid breeding and micropropagation of planting material have contributed to the success of that crop (Basri et al. 2005). Other biotechnologies such as the production of doubled haploids in rapeseed (Chen et al. 1994) helped to accelerate the breeding progress. The perhaps most prominent examples of genetic engineering and molecular genetics in oilseeds are glyphosate tolerant soybean and the integrated soybean linkage map (Table 1.3), but genetic engineering also has a significant impact in oilseed rape (herbicide tolerance, engineering fatty acid biosynthesis pathways; Snowdon et al. 2007) and in cotton (*Bacillus thuringiensis* toxin mediated insect resistance; Christou et al. 2006) at present.

Table 1.3 Recent milestones in oil crop breeding with relevance to world oil crop production

Milestone	Year	Comment	References
Low erucic acid rapeseed	1959	Character found in German spring-type rapeseed cv. Liho, allowed for the development of high oil quality rapeseed cultivars and subsequent conversion to 'canola' quality cultivars in Canada and Europe in the 1970s	Stefansson et al. (1961)
Low glucosinolate rapeseed	1968	Found in the Polish spring-type rapeseed Bronowski, lead to release of low glucosinolate meal cultivars from the 1970s on (00-quality cultivars)	Josefsson and Appelqvist (1968) and Lein (1970)
Cms sunflower	1968	Source of cms from interspecific cross *Helianthus petiolaris* x *H. annuus*, introduction of hybrid cultivars in the 1970s	Leclercq (1969)
Cms rapeseed	1968	Cytoplasmic male sterility found in Japanese radish (Ogura-cms), later transferred to *Brassica napus* in France; cms in Chinese rapeseed cv. Polima; at present the two major cms sources for hybrid seed production in oilseed rape	Ogura (1968) and Fu (1981)
High oleic sunflower	1976	Induced mutant in sunflower variety VNIIMK 8 931, base of cultivar Pervenets, from which high oleic sunflower lines with over 90% oleic acid and high oleic hybrids were developed	Soldatov (1976) and Burton et al. (2004)
Low linolenic acid linseed	1986	Two induced mutants from cv. Glenelg with a combined linolenic acid content below 2% for linseed oil with improved oxidative stability	Green (1986)
Low linolenic soybean	1986	Three induced mutant allels with reduced linolenic acid content combined together for a 1% linolenic acid soybean oil with better stability and less trans fatty acid formation	Fehr et al. (1992) and Ross et al. (2000)
Glyphosate tolerant soybean	1996	Genetically engineered soybean (*Roundup Ready* soybean) containing EPSP synthase from *Agrobacterium* sp. with tolerance to the herbicide glyphosate, first commercially grown in 1996, reached over 50 million hectares in 2005	Padgette et al. (1995)
Integrated soybean linkage map	1999–2004	Integrated high-density soybean linkage map with more than 1800 genetic markers (mainly SSRs), useful in fine-mapping genes, map-based cloning or QTL analysis	Song et al. (2004)

1.4 Specific Breeding Objectives

Apart from agronomic performance and resistances, oil content of seed or fruit is the breeding objective economically most important to growers and primary processors. While breeding for oil quality, i.e. fatty acid composition, has been the subject of earlier reviews (e.g. Velasco and Fernández-Martínez 2002) and is being dealed with in dedicated crop chapters, various aspects of oil content will be presented here. Additionally, newly arising breeding objectives of altering seed composition for health and industrial applications will as well be covered within the present section.

1.4.1 Oil Content

1.4.1.1 Oil Bodies and the Cytology of Oil Content

Most storage lipids of oilseeds are composed of triacylglycerols which are synthesized during seed filling. De-novo biosynthesis of fatty acids has been well presented in earlier reviews (e.g. Stumpf 1989; Harwood and Page 1994), whereas newer reviews also cover the potentials of genetic engineering fatty acid synthesis in oil plants (Dyer and Mullen 2005; Napier 2007; Singh et al. 2005).

Fatty acid synthesis is located in plastids of cells in developing embryos, from where fatty acids activated with coenzyme A are released and accumulate in a compartment formed by layers of the endoplasmatic reticulum. Inside the endoplasmatic reticulum, fatty acids may undergo different modifications and finally are esterified to form triacylglycerols. Due to their hydrophobic nature, the accumulation of triacylglycerols results in bulges of the endoplasmatic reticulum from where oil bodies (oleosomes) are developing (Dyer and Mullen 2005) which are the microscopically visible oil bearing structures in mature seeds. Wältermann and Steinbüchel (2005) have illustrated the most widely accepted model of oil body formation in oilseeds (Fig. 1.2). From a bulge formed by triacylglycerols, an oil body is developing and surrounded by a monolayer of phospholipids, which is derived from the outer leaflet of the endoplasmatic reticulum. Subsequently, oleosine protein units are embedded in the phospholipid layer, and the oil body is separating from the endoplasmatic reticulum. The central domain of the oleosine protein is hydrophobic and therefore contacting the lipid matrix, whereas both termini are directed towards the cytoplasm. Oleosine proteins are present in all oilcrops with seeds undergoing dehydration during seed maturation but are not found in oil bodies of non-desiccating species such as olive, avocado or other tropical oil plants (Murphy and Vance 1999; Wältermann and Steinbüchel 2005). The size of oil bodies is dependent on the plant family; the diameter of oil bodies is between 0.3 and 0.8 μm in Brassicaceae oilseeds, between 0.5 and 2.0 μm in cotton, linseed and maize, and often above 2 μm in poppy, sunflower and sesame (Menge and Seehuber 1988; Tzen et al. 1993; Mantese et al. 2006);

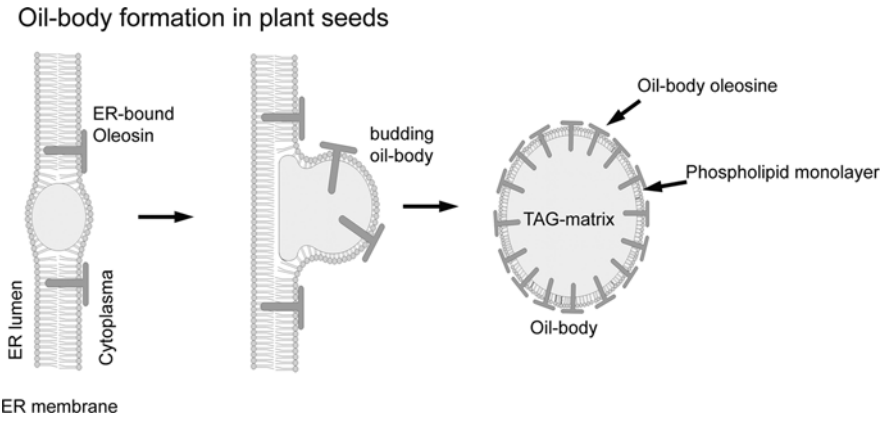

Fig. 1.2 Model of oil body development in oilseeds (from: Wältermann and Steinbüchel 2005; image kindly provided by the authors and used with permission from the American Society for Microbiology)

very large oil bodies (5–50 μm in diameter) are found in non-desiccating species (Murphy and Vance 1999). Oleosines regulate the size of oil bodies, they provide stability during desiccation and rehydration (Peng et al. 2003; Murphy and Vance 1999) and might be a target to genetic modification of lipid accumulation (Siloto et al. 2006) and subsequently oil content.

1.4.1.2 Botanical Features of Oil Content

Storage lipids are synthesized, stored and later re-metabolized in the same tissues within seeds or fruits, as they cannot be translocated within a plant because of their hydrophobic nature. As storage lipids are a seedlings major source of energy during germination and emergence, oil bodies are concentrated in embryonic tissues, i.e. parenchymatic cells of cotyledons and the embryo axis in oilseeds, or in the embryo (mainly the scutellum) of cereals, whereas endosperm tissue is devoid of storage lipids except for castor and few other species. The basis of genetic variation in oil content may therefore be variation in size or density of oil bodies, or variation in the proportion of embryonic tissue containing storage lipids relative to total seed or fruit mass which is most relevant in practical breeding for high oil content.

In sunflower, Mantese et al. (2006) investigated the temporal and histological patterns of lipid accumulation in genotypes with achene oil content ranging from 300–330 g/kg (low oil content) up to 450–550 g/kg (high oil content). They reported a tendency of a slightly larger oil body diameter in high oil content genotypes as compared to a low oil content genotype. While absolute oil mass of embryo was similar in high and low oil content genotypes, embryos of low oil genotypes were larger and thus had a lower density of oil bodies; moreover, in

cotyledon transsections of low oil genotypes a significantly larger cell area was occupied by protein bodies than in high oil genotypes.

While variation in size and density of oil bodies would contribute to the increase of oil content in small increments, major steps towards improvement of oil content have been achieved in many oilseeds through selection for reduced pericarp or thin testa mutants, as shown in Fig. 1.3 for sunflower, rapeseed, linseed, poppy and oil pumpkin, respectively.

In sunflower (Fig. 1.3A), confectionary genotypes have large achenes with a thick pericarp (hull) and an oil content of 200–300 g/kg, whereas oilseed

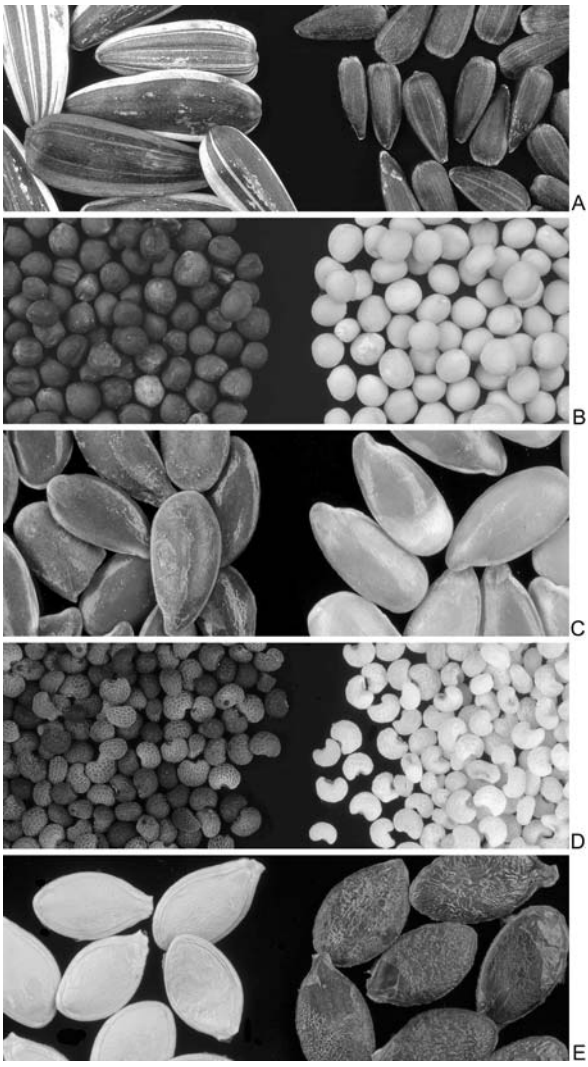

Fig. 1.3 Low (*left*) and high (*right*) oil content accessions of sunflower (**A**), oilseed rape (**B**), linseed (**C**), poppy (**D**) and oil pumpkin (**E**), respectively, differing in testa or pericarp thickness

cultivars have small achenes with thinner pericarp and an oil content of 400–550 g/kg (Mantese et al. 2006; Tang et al. 2006). It has been estimated that prior to 1970 two-thirds of increase in sunflower oil content resulted from a reduction of hull percentage and one-third from increases in kernel (seed) oil content, while over the last decades almost all of the increase in achene oil content has been achieved through an increase in kernel oil content (Miller and Fick 1997). In sunflower cultivars released in Argentine from 1930 to 1995, achene oil content was increased from 350 to 550 g/kg, while the ratio of kernel to achene weight was increased from 0.6 to 0.8 by selection for high oil content (López Pereira et al. 2000). In safflower, another Asteraceae oil crop, increases in oil content from 390 to 470 g/kg were similarly achieved through selection of achenes with thinner hulls (Knowles 1983).

In oilseed rape (Fig. 1.3B), the yellow seed character was introduced through resynthesis from a *Brassica rapa* source (Liu et al. 2005). The oil content of yellow-seeded lines with thin testa is 445 g/kg, whereas in comparable lines with black seeds an oil content of 399 g/kg has been reported (Badani et al. 2006b). Moreover, a major QTL for yellow seed colour was located in the same position as a QTL for reduced fibre content in different populations, and candidate genes for the yellow seed coat character have been proposed from the flavonoid biosynthesis pathway (Badani et al. 2006a), as the black seed colour of oilseed rape is made up by proanthocyanidins (condensed tannins).

In linseed, the yellow seed character (Fig. 1.3C) is known to be associated with higher oil content and larger seed weight, but has shown agronomic disadvantages such as a lower seed germination rate due to seed injury and a higher incidence of root rot (Culbertson et al. 1960; Bradley et al. 2007). In a large linseed germplasm collection, yellow seed is also associated with a significantly higher oil content and a larger seed weight than brown seed (Diederichsen and Raney 2006). The yellow seed character is controlled by several independent loci (Mittapalli and Rowland 2003), but causal relations for the association between yellow seed and high oil content are missing. Similar to other oilseeds, yellow seed colour in linseed may be due to a thinner seed coat of yellow seeds as compared to brown seeds. This view is also supported by the finding that linseed mucilage, which consists of polysaccharides located in the epidermis of the seed coat, is present in lower concentrations in yellow-seeded accessions than in brown-seeded ones (Diederichsen et al. 2006). Similarly, yellow or white seeds of poppy (Fig. 1.3D) are superior in oil content to blue seeds (Azcan et al. 2004; Bernáth 1998), which may as well be due to a thinner seed coat. In oil pumpkin (Fig. 1.3E), a mutation prohibiting the lignification of testa (hull) gave rise to utilising pumpkin as an oilseed crop instead as a vegetable in some Central European countries. Oil content of seeds with thick, lignified testa is below 300 g/kg, whereas oil content of cultivars utilizing the thin testa mutation (Styrian oil pumpkin cultivars) is between 350 and 500 g/kg (Idouraine et al. 1996; Murkovic et al. 1996). Lignification is controlled by one major gene with the thin testa character being recessive, and

several minor genes may cause partial lignification of thin testa genotypes (Teppner 2004; Zraidi et al. 2003).

In maize, 100 generations of selection for either high or low oil content yielded strains with an oil content of above 20% or below 1%, respectively, in the Illinois long-term selection experiment for oil and protein (Dudley and Lambert 2004). As the oil-bearing tissue of a maize seed is the embryo, high oil content was associated with an enlargement of the scutellum (Moose et al. 2004). In another maize synthetic (Lambert et al. 2004), high oil content was associated with a reduced starch content, whereas protein content remained unaffected; moreover, seed weight was reduced, and the proportion of the embryo was increased, while endosperm was decreased through selection for high oil content.

As cotyledons hold the oil-bearing tissues in most of the annual oilseeds, selection for cotyledon size, as demonstrated for *Brassica rapa* (Tel-Zur and Goldman 2007), might be an alternative route to improve seed oil content.

1.4.1.3 Genetics of Oil Content

Oil content is a quantitative character controlled by both the genetics of a cultivar and the environment. While the genetics of particular fatty acids had been well characterised in many oil crops, information about the inheritance of oil content was meagre for a long time. As an example, oil content of rapeseed was assumed to be controlled by mainly additive gene action with dominance being not significant and epistasis absent, and the number of genes involved in oil content was estimated to be lower than that for seed yield (Röbbelen and Thies 1980). During the last decade, however, mapping of quantitative trait loci (QTL) has brought more insight into the genetics of oil content and the functional principles behind particular QTL.

In soybean, 69 QTL for oil content were listed in the USDA SoyBase database (http://soybeanbreederstoolbox.org, October 2007) with most listings occurring for linkage groups A1, E, I and L. While most putative QTL do have small effects on oil content, one to four major QTL explain more than 10% of the phenotypic variation in different segregating populations (Lee et al. 2007). Three to eight QTL affecting oil content have also been reported for oilseed rape (Ecke et al. 1995; Burns et al. 2003) and other cruciferous oil crops such as *Brassica juncea* (Mahmood et al. 2006) or *Camelina sativa* (Gehringer et al. 2006), while one or two of up to 14 QTL explained more than 10% of oil content variation in oilseed rape (Delourme et al. 2006). Additive gene action has been proposed as the way of action for most QTL influencing oil content. In addition, Leon et al. (2003) reported dominant QTL effects for oil content in a bi-parental sunflower population. Digenic epistasis in oil content QTL has been described for oilseed rape (Zhao et al. 2005; Delourme et al. 2006), *Brassica juncea* (Mahmood et al. 2006), soybean (Lark et al. 1994), or oat (Zhu et al. 2004). In the maize long-term selection for oil and protein, large numbers of epistatically acting QTL for oil,

protein and starch content suggest the importance of epistasis for continuing selection response, particularly at lower levels of genetic variability (Dudley 2008).

The negative correlation between seed oil and protein content known from various oilseeds is also evident on the QTL level. QTL regions associated with oil content are frequently found to control protein as well and vice versa. This association may be due to tight linkage between oil and protein alleles in repulsion phase or to pleiotropic effects. In soybean, Lee et al. (2007) estimated that about 58% of oil content QTL are also associated with protein across a number of studies. Chung et al. (2003) described a QTL affecting oil content, protein content and grain yield of soybean and proposed the presence of a single QTL pleiotropically affecting both oil and protein. Nichols et al. (2006) narrowed down an oil and protein QTL region on soybean linkage group I to a 3-cM marker interval by fine-mapping, but still could not discriminate between either pleiotropy or close linkage of two loci affecting both traits. In oilseed rape, conditional mapping of QTL for oil content (Zhao et al. 2006) allowed for discriminating between true oil or protein QTL as well as the identification of additional oil QTL as compared to unconditional mapping.

Knowledge on the functional genetic mechanisms and regulatory metabolic factors controlling oil content is rather limited. In Brassicaceae, genes coding for erucic acid content may be considered 'candidate genes' for oil content, because the increased chain length and molecular weight of the erucic acid molecule (C22-body) as compared to C18-fatty acids causes an increase of total oil content. In oilseed rape, two QTL affecting oil content were at the same time associated with the two genes for erucic acid content (Ecke et al. 1995) in a cross between a high and a low erucic acid line. Subsequently, these genes were characterised as fatty acid elongase genes homologous to the *Arabidopsis FAE1* gene coding for the elongation of C18:1 (oleic acid) to C22:1 (erucic acid) in oilseed rape (Fourmann et al. 1998) and in *Brassica juncea* (Gupta et al. 2004). Contrary to erucic acid, palmitic acid (C16:0) has a lower molecular weight than C18-fatty acids, which may partly explain its negative correlation with oil content in oilseed rape, sunflower or soybean (Möllers and Schierholt 2002; Velasco et al. 2007; Hartmann et al. 1996). Differential gene expression studies using oilseed rape lines either high or low in oil revealed a major involvement of genes related to chloroplast function (photosynthesis) and sucrose metabolism in the expression of oil content (Li et al. 2006b). In oat, a plastidic acetyl-CoA carboxylase gene which catalyzes the initial step of de novo fatty acid synthesis was identified as a candidate gene strongly affecting oil content (Kianian et al. 1999). In an *Arabidopsis* transformation experiment, Jako et al. (2001) showed an increase in oil content through over-expression of a diacylglycerol acyltransferase. The metabolic complexity of oil content as a trait is also depicted on the proteomics level, where proteins related to energy, carbohydrate and amino acid metabolism are prominently expressed during

seed filling in oilseed rape and sunflower (Hajduch et al. 2006, 2007), which may contribute to identifying high level genes regulating oil content.

1.4.1.4 Breeding for Oil Content

Due to the diversity in reproductive systems of different oil crop species, cultivars can be ascribed to each of the four classical plant breeding categories, i.e. clones (olive, clonally propagated oil palm), allogamous populations (coconut, oil palm, castor, outcrossing brassicas), autogamous pure lines (soybean, linseed, peanut, poppy, sesame), and hybrids (sunflower, oilseed rape, oil palm, castor). As oil content is mainly controlled by additive gene action, the transition from open-pollinated or pure line types of cultivars to hybrids such as in sunflower or oilseed rape did not affect oil content to a great extent. In oilseed rape, considerable heterosis is present in grain yield, whereas heterosis is not relevant for oil content (Qian et al. 2007). In soybean, a strictly autogamous species, heterosis in testcrosses was high for maturity date, plant height and grain yield, but low for oil and protein content (Lewers et al. 1998). Thus, the additional gain from hybrid oil crop cultivars is primarily due to higher grain yield and a subsequent oil yield increase rather than a higher oil content. In oil palm, contrary to annual oilseeds, not fully inbred parents are used to produce heterogeneous hybrids; a transition from seed to clonal propagation through in vitro culture techniques would allow for the multiplication of high-yielding individual palm trees thus enhancing oil yield per unit area (Soh et al. 2003).

Oil crop breeding is a complex undertaking, and selection for oil content needs to be considered in the context of various other traits and their biological and economic values. In most breeding populations, the correlation between grain yield and oil content is positive or not significant, whereas the correlation between grain yield and protein content is negative. Additionally, correlations between oil content and time to flowering, seed weight or fatty acid concentrations may also be of relevance, particularly in recurrent selection for oil content or in trait introgression programs. Oil content usually appears as quantitative character with medium to high heritability in most populations, which ensures a significant selection response.

On the technical level of selection, efficient analytical tools such as nuclear magnetic resonance (NMR) or near-infrared reflectance spectroscopy (NIRS) have been established for non-destructive measurement of oil content. In addition to the widely used NIRS-based prediction of oil, protein and moisture content, NIRS calibrations have been developed for measuring amino and fatty acids (Pazdernik et al. 1997; Velasco et al. 1999; Kovalenko et al. 2006) and antinutritional components such as glucosinolates (Font et al. 2006) or phenolics (Velasco et al. 1998). Moreover, NIRS procedures for analyzing single seeds for oil content or fatty acid concentration (Jiang et al. 2007; Tillman et al. 2006; Velasco et al. 1999, 2004) have

been presented. Thus, high-throughput technologies for screening large numbers of samples for oil content and related quality features on the basis of individual seeds, individual plants (e.g. Fasoula and Boerma 2005) or small plots such as hill or single-row plots (Pazdernik et al. 1996) are available for selection and quality monitoring at various stages of breeding programs.

1.4.2 Altered Seed Composition for Health and Industrial Applications

Besides altering the relative amounts of oil and protein in the seed of oil crops, considerable efforts have been made by oil crop breeders to alter the fatty acid profile of the oil. There are five major fatty acids found in most of the annual oilseeds, palmitic, C16:0; stearic, C18:0; oleic, 18:1; linoleic, C18:2; and linolenic acid, C18:3. Additional fatty acids are found in specific oil crops such as erucic acid C22:1 in oilseed rape or ricinoleic in castor bean. The manipulation of relative proportion of the fatty acids has often been practised to address issues of the healthfulness of oil by reducing the saturated fats (e.g. C16:0) or increasing the levels of monounsaturated fats such as C18:1. Mutagenesis, natural variation, recurrent selection and genetic engineering have been the approaches that have been used by oil crop researchers over the past several decades. It is noted that, although some of these EMS-derived mutations such as the low linolenic have been developed more than 30 years ago (Rajcan et al. 2005; Röbbelen and Nitsch 1975; Fehr 2007), many of them have gained in market importance only recently. The main interest in the low linolenic acid oils (with less than 3% of C18:3) is due to the lack of need for partial hydrogenation, which in high C18:3 oils results in the production of the by-product trans-fatty acids that have been shown as more detrimental to heart health than saturated fat. The low C18:3 oil only became economically attractive when the Food and Drug Administration regulated the compulsory food labelling of trans fat content in the USA, which in turn prompted considerable interest and market demand by food manufacturers. Similarly, while high oleic sunflower mutants have been available for a long time, the mid-oleic NuSun sunflower only gained market popularity when such hybrids developed by the USDA (Miller and Vick 2002) became readily available and a solid customer base had been generated. It would appear that healthier oils that have been developed as result of mutagenesis and selection work by oil crop breeders often have to wait until the market is ready for them, which sometimes political decisions rather than biological or health considerations. It is reasonable to expect that other fatty acid profiles that could address health issues, stability of oil or provide feedstock for specific food or industrial products will gain market acceptance after years of selection work by oil crop breeders. Some of the novel profiles that may be of interest in the future could be a high stearic oil for margarine production, low palmitic

combined with low linolenic for a healthier and more stable oil, high palmitic oil for biodiesel engines, high polyunsaturates for polyol and other biomaterials industries, etc.

Numerous clinical studies have been conducted that confirm the health benefits of non-oil nutritional components of oil crop seeds such as soybean protein (or peptides as part of it), or nutraceutical components such as glucosinolates of oilseed rape, lignan of linseed, isoflavones and saponins of soybean. The effects of these compounds include a reduction of heart and coronary disease, osteoporosis, certain types of cancer, e.g. prostate and breast cancer, and menopausal symptoms in women. These findings have opened a new avenue of research for plant breeders and geneticists in their efforts to widen the array of uses for oil crops and/or their products in support of the nutritional and nutraceutical industries. Recent efforts by plant breeders have concentrated around improving their understanding of the genetic control of the already recognized functional food components such as isoflavones (Primomo et al. 2005), tocopherols (Wohleser 2007) or saponins (Rupasinghe et al. 2003). The options for breeders and industry are plentiful and seemingly limited only by the type and variation of compounds in the seeds as well as market considerations. Faced with stagnating commodity prices, oil crop breeders are looking toward nutraceuticals, biobased industrial feedstocks and biofuels as means to alleviate low and fluctuating profitability in agriculture. The development of value-added oil crops and products for the current and emerging niche markets appears an attractive alternative for breeders and producers.

1.5 Perspectives in Oil Crop Breeding

1.5.1 Technology

On the level of technology, new tools are on the horizon which may affect strategies and efficiency of oil crop breeding in the near future.

Mutation induction is a well-established technique with special relevance to oilseed crops: At present, numerous mutants induced and isolated decades ago are being incorporated in widely grown cultivars to improve their fatty acid composition and nutritional value (Bhatia et al. 1999); while phenotyping large populations for a desired mutation is a major bottleneck in mutation breeding programs, new approaches such as TILLING combine conventional mutation induction and PCR-based high throughput reverse genetics mutant identification (Henikoff et al. 2004; Cooper et al. 2008); thus, numerous new alleles could be isolated at a given gene locus and phenotyped for their usefulness to breeding thereafter.

Although genetic markers are widely applied in oil crop breeding at present, their utilization could be further stimulated by the wider availability of high-throughput methods, SNP-markers and other techniques. Monitoring of

genetic diversity, prediction of hybrid performance, QTL analysis, marker assisted introgression and transfer of specific alleles, or marker assisted selection for traits difficult to phenotype such as disease/pest resistance, seed quality or health components would undoubtedly increase the efficiency of oil crop breeding programs.

Other biotechnologies such as doubled-haploids for rapid recovery of homozygous recombinants or micropropagation of superior planting materials in perennial crops such as the oil palm are well established techniques in particular applications. However, while doubled-haploids are widely used in cereals, oilseed rape and numerous other crops (Forster et al. 2007), species such as the soybean are considered recalcitrant to haploid production, and almost no research is devoted to that area despite its huge potential.

Oil crops are the earliest and major group of genetically modified (GM) crops grown world-wide: The total acreage of GM crops reached over 114 million hectares in 2007 (James 2007) with soybean, maize, cotton and canola being the lead crops, and herbicide tolerance and insect resistance being the major traits targeted. Despite of the benefits attributed to biotech crops, consumer acceptance of GM crops and products is low; wider evidence on crop biosafety and the development of 'second generation biotech crops' (Napier 2007) with new traits beneficial both to the environment and to consumers as well as farmers are considered crucial with respect to public opinion on GM crops. Meanwhile, separate breeding programs are maintained in parallel for GM and GM-free cultivar development in some crops to serve the different market requirements.

1.5.2 Biology

On the level of biology, issues such as maintenance of genetic diversity, the development of new oil crops, stability of quality features in crop harvests, or adaptation of oil crops to particular growing conditions such as organic farming may gain importance for future oil crop breeding.

Genetic diversity is comparatively low in most oil crops due to their specific crop and breeding histories. While this may not be a problem for immediate breeding work, it could negatively affect the long-term breeding progress, impede the adaptation to new environments or enhance crop vulnerability with respect to epidemic pests or diseases. Introgression of wild type alleles by backcrossing is efficient with respect to a specific target character such as the protein content of soybean (Sebolt et al. 2000), but will not increase overall genetic diversity. Wide crosses, recurrent programs, pre-breeding on a wild species level or hybridizations between wild and domesticated species for transferring complex characteristics such as the outstandingly high oil content found in some wild sunflower species (Seiler 2007) are long-term concepts for

increasing diversity while being challenged by the need to maintain an acceptable level of agronomic performance.

Instead of transferring various traits into few established oil crop species, development of new crops with specific qualities or the revival of forgotten and underutilized species represent alternative strategies with the long-term goal of enhancing agro-biodiversity at large. While various different species of that kind have been discussed during the past decades, few have appeared on the crop production and marketing level so far, and considerable research input may be needed for others.

1.5.3 Utilization

Oil crop utilization perspectives are most difficult to predict due to increasing market volatility of agricultural commodities in general and a high level of substitution among oils of different species.

Nevertheless, oil crop breeding for product quality features has been successful in generating diversified markets for vegetable oils. In food utilization of oils, diversification of products through breeding is constantly producing new features such as improved shelf life, suitability for frying, reduction of trans-fatty acid generation during hydrogenation, human blood cholesterol reducing properties, anti-oxidant and various other health promoting properties. In non-food utilization, demands from oleochemistry have prompted for the development of crops producing long- or mid-chain fatty acids as well as hydroxy and epoxy fatty acids, while many other properties may be realized by genetic engineering approaches (e.g. Dyer and Mullen 2005). Demands for energetic utilization of vegetable oils, e.g. for bio-diesel production, have been less specific in terms of oil quality so far, and the future position of bio-diesel production is unpredictable because of the rise of ethanol producing crops as well as high competition for land due to increasing world market prices for food and agricultural commodities. Apart from improving oil qualities itself, significant plant breeding efforts will also have to be devoted to the wide range of possible by-products of oil crop production such as protein meals for feeding or other industrial applications, as successful marketing of by-products has become a key factor for economic success of oil crop processing.

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Chapter 2
Modifying Vegetable Oils for Food and Non-food Purposes

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2.1 Introduction

Oils and fats are an important source of energy for the human diet and also contribute significantly to the sensory characteristics of food. Many oils are also used for non-food applications, although industrial use currently accounts for only a small proportion of the world vegetable oil production, less than 5% of total production, mostly for biodiesel. About 80% of edible oils are derived from plant sources and temperate annual oil seeds (soy, rapeseed, sunflower and peanut) account for about 60% of this total. Soybean oil is by far the dominant oil in this category, accounting for over half of the world vegetable oil production.

Improving the functional and nutritional qualities of vegetable oils has garnered much attention over the last 15 years or so. This chapter will describe some of the attempts to genetically improve plant seed oils, with special emphasis on soybean oil, for food and non-food uses.

2.2 Modulating the Fatty Acid Content of Plant Oils for Food Uses

2.2.1 Fatty Acid Profile and Oil Functionality

The fatty acid profile plays a significant role in both the nutritional properties and end-use functionality of edible plant oils. With its high percentage of polyunsaturated fatty acids (>65%), the major food oils derived from seeds are relatively oxidatively unstable, which limits their utility in food applications. Historically, oil processors have used partial hydrogenation as a means to improve upon the oxidative stability of plant oils. This process chemically shifts the fatty acid profile towards increased saturated and monounsaturated, and

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concomitantly reduces the polyunsaturated fatty acids. Partial hydrogenation also significantly improves solid fat functionality, which is especially useful for baking and similar applications. This improved functionality comes at a cost to nutritional quality due to the presence of high concentrations of *trans* fatty acids found in partially hydrogenated oils. *Trans* fatty acids have been correlated with cardiovascular disease (Mensink and Katan 1990; Korver and Katan 2006). Intake of other dietary fatty acids can have a strong influence on overall health. For example, saturated fatty acids such as palmitic acid, tend to raise LDL-cholesterol whereas stearic acid has neutral effect (Minihane and Harland 2007). Hence, the development of alternative vegetable oil feed stocks that can simultaneously improve functionality while maintaining nutritional quality holds great market potential to both the frying and baking industries.

Commodity soybean oil presently is composed of 14% saturated fatty acids, 18% monounsaturated fatty acid, and 65% polyunsaturated fatty acids. Through targeted genetic alterations, designer soybean oils are being developed to address both oxidative stability and nutritional quality. These include oils high in oleic acid and low in saturated and polyunsaturated fatty acids, oils elevated in stearic acid, and combinations of reduced polyunsaturate content with elevated stearic acid.

2.2.2 Conventional Approaches to Fatty Acid Modification

Oleic acid is metabolized to linoleic acid by a single desaturation step carried out by a Δ12-desaturase encoded by the *FAD2* gene (Heppard et al. 1996). In soybean there are at least six *FAD2* genes that fall into two classes, *FAD2-1* and *FAD2-2*. The *FAD2-1* class is primarily embryo-specific, while the *FAD2-2* class is generally constitutive, expressed during both vegetative and seed developmental stages (Tang et al. 2005). Soybean breeders have made great strides to move an elevated oleic acid phenotype into elite genotypes by exploiting natural variation in oleic acid levels among various sources of soybean germplasm (Takagi and Rahman 1996; Rahman et al. 2001; Alt et al. 2005a,b). Conventional approaches to raise the oleic acid content in soybean oil has led to the development of "mid-oleic" phenotype, in which seed storage lipids range in oleic acid from 30 to 70%. The conventional approach to develop the mid-oleic phenotype has some drawbacks. First, the genetics of the phenotypes require the stacking of multiple loci (Alt et al. 2005a, b), which may complicate the breeding process. Secondly, the "mid-oleic" phenotype is affected by environment, typically requiring growth in warmer climates for stability of the elevated oleic acid trait to be maintained. This is due to the temperature effect on the desaturase activity and expression (Heppard et al. 1996; Tang et al. 2005). The third drawback associated with the conventional breeding approach for a "mid-oleic" soybean is the germplasm that expresses this phenotype is associated with yield drag (Primomo et al. 2002).

In soybean seed the content of palmitate is regulated by a palmitoyl thioesterase encoded by *FatB* genes (Kinney 1997). Low palmitic acid soybean genotypes have been reported (Bubeck et al. 1989; Primomo et al. 2002). The low palmitic acid phenotype in soybean has recently been associated with allelic variation in *FatB* (Cardinal et al. 2007). Importantly, as the conventionally bred "mid-oleic" soybean, the low palmitic acid genotypes seem to suffer from yield penalty (Rebetzke et al. 1998; Cardinal and Burton 2007).

The concentration of the cardiovascular "neutral" saturated fatty acid, stearate, in soybean is controlled by both desaturase (Cheesbrough 1990) and thioesterase activities (Pantalone et al. 2002). The genetic locus controlling elevated stearic acid levels in soybean, is designated *Fas*, and allelic variation at this locus manifests stearic acid levels ranging from wild type concentrations of 3%, up to high stearate germplasm with 35% (Bubeck et al. 1989; Rahman et al. 1995; Pantalone et al. 2002).

Significant amount of progress has been made in developing novel soybean germplasm, with altered fatty acid profiles, using conventional breeding strategies. However, as mentioned above there tends to be a yield drag associated with these improved oil traits. This may be due to the allelic variants selected for altered fatty acid profile during both vegetative and embryogenesis development, thereby increasing the probability of a negative agronomic effect associated with the mutant allele governing the novel oil phenotype. Moreover, while soybean output traits, such as "mid-oleic", low palmitic acid, and elevated stearate have value, there are applications in which stacking of these traits would offer expanded functionality. Given the genetic complexity regulating the "mid-oleic" trait in soybean, combining this phenotype with low palmitic and/or elevated stearic acid traits, into elite soybean genotypes, would be challenging. High oleic acid mutants, with an oleic content ranging from 60 to 90%, have also been developed in corn, peanut, canola and sunflower. These mutants all have defective *FAD2* genes (Perez-Vich et al. 2002; Patel et al. 2004; Hu et al. 2006; Belo et al. 2008). As in soybean, many of these plants have a number of *FAD2* genes all which contribute to seed linoleic acid content (Tang et al. 2005), which means that the development of mutant lines with useful oleic acid contents often requires combining several mutant loci (Mikkilineni and Rocheford 2003).

2.2.3 *Novel Fatty Acid Profiles in Soybean Derived from the Tools of Biotechnology*

Implementing the tools of biotechnology novel oil traits can be achieved in a seed-specific fashion, with a single dominant allele. This approach simplifies breeding and reduces the probability of agronomic performance being compromised.

Targeted perturbation of *FAD2* alleles in a seed-specific fashion in soybean has been shown to produce a high oleic acid (75–85%) phenotype in the seed oil (Kinney and Knowlton 1997; Mazur et al. 1999). Modulation of *FAD2* expression using this transgenic approach was carried out by introducing transgenic elements designed to induce post-transcriptional gene silencing (Cerutti 2003).

High oleic acid soybean derived from down-regulating *FAD2*, concomitantly reduces polyunsaturated fatty acids to below 6% (Kinney and Knowlton 1997), in addition palmitic acid was reduced to approximately 7–8% (a 20% reduction in palmitate).

For some uses, such as salad oils, it is desirable to reduce saturated fatty acids, especially palmitic acid as much as possible. It has been shown that the *FATB* class of acyl:ACP thioesterases control the release of some saturated fatty acids, including palmitic, into the cytoplasm, making them available for oil biosynthesis (Dörmann et al. 2000). Seed-specific silencing of *FATB* genes in soybean leads to a major reduction in total saturated fatty acids, from about 15% to less than 6% (Kinney 1996). When this gene was combined with a silenced *FAD2*, oleic acid contents of over 90% were observed (Buhr et al. 2002). Importantly, field trials with soybean having this novel oil phenotype, conducted across multiple environments, revealed that the fatty acid content was not affected by temperature and the agronomic performance was not compromised.

For certain food applications, such as baking, solid fat functionality is needed. This functionality is currently provided by animal fats, palm oil and hydrogenated vegetables oils, and biotechnology has the potential to provide a healthy alternative to these fats. It is thought that oils rich in stearic acid could provide this important function without compromising human health because, unlike other saturated fatty acids, stearic is not considered to play a significant role in cardiovascular disease (Mensink 2005; DiRienzo et al. 2008).

High stearic acid vegetable oil has been developed by targeting either down-regulation of desaturase activity (Liu et al. 2002) or heterologous expression of a stearoyl-ACP thioesterase (Hawkins and Kridl 2002). Soybeans seeds have several Δ9 desaturase (SAD) genes expressed in their seeds and silencing one of these genes (*SAD3*) in a seed-specific manner resulted in soybean oils containing 20–30% stearic acid when compared with just a few percent in commodity soybean (Booth et al. 2006). The seeds of mangosteen (*Garcinia mangostana*) contain stearate as the predominant fatty acid (45–50% of total fatty acids), a result of a stearate-specific thioesterase. When the mangosteen gene was expressed in transgenic canola seeds, stearate contents of 20–30% were reported (Hawkins and Kridl 1998). Similar increases in stearate have been observed when this gene was expressed in soybean seeds (Kridl 2002).

To replace the solid-fat functionality of many hydrogenated oils the stearate needs to be a major component of the seed oil, around 30% of the total fatty acids (Lumor et al. 2007) and germination problems are often seen when the stearic acid content of seeds is elevated to these levels (Roberts et al. 2006; Clemente

unpublished data). However, by combining the silencing of *SAD3* with silencing of *FAD2* in soybean it has been possible to make viable seeds that have an oleic content in the 50–60% range with stearic around 15–20% (Booth et al. 2002). This combination of stearic and oleic confers the desired solid fat functionality without compromising the seed. Improved functionality has also been obtained by combining increased stearic acid with low linolenic acid oils (DiRienzo et al. 2008). Healthy oils from these types of seeds have the potential to replace solid fats in a wide range of baking and heavy-duty frying applications.

The introduction of modified seeds oils such as high oleic acid soybean oil and high oleic/high stearic oils will go a long way to removing the undesirable saturated fatty acids and *trans* fatty acids from the human diet and help promote cardiovascular health (Korver and Katan 2006; Lichtenstein et al. 2006; Mozaffarian and Willett 2007).

2.3 Next Generation Edible Oils: Producing Long Chain ω-3 Fatty Acids in Seed Oils

2.3.1 Engineering Complex Pathways into Plant Seeds

The efforts toward genetic engineering plants to alter seed oil compositions described above focused on modifying existing biosynthetic pathways at a single enzyme step. With advancements in plant transformation and genomic technologies, expression of significantly more complex gene combinations, including whole metabolic pathways from heterologous sources, are now being explored and thus, the flexibility to produce highly functionalized, higher-value metabolic end-products is also becoming possible. A good example of valuable but metabolically complex end products that are of current significant interest are the long-chain polyunsaturated fatty acids (LCPUFA) including the ω-3 LCPUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The importance of these fatty acids in human health and nutrition and current efforts to produce them in oil seed crops will be discussed.

EPA is a 20 carbon fatty acid having 5 double bonds while DHA is a 22 carbon fatty acid having 6 double bonds. Both are considered ω-3 fatty acids in that they have a double bond occurring between the third and fourth carbon from the methyl end. Arachidonic acid (ARA) is similar to EPA in that it is a 20 carbon fatty acid but lacks the ω-3 double bond and the double bond closest to the methyl end of the fatty acid occurs between the sixth and seventh carbon, thus making ARA an ω-6 fatty acid. Most naturally occurring fatty acids, and all that are relevant to this discussion, have double bonds having a *cis* configuration. The chemical structure of the fatty acid (i.e. chain length; number, position and stereochemistry of double bonds) is directly related to its effect on human physiology including potential health properties and a list of the fatty acids relevant to this discussion along with their chemical structures is shown in Table 2.1

Table 2.1 Nomenclature and chemical structures of fatty acids

Saturation	Common name	Abbreviation	Carboxyl nomenclature	ω nomenclature
Saturated	myristic	MA	14:0	14:0
	palmitic	PA	16:0	16:0
	stearic	SA	18:0	18:0
Monounsaturated	oleic	OA	18:1(Δ9)	18:1 ω-9
	eicosanoic	EA	20:1(Δ11)	20:1 ω-9
Polyunsaturated	taxoleic	TXA	18:2(Δ5,9)	18:2 ω-9
	linoleic	LA	18:2(Δ9,12)	18:2 ω-6
	γ-linolenic	GLA	18:3(Δ6,9,12)	18:3 ω-6
	pinolenic	PNA	18:3(Δ5,9,12)	18:3b ω-6
	α-linolenic	ALA	18:3(Δ9,12,15)	18:3 ω-3
	stearidonic	STA	18:4(Δ6,9,12,15)	18:4 ω-3
	eicosadienoic	EDA	20:2(Δ11,14)	20:2 ω-6
	dihomo-γ-linolenic	DGLA	20:3(Δ8,11,14)	20:3 ω-6
	sciadonic	SCI	20:3(Δ5,11,14)	20:3b ω-6
	eicosatrienoic	ERA	20:3(Δ11,14,17)	20:3 ω-3
	arachidonic	ARA	20:4(Δ5,8,11,14)	20:4 ω-6
	eicosa-tetraenoic	ETA	20:4(Δ8,11,14,17)	20:4 ω-3
	juniperonic	JUP	20:4(Δ5,11,14,17)	20:4b ω-3
	eicosa-pentaenoic	EPA	20:5(Δ5,8,11,14,17)	20:5 ω-3
	docosa-tetraenoic	DTA	22:4(Δ7,10,13,16)	22:4 ω-6
	docosa-pentaenoic	DPAn-6	22:5(Δ4,7,10,13,16)	22:5 ω-6
	docosa-pentaenoic	DPA	22:5(Δ7,10,13,16,19)	22:5 ω-3
	docosa-hexaenoic	DHA	22:6(Δ4,7,10,13,16,19)	22:6 ω-3
	tetracosa-pentaenoic	TPA	24:5(Δ9,12,15,18,21)	24:5 ω-3
	tetracosa-hexaenoic	THA	24:6(Δ6,9,12,15,18,21)	24:6 ω-3

Although the effects of fatty acids on human health may be variable in different human populations depending on age, sex, race and genetic background, studies have shown that consumption of fats high in short-chain, saturated fatty acids leads to increases in low-density lipoprotein (LDL) and may lead to increased risk of cardiovascular disease (Schaefer 1997). Stearic acid (SA), an 18 carbon saturated fatty acid, is neutral in its effect on blood lipids (Kris-Etherton et al. 2005; Davis and Kris-Etherton 2003; Schaefer 1997) and monounsaturated and polyunsaturated fatty acids are inversely correlated with coronary heart disease (Binkoski et al. 2005;Davis and Kris-Etherton 2003; Kris-Etherton 1999). Given these trends, reduced consumption

of short-chain, saturated fat and increased consumption of foods rich in mono- and polyunsaturated fats and oils should lead to an overall healthier state.

Vegetable oils such as soybean and canola oils are rich sources of polyunsaturated fatty acids (ω-3 and ω-6). The polyunsaturated fatty acids linoleic acid (LA) and α-linolenic acid (ALA) commonly found in many oilseeds are not only considered healthy but are, in fact, essential fatty acids since humans lack the enzymes necessary to produce them. Humans are biosynthetically capable of producing oleic acid (OA) de novo from glucose or other basic carbon sources but are incapable of further desaturating OA to LA, and LA to ALA as they are missing the Δ12 and Δ15 desaturase (ω-3 desaturase) enzymes, respectively. Oilseed plants have Δ12 and Δ15 desaturases which act on phospholipid-bound OA. In the body, LA and ALA are further converted to ARA and EPA, respectively and EPA is a precursor to DHA (Sprecher 2000).

The eicosanoid family of metabolites which include prostaglandins, leukotrienes and thromboxanes (Funk 2001; Smith 2005) are formed directly from ARA and EPA. These molecules are key control points for metabolic processes such as inflammatory responses, blood clot induction and regulation of blood pressure (Yaqoob 2003). Inflammatory response is regulated by the balance of these types of eicosanoids with ARA- and EPA-derived eicosanoids producing a pro- and anti-inflammatory response, respectively (Calder 2003; Simopoulos 2006; Smith 2005). When EPA is not consumed directly in the diet, the ratio of LA to ALA consumed has a direct affect on the concentration of ARA and EPA because humans lack an ω-3 desaturase that would convert LA to ALA or ARA to EPA. Therefore, the essential need for LA and ALA in the diet is in part due to their importance as intermediates of LCPUFAs and eicosanoid biosynthesis, which are crucial for normal human physiology.

Although inflammatory responses are critical in many human defense and healing mechanisms, prolonged induction of an inflammatory state could lead to health problems. In Western society, consumption of foods rich in LA, such as vegetable oils or grain-fed meat and poultry, has increased substantially over the past 60 years (Hibbeln et al. 2004). This increase combined with increased consumption of hydrogenated fats where ω-3 fatty acids have been selectively removed, has resulted in a substantial shift in the balance of ARA and EPA in the blood stream with numerous potential negative consequences (Hibbeln et al. 2004; Simopoulos 1999; Wada et al. 2007) including those disease states related to excessive inflammation or auto-immune conditions. For example, inflammatory bowel disease has been linked to increased concentrations of ARA (Ramakers et al. 2007).

Consumption of EPA and/or DHA has also been shown to be beneficial in other areas of human health such as cardiovascular disease and mental health (Ruxton et al. 2007). Benefits of EPA/DHA in decreasing death rates after a

myocardial infarction have been described while consumption of ALA is not as effective (Breslow 2006; von Schacky and Harris 2007). EPA and DHA are protective at doses <1 g/d due to suppression of fatal arrhythmias and at doses >3 g/d, EPA plus DHA can improve cardiovascular disease risk factors, including decreasing plasma triacylglycerides, blood pressure, platelet aggregation, and inflammation, while improving vascular reactivity (Breslow 2006; von Schacky and Harris 2007). Low levels of EPA and/or DHA have been linked to higher instances of depression and increased consumption of EPA and/or DHA can attenuate symptoms of depression and bipolar disorder (Sontrop and Campbell 2006). DHA is an important component of cell membrane phospholipids (Horrocks and Farooqui 2004). Mammalian retinal and brain membranes are enriched in DHA and it is important for the cognitive development of infants (Fleith and Clandinin 2005; Iribarren et al. 2004; Stoll et al. 2001; Willatts and Forsyth 2000). The use of DHA in treating brain-related diseases such as Alzheimer's disease have also been suggested (Oksman et al. 2006).

The best direct source of EPA and DHA in our diet comes from the consumption of marine fish or their oils. In fact, the American Heart Association has recommended at least two weekly servings of fish as part of a heart-healthy diet. Many consumers are also complementing diet with fish oil supplements. But, while increasing consumer awareness towards the health benefits of fish oil, ω-3 fatty acids, EPA and DHA is generally good from a health perspective, the increasing demand for fish and fish oil products is placing further stress on limiting fish populations, many of which are already in jeopardy (Pauly et al. 2002). Additionally, many fish species and resulting fish oil derived from them are tainted with contaminants and some consumption restriction recommendations have been made (Mozaffarian and Rimm 2006). In fact, the main source of LCPUFA in fish originates in marine microorganisms such as diatoms, golden-brown algae, green algae, blue-green algae, microbial fungi and dinoflagellates, all of which are rich in LCPUFA (Radwan 1991; Shaw 1966). These organisms are ingested by small fish which in turn are eaten by larger fish and other animals. Sustainable alternatives to fish oils where LCPUFAs are produced through fermentation of microalgae are free from the contaminants found in fish oils but their widespread use is limited to high value applications such as infant formula and medical foods (Boswell et al. 1996) due to their high cost of manufacture.

2.3.2 LCPUFA Production in Plants

In nature, there exist two main biochemical pathways to ARA, EPA and DHA. These are the aerobic desaturase/elongase-type pathway and the anaerobic polyketide synthase (PKS) pathway (Sperling et al. 2003; Bentley and Bennett 1999). The aerobic desaturase/elongase-type pathway can further be divided into two classes based on whether the first step of the pathway is elongation or desaturation.

2.3.3 EPA Production in Plants via the Δ6 Desaturase Pathway

In animals, including fish and humans, and the majority of marine micro-organisms studied thus far, EPA is generated by a Δ6 desaturase pathway (Sayanova and Napier 2004) where a double bond is first added to ALA by a Δ6 desaturase to form stearidonic acid (STA), followed by elongation of STA to eicosatetraenoic acid (ETA) catalyzed by a Δ6 fatty acid elongase and lastly the formation of another double bond in ETA by a Δ5 desaturase to form EPA. Some micro-organisms that produce ARA do so by an analogous pathway as for EPA but the desaturases and elongases utilize the ω-6 forms of these substrates when they are present. Therefore, LA can be converted to γ-linolenic acid (GLA) by the Δ6 desaturases, GLA can be converted to dihomo-γ-linolenic acid (DGLA) by the elongases and DGLA can be converted to ARA by the Δ5 desaturases. The substrate preference for either ω-6 or ω-3 substrates varies depending on the species from which the enzymes are derived and some species produce both ω-6 and ω-3 fatty acids (Sayanova et al. 2006; Sayanova and Napier 2004). In addition, some organisms can convert ω-6 fatty acids (C18 and/or C20) to ω-3 fatty acids through the action of an ω-3 fatty acid desaturase (Damude et al. 2006; Oura and Kajiwara 2004; Pereira et al. 2004b; Sakuradani et al. 2005; Spychalla et al. 1997). The ratio of products produced in any given pathway will, therefore, be a function of both the substrate specificity of the enzymes used and the concentrations of either ω-3 or ω-6 substrates available in the host.

Most oilseed plants that produce polyunsaturated fatty acids also produce a very high concentration of LA, which results in a ratio of ARA to EPA that is too high when converted in the body. One exception to this is linseed oil, which has ALA concentrations of approximately 50–60% of the total fatty acids. A transgenic approach to make higher levels of ALA in soybean by expressing a bifunctional Δ12/Δ15 desaturase from *Fusarium moniliforme* resulted in ALA concentrations as high as 72% in seed (Damude et al. 2006). In a similar attempt to make pork healthier, transgenic pigs were produced, which expressed a "humanized" *Caenorhabditis elegans* ω-3 desaturase (Lai et al. 2006) and this resulted in pork fat with higher concentrations of ALA.

The first step of the Δ6 desaturase pathway is the conversion of ALA to stearidonic acid (STA). In humans, the Δ6 desaturase is rate-limiting (Burdge et al. 2002; James et al. 2003). Thus, even when ALA concentrations in the diet are high, ALA is poorly converted to EPA in healthy subjects (James et al. 2003; Miles et al. 2004). Further, Δ6 activity has been shown to decline with age (Bourre et al. 1990).

By-passing the ineffective Δ6 desaturase by consuming oils rich in STA directly has been proposed as an indirect way of more effectively obtaining needed levels of EPA and DHA. Some plants such as hemp, borage, black

currant and evening primrose express a Δ6 desaturase and produce GLA and STA in their seed oils naturally, and these oils are currently marketed and sold for their proposed health benefits (Barre 2001). But, their cultivation is carried out on a scale that is relatively small and yields are poor making the oils produced expensive.

Producing STA in commercial oilseed crops has also been proposed and requires the minimal expression of a single Δ6 desaturase. The synthesis of GLA and STA in a plant (tobacco) was first demonstrated using the Δ6 desaturase from *Synechocystis* expressed constitutively under control of the 35S promoter (Reddy and Thomas 1996). Subsequent expression of other Δ6 desaturases in tobacco, *Brassica juncea* and soybean further improved yields of both GLA and STA (Sayanova et al. 1997; Hong et al. 2002; Qiu et al. 2002; Sato et al. 2004). STA production was further optimized in soybean using seed-specific expression of the borage Δ6 desaturase and the *Arabidopsis* Δ15 desaturase giving STA contents of as high as 30% (Eckert et al. 2006). Both GLA and STA production in oilseeds are under active commercial development with reports of GLA synthesis in safflower yielding up to 73% GLA (Knauf et al. 2006) and STA synthesis in soybean giving greater than 20% STA with about 5–6% GLA (Wilkes 2007).

Producing GLA and STA in the seeds of commercial oilseed plants are significant achievements in their own right, but STA still needs to be consumed in larger amounts to have the same efficacy as EPA (Miles et al. 2004). In addition STA does not appear to be converted by the body to DHA, an n-3 LCPUFA that is very important for cognitive function (James et al. 2003). Thus, direct consumption of EPA and DHA in the diet remains preferred and requires the expression of the remaining pathway genes in plants.

In the Δ6 pathway to EPA, STA is elongated by two carbons to ETA via a microsomal fatty acid elongation complex (Metz et al. 2001), which is similar to that in the plastid but which uses acyl-CoA substrates instead of acyl-ACP substrates. With the exception of a recently characterized elongase from the marine parasitic protozoon *Perkinus marinus*, all of the elongases that are involved in LCPUFA biosynthetic pathways are of the ELO/SUR4 gene family (Venegas-Caleron et al. 2007). Of the remaining proteins involved in elongation, putative beta-ketoacyl-CoA reductases (Beaudoin et al. 2002; Han et al. 2002) and enoyl-CoA reductases (Gable et al. 2004; Paul et al. 2007) have been suggested for yeast and plants. Recently, the PHS*1* gene has been suggested to be responsible for the dehydration reaction in yeast (Denic and Weissman 2007). Further desaturation of ETA by a Δ5 desaturase generates the final EPA product.

Production of EPA in a plant using the Δ6 desaturase pathway was first demonstrated in soybean (Kinney et al. 2004) and is, to date, the highest abundance of EPA achieved in any plant tissue. In the study, a Δ6 desaturase, elongase and Δ5 desaturase gene from *Mortierella alpina* were used along with an *Arabidopsis* fad3 gene (Yadav et al. 1993) and a

S. diclina Δ17 desaturase (Pereira et al. 2004b) and each were expressed under the control of different, strong, seed-specific promoters. EPA contents in embryos as high as 13% in embryos and 20% in seed were achieved with little to no ARA produced due mainly to the presence of the highly efficient Δ17 desaturase used. The ω-3 to ω-6 fatty acid ratio increased from 0.2:1 (the normal soybean ratio) to 1.5:1 (a ratio close to that of many fish oils) and overall elongation was 65% suggesting a highly efficient transfer to CoA pools for subsequent elongation. Unexpectedly, docoaspentaenoic acid (DPA), DHA precursor, was also found in the high EPA lines as abundant as 4%. DPA resulted from the additional activity of the *M. alpina* Δ6 elongase towards the Δ5 fatty acid EPA. This same elongase had almost no Δ5 EPA-elongating activity when expressed in yeast (Parker-Barnes et al. 2000). In the same study, some events where the *S. diclina* Δ17 desaturase was not functioning contained ARA concentrations as high as 26% in seed were obtained (Damude unpublished data).

ω-3 to ω-6 ratios were further improved when the *Arabidopsis* ω-3 desaturase was replaced with a novel, bifunctional Δ12/Δ15 desaturase from *Fusarium moniliforme* (Damude and Yadav 2005; Damude et al. 2006). The *Fusarium* Δ15 desaturase had broad substrate specificity for numerous ω-6 fatty acids including LA>GLA>DGLA>ARA (Damude et al. 2006), and when co-expressed with the LCPUA pathway, led to an overall ω-3 content as high as 57% in soybean embryos with up to 16% EPA.

Interestingly, a recent similar attempt at producing ARA using the Δ6 desaturase pathway in soybean (Chen et al. 2006) resulted in low concentrations of ARA (2.1% of total lipids) in embryos and even lower concentrations (0.5–0.8%) in seed. In this approach, a *FAD3* down-regulation cassette was combined with the *Mortierella alpina* Δ6 desaturase, elongase and Δ5 desaturase genes, under control of the strong seed-specific β-conglycinin promoter. Use of the same promoter multiple times was suggested to cause poor expression of the genes and led to low ARA concentrations.

The initial demonstration of EPA in soybeans using a Δ6 desaturase pathway (Kinney et al. 2004) was followed closely by another report (Abbadi et al. 2004) where ARA and EPA were produced (less than 2% total) in tobacco and flax seeds using genes from the diatom *Phaeodactylum tricornutum* and the moss *Physcomitrella patens* (Abbadi et al. 2004). In this study, the EPA pathway intermediates GLA and STA predominated at approximately 30% with only low concentrations of elongated fatty acids such as DGLA, ETA, ARA and EPA. Results indicated the elongation step was severely limited (10% total elongation) in both tobacco and flax. The elongation bottleneck was shown to be the result of low incorporation of the substrates for elongation, GLA and STA, into the acyl-CoA pools from the phospholipid pools from where they were produced. This poor exchange resulted in the incorporation of GLA directly into triglyceride mostly through the direct conversion of phosphatidylcholine (PC) containing

GLA to DAG followed by acylation to TAG. A similar direct incorporation of phospholipid backbones containing the unusual fatty acid eleostearic acid directly into TAG was seen when a *Momordica charantia* fatty acid conjugase was expressed in *Arabidopsis* (Cahoon et al. 2006).

In another study, a full complement of Δ6 desaturase pathway genes has been expressed in *Brassicca juncea* (Wu et al. 2005) and inclusion of a Δ17 desaturase significantly increased EPA production (up to 15%) with a substantial decrease in ARA. In all of these studies described above, the Δ6 and Δ5 desaturases included utilized acyl-phosholipid substrates and in some cases transfer of the acyl groups to acyl-CoA pools was shown to be limiting. One other Δ6 desaturase pathway study in *Arabidopsis* seed used a Δ6/Δ5 desaturase from a species of zebrafish (*Danio rerio*) which utilizes acyl-CoA substrates along with a *Caenorhabditis elegans* elongase (Robert et al. 2005). It was thought that a complete pathway with preference for acyl-CoA substrates would improve flux since reduced exchange between acyl-CoA and phospholipid pools would be required. Using this approach, an EPA content of only 2.5% was achieved.

2.3.4 EPA Production in Plants via the Δ9 Elongase Pathway

Species from the *Prymnesiophyceae* (e.g. *Pavlova*, *Isochrysis*) *Acanthamoebidae* (e g. Acanthamoeba) and *Euglenophyceae* (e.g. *Euglena*) families of LCPUFA-producing microorganisms, have been shown to use a slightly different aerobic pathway to produce EPA (Sayanova and Napier 2004). In this alternate Δ9 elongase pathway, ALA is first elongated by a Δ9-specific elongase to eicosatrienoic acid (ERA), followed by Δ8 desaturation to ETA. As in the more common Δ6 pathway, these fatty acids are then desaturated to EPA respectively by a Δ5 desaturase.

The earliest published report of synthesis of EPA in plants described expression of this alternate Δ9 elongase pathway in *Arabidopsis* leaf tissue. A Δ9 elongase from the microalgae *Isochrysis galbana*, a Δ8 desatuarase from the *Euglenophyceae*, *Euglena gracilis*, and a Δ5 desaturase from the microbial fungus *Mortierella alpina* were expressed constitutively in the model plant *Arabidopsis* (Qi et al. 2004). EPA contents as high as 3.0% with ARA contents up to 6.6% were achieved in *Arabidopsis* leaves with ratios of ω-3 to ω-6 ratio fatty acids (2.2:1) similar to that commonly found in fish oils (Sargent 1997). Total elongation of fatty acids was good at 36% demonstrating a relatively high availability of LA and ALA in the acyl-CoA pool of *Arabidopsis* leaves. It was suggested that the bottleneck of acyl-transfer between acyl-CoA and phospholipid pools was relieved by placing the acyl-CoA-requiring elongation step first in the pathway followed by two acyl-phospholipid-requiring desaturation steps.

In a case of premature desaturation, the fatty acid by-products sciadonic acid (SCI) and juniperonic acid (JUP) were also formed in this Δ9 elongase pathway. This resulted from the direct action of the Δ5 desaturase on EDA and ERA before the Δ8 desaturation step. Interestingly, the species from which the Δ9 elongase and Δ8 desaturase were taken do not produce significant levels of these fatty acid by-products suggesting different specificities of the host Δ5 desaturase or more efficient flux through the pathway.

Some plant gymnosperm species (e.g. *Pinaceae* family) naturally produce seeds with twenty carbon fatty acids having Δ5 double bonds such as sciadonic acid (SCI) and juniperonic acid (JUP), suggesting the presence of C20 elongases and Δ5 desaturases (Wolff et al. 2000).

2.3.5 DHA Production in Plants via the Aerobic Elongation/ Desaturation Pathways

In animals, generation of DHA from EPA occurs by the Sprecher pathway whereby EPA is elongated twice by the fatty elongation complex (Sprecher et al. 1999) and the first enzyme of elongation is specific for the co-acylated form of EPA. The 24 carbon fatty acid tetracosapentaenoic acid (TPA) generated by elongation is then desaturated by the rate-limiting Δ6 desaturase to produce tetracosahexaenoic acid (THA). Chain reduction by 2 carbons via beta-oxidation forms DHA (Wallis et al. 2002).

In microorganisms, DHA is synthesized by a much simpler pathway from EPA where initial elongation to docosapentaenoic acid (DPA) by the fatty acid elongation complex is followed by a Δ4 desaturase to directly produce DHA without the need for further elongation, Δ6 desaturation or beta-oxidation (Sayanova and Napier 2004).

The first successful production of DHA in plants was carried out in soybean (Kinney et al. 2004). The Δ5 elongase from *Pavlova* sp. (Pereira et al. 2004a) and the Δ4 desaturase from *Schizochytrium aggregatum* (Mukerji et al. 2002) was added alongside the Δ6 desaturase EPA biosynthetic pathway, and soybean somatic embryo oil with up to 3.3% DHA was achieved. To this date this continues to be the highest concentration of DHA made in any plant. It was difficult to separate whether the additional *Pavlova* elongase or the dual specificity of the *M. alpina* Δ6 elongase for Δ5 substrates contributed to the elongation of EPA but given the high levels of DPA achieved with only the *M. alpina* elongase, it is most likely the latter. The *S. aggregatum* Δ4 desaturase used was highly active in plants with, in some cases, close to 100% conversion of DPA to DHA.

Subsequently, DHA has been produced in other plants including up to 1.5% in *Brassica* seed by combining EPA biosynthetic genes (Δ6 desaturase pathway) with the Δ4 desaturase from *Thraustochytrium* sp., and an elongase from the fish *Oncorhynchus mykiss* (Wu et al. 2005) and up to 0.5% in

Arabidopsis seeds by re-transforming an *Arabidopsis* plant making EPA with a Δ5 elongase and a Δ4 desaturase from *Pavlova salina* (Robert et al. 2005).

2.3.6 DHA Production in Plants via the Anaerobic Polyketide Synthase Pathway

Many marine microbes synthesize EPA or DHA using the anaerobic polyketide synthase pathway (Metz et al. 2001), which are similar to the fatty acid synthase complex in plants. These enzymes can be formed from multiple large proteins which contain many multi-domained subunits and where each domain carries out a different chemistry. Double bonds are produced in the growing fatty acid chain by a dehydrase-isomerase mechanism similar to *FabA* in *E. coli* and do not require oxygen as do fatty acid desaturases (Metz et al. 2001). Interestingly, in some organisms both a PKS and a fatty acid synthase pathway for EPA or DHA synthesis may be present. For example, a complete PKS-type DHA-synthase has been cloned and characterized from a number of *Thraustochytid* species, as have various fatty acid desaturases and elongases (Metz et al. 2004; Qiu et al. 2001).

Successful expression of PKS genes in yeast and plants has been reported (Metz et al. 2006, 2007). Genes encoding the three subunits (ORFA, B, C) of a *Schizochytrium* PKS were co-expressed with a phosphopantetheinyl transferase (PPT) from *Nostoc*, essential for activating the ACP domains of the DHA-synthase PKS individually under control of the linin seed-specific promoter from flax. Expression in the plastid was achieved by fusion with a *Brassica* acyl-ACP thioesterase plastid targeting signal. *Arabidopsis* seeds were obtained having up to 0.8% DHA with an additional 1.7% DPAn-6, which is also observed in *Schizochytrium*. Further increases in DHA content in *Arabidopsis* seed were made by co-expressing a PKS with either an acyl-CoA synthetase (ACSI or ACSII) from *Schizochytrium*, or RNAi constructs for down-regulation of the *Arabidopsis* KASII or KASIII genes. Analysis of the fatty acid profiles of single seed expressing the PKS, PPT and the KASIII RNAi construct showed levels of DHA and DPAn-6 as high as 2.4 and 1.8%, respectively.

2.4 Modifying Vegetable Oils for Non-food Purposes

2.4.1 Non-food Uses of Plant Oils

Consumption of soybean oil for industrial uses has undergone a dramatic increase between 2003 and 2006. During this period, the use of soybean oil for industrial applications increased from 552 million pounds to 2379 million pounds in the United States (SoyStats^{TM} 2007). Currently, 12% of U.S.

soybean oil consumption is directed to industrial uses. The large majority of this increase has resulted from demand for soybean oil for biodiesel production. Given the rising petroleum prices, it is likely that this demand will increase into the foreseeable future, not only for biodiesel but for the production of industrial materials such as lubricants, paints, and plastics that have historically been derived from crude oil.

2.4.2 High Oleic Acid Soybean Oil

Nearly all of the soybean oil that is now used for industrial applications is conventional soybean oil that lacks any genetic modification of its fatty acid composition. However, through the use of breeding and biotechnology, it is possible to generate fatty acid profiles that improve the functionality of soybean oil for industrial uses, including biodiesel. Soybeans with increased oleic acid content have received the most attention for use in a variety of industrial applications (Kinney and Clemente 2005). Enhancement of oleic acid content can be achieved by breeding different mutant alleles for Δ12 oleic acid desaturase (*FAD2*) genes (Burton et al. 2004). Typical FAD2 enzymes convert oleic acid to linoleic acid (Okuley et al. 1994). This enzyme uses an oleic acid molecule principally to phosphatidylcholine (PC) as its substrate. By mutating or suppressing the expression of *FAD2* genes, soybean seeds accumulate oleic acid, rather than linoleic acid, as the major component of the seed oil. The largest increases in oleic acid have been obtained through a biotechnological approach by suppression of the *FAD2-1* gene in combination with down regulation of palmitoyl-acyl carrier protein thioesterase (*FatB*) genes (Buhr et al. 2002). Through the use of this approach, high oleic (HO) acid oils containing as much as 90% oleic acid have been achieved. The suppression of *FatB* expression also reduced the palmitic acid content of these oils to <4% of the total fatty acids (Buhr et al. 2002), resulting in oils with less saturated fatty acids.

HO soybean oils are not only enriched in oleic acid but also have reduced amounts of the polyunsaturated fatty acids linoleic and linolenic acids. For example, HO oils with as much as 90% oleic acid have <4% each of polyunsaturated fatty acids and palmitic acid (Buhr et al. 2002). It is likely that most of the remaining polyunsaturated fatty acids can be removed by breeding into 1% linolenic acid soybean mutant lines (Ross et al. 2000). The combination of high oleic acid and low polyunsaturated fatty acid content results in a liquid oil with greatly improved oxidative stability. HO soybean oil with 85% oleic acid, for example, has an oxidative stability index value that is nearly 12-fold higher than that of conventional soybean oil (Knowlton 1999). This property is critical for the use of vegetable oils in lubricants, including motor and hydraulic oils (Erhan et al. 2006; Sharma et al. 2005). HO soybean oil also displays superior

properties in biodiesel formulations compared to conventional soybean oil (Kinney and Clemente 2005; Tat et al. 2007). The reduced content of polyunsaturated fatty acids in HO soybean oil results not only in enhanced oxidative stability but also less emission of nitrogen oxides (NOx) (Tat et al. 2007). In addition, the cold point properties are also improved in HO soybean oil due primarily to its lower content of palmitic acid (~4% in HO soybean oil versus ~12% in conventional soybean oil) (Tat et al. 2007). For example, methyl esters generated from HO soybean oil have cloud and pour points of –5 and –9°C, respectively. By comparison, the cloud and pour points of methyl esters produced from conventional soybean oil are 1 and 0°C, respectively (Tat et al. 2007). Undoubtedly, soybean oils with additional novel industrial functionalities can be generated by crossing of HO soybeans derived from biotechnology with soybeans with altered contents of other fatty acids (e.g. high stearic acid) derived from breeding of mutant alleles.

2.4.3 Metabolic Engineering of Soybean for the Production of Oils with High-Value Industrial Fatty Acids

The improved properties of HO soybean oils for industrial applications demonstrate the utility of altering the relative proportions of the five fatty acids typically found in soybean seeds. Another approach for generating soybean oil with improved industrial functionality is to introduce genes that produce fatty acids with novel structures. Such genes have typically been isolated from non-agronomic species that accumulate unusual types of fatty acids in their seed oils (Voelker and Kinney 2001). The enzymes encoded by such genes have been useful for producing fatty acids with carbon chain modifications such as hydroxy or epoxy groups or for producing fatty acids with novel double bond positions and configurations or for even producing fatty acids with chain lengths greater than 18 carbon atoms. Many of these genes encode enzymes that are related to FAD2 but have evolved new enzymatic properties (Cahoon and Kinney 2005). One example is the oleic acid hydroxylase from castor (*Ricinus communis*) (van de Loo et al. 1995). This enzyme uses the same oleoyl-phosphatidylcholine substrate as the typical FAD2 but introduces a hydroxyl group rather than a *cis* double bond at the Δ12 position. The hydroxylated fatty acid product ricinoleic acid (OH-18:1Δ9) has a wide-range of useful industrial properties. These include uses in lubricants, hydraulic fluids, surfactants, cosmetics, and nylon production. The hydroxyl group of ricinoleic acid may also increase the lubricity of fatty acid methyl esters in biodiesel applications (Kinney and Clemente 2005). In an attempt to capture these desirable industrial properties in soybean, research has been conducted to introduce the castor hydroxylase gene under control of a seed-specific promoter to soybean (Kinney and Clemente 2005). These studies have resulted in the generation of soybeans that accumulate ricinoleic acid to approximately 15% of

the total fatty acids of the seed oil. Oils extracted from these engineered seeds are currently being evaluated to determine the value of the increased lubricity associated with fatty acid hydroxylation and the improved oxidative stability associated with the increased oleic acid content (Clemente unpublished results).

Fatty acid epoxidation is another modification that has received interest for the improvement of the industrial value of soybean oil. Chemical epoxidation of soybean oil is currently used to generate plasticizers and precursors such as polyols for the production of coatings, adhesives, and biopolymers (Liu et al. 2006). Epoxidation involves the reaction of the double bonds of the fatty acids of soybean oil with hydrogen peroxide under acidic conditions (Vlcek and Petrovic 2006). This reaction is non-specific for conversion of the Δ9, 12, and 15 double bonds that can be found in the fatty acids of soybean oil. A number of non-agronomic plant species have evolved enzymes that allow for the specific conversion of the Δ12 double bond of linoleic acid to form the epoxy fatty acid vernolic acid (Voelker and Kinney 2001). Vegetable oils enriched in vernolic acid have received interest not only for existing applications for of epoxidized soybean oil but also for paint solvents with low content of volatile organic compounds (VOCs) solvent for paint (Bhardwaj et al. 2007). Novel chemistries for conversion of vernolic acid to industrially useful materials have also been explored (Ayorinde et al. 1997). The Δ12 epoxy group of vernolic acid can be produced from linoleoyl-PC by the activity of a divergent FAD2 epoxygenase (Lee et al. 1998) or from a structurally unrelated cytochrome P450 epoxygenase (Cahoon et al. 2002). A gene from *Vernonia galamensis* for a FAD2 epoxygenase has been introduced into soybean to produce oils containing approximately 7% vernolic acid (Hitz 1998). Similar levels of vernolic acid have been produced in soybean somatic embryos by expression of a cytochrome P450 epoxygenase from *Euphorbia lagascae* (Cahoon et al. 2002).

A major industrial use of soybean oil is as a component of soy ink (Erhan and Bagby 1991). Soy ink is widely used for the color print of newspapers. A limitation of soybean oil for newspaper print ink is its relatively slow drying rate. To improve its drying properties, soybean oil can be supplemented with tung oil, which contains high levels of fatty acids with conjugated double bonds (or "conjugated fatty acids"). The double bonds of conjugated fatty acids are positioned at adjacent carbon atoms whereas the double bonds of linoleic and linolenic acids, the major polyunsaturated unsaturated fatty acids of soybean oil, are separated by methylene groups. To date, soybeans have been engineered to produce two isomers of conjugated fatty acids eleostearic acid and calendic acid (Cahoon et al. 1999, 2006). Both fatty acids are produced by the activity of FAD2-related enzymes termed "fatty acid conjugases" (Cahoon et al. 1999, 2001; Qiu et al. 2001). These enzymes convert an existing *cis*-double bond of linoleic acid bound to PC into two conjugated *trans*-double bonds to generate a conjugated trienoic fatty acid (Cahoon and Kinney 2005). The fatty acid conjugase that produces calendic acid converts the Δ9 double bond of linoleic acid to Δ8-*trans* and Δ10-*trans* double bonds; whereas, the fatty acid conjugase that generates eleostearic acid converts the Δ12 double bond of

linoleic acid into Δ11-*trans* and Δ13-*trans* double bonds (Cahoon and Kinney 1995). A Δ9-modifying conjugase cDNA from *Calendula officinalis* has been engineered in soybean to produce calendic acid at levels of 10–25% of the fatty acids of the seed oil (Cahoon et al. 2006). Similarly, Δ12-modifying conjugase cDNAs from *Momordica charantia*, *Impatiens balsamina*, and *Chrysobalanus icaco* have been introduced into soybean somatic embryos to generate oils with up to 20% eleostearic acid (Cahoon et al. 1999, 2006, 2007a).

From a technical standpoint, the metabolic engineering of genes from other species has successfully resulted in the production of soybean oil with novel fatty acid compositions. However, in most all cases to date, the amounts of these fatty acids obtained in soybean oil have been considerably lower than those in oils from seeds that naturally accumulate unusual fatty acids. For example, castor bean accumulates ricinoleic acid to levels of approximately 90% of its seed oil. However, soybeans engineered to express the castor bean hydroxylase accumulate ricinoleic acid to amounts of about 15% of the seed oil (Kinney and Clemente 2005). The inability to achieve high levels of unusual fatty acid accumulation in engineered soybean seeds has been the major technical hurdle that has limited the adoption of this technology for producing new types of industrial oils in soybean. In the case of fatty acids derived from divergent FAD2s, bottlenecks associated with unusual fatty acid accumulation in engineered oilseeds appears to result from defects in the efficient flux of unusual fatty acids from PC following their synthesis on this lipid (Cahoon et al. 2006, 2007b). This is exemplified by phenotypes observed in seeds engineered to express fatty acid conjugases. In *Arabidopsis* and soybean seeds that express Δ9- and Δ12-type conjugases, conjugated fatty acids not only accumulate in TAG but are also present in PC at the same or higher relative amounts (Cahoon et al. 2006). For example, soybean seeds that express the *C. officinalis* conjugase accumulate calendic acid to amounts of about 20% of total fatty acids in triacylglycerols (Cahoon et al. 2006). Calendic acid also aberrantly accumulates to levels of about 25% of the fatty acids in PC in these seeds. By contrast, calendic acid is found in *C. officinalis* seeds at levels of approximately 55% of TAG fatty acids, but $<1\%$ of the fatty acids of PC. As such, it appears that *C. officinalis* has evolved a mechanism for limiting calendic acid aberrantly accumulation in PC that is absent in soybean seeds. Seeds of other species that naturally produce conjugated fatty acids have also apparently evolved the metabolic capacity to efficiently remove these fatty acids from PC, as conjugated fatty acids are rarely found at levels of $>2\%$ in PC, even in species that accumulate conjugated fatty acids to $>80\%$ of the fatty acids of their seed oil (Cahoon et al. 2006). Current research is focusing on the identification of divergent types of phospholipolytic enzymes from plants that naturally accumulate unusual fatty acids from the activity of FAD2-related enzymes. It is presumed that such enzymes have evolved for the efficient metabolism of unusual fatty acids, and that co-expression of these metabolic enzymes

along with FAD2 biosynthetic enzymes will enable the production of economically-relevant levels of industrial fatty acids in soybean seeds.

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Chapter 3
Soybean

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3.1 Introduction

Soybean [*Glycine max* (L.) Merr.] is the leading oilseed crop produced in the world (Wilcox 2004). Over the past three decades, world production of soybean has almost tripled, from 73,854,802 Mg in 1977 to 216,144,262 Mg in 2007 (FAOSTAT 2008). The majority of soybean is grown in North and South America, China and to a smaller extent in many other countries on every continent. During the past five decades, the USA has been the world's leading producer of soybean representing 33% of the world production, followed by Brazil with 27%, Argentina with 21%, China with 7.2%, India with 4.4%, Paraguay with 1.8% and Canada with 1.3% (FAOSTAT 2008). During the 1990s and 2000s production in Brazil and Argentina has seen a tremendous increase, reaching almost 50% of the total world production when combined in 2007. Increasing world population, constant need for animal feed and over 300 different soybean products contribute to the strong demand for soybean in world markets. From 1992 to 2007, soyfood sales increased from $300 million to nearly $4 billion (Soyatech Inc. 2008) with most of the recent increase coming from soymilk. In 2008, 85% of the consumers in the USA rated soybean as healthy, which was up 3% from 2006 (United Soybean Board 2008). Healthy aspects of soyfoods go beyond the oil and protein and include minor compounds with nutraceutical properties such as isoflavones, saponins and tocopherols (Rajcan et al. 2005). Clearly, soybean production, consumer acceptance and consumption in non-traditional regions of the world are on the rise. Because of the high concentration of protein (40%) and oil (20%) typically found in the seed, soybean is used both for oil production and protein processing. Most of the soybean is used for oil extraction and for animal feed processed from the remaining meal after the oil is crushed. Additionally, a significant and increasing portion is also used as food, including traditional

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uses originating in Asia such as tofu, miso, edamame, soy sauce, soymilk, natto and newer uses in Western culture such as soy pudding, soy granola bars, soy nuts and other products.

Typically, soybean oil consists of approximately 10% palmitic (16:0), 4% stearic (18:0), 22% oleic (18:1), 54% linoleic (18:2) and 10% linolenic (18:3) acid (Wilson 2004), making it one of the healthiest vegetable oils. In addition to oil, the protein contains all essential amino acids and, therefore, is a prime source for both animals and humans. However, soymeal remains deficient in the sulfur containing amino acids, methionine and cysteine, and breeders are currently striving to enhance their concentrations (Panthee et al. 2006).

Major improvements in soybean seed yield occurred during the past several decades. In the USA soybean yields have risen by 22.6 kg ha^{-1} yr^{-1} from 1924 to 1997, however, during the 25 years from 1972 to 1997, the increase has been faster, 31.4 kg ha^{-1} yr^{-1} (Specht et al. 1999). During the same period in Ontario, Canada, changes in some physiological traits have contributed to a 0.5% yield increase yr^{-1}, i.e. improvements in lodging resistance, N_2 fixation, and stress tolerance among other traits (Specht et al. 1999; Voldeng et al. 1997).

Breeding has played a dominant role in yield improvements coupled with production practices such as increased planting density, which resulted from new cultivars possessing smaller leaf area index and higher photosynthetic rates (Cober et al. 2005), particularly in short-season soybean. The objective of this chapter is to discuss a wide range of topics from the origin of the species and historical perspectives up to current as well as future breeding goals and the use of new biotechnological tools in soybean improvement. Unfortunately, it is not possible to cover every trait under improvement. For a detailed review, the reader is referred to the most recently published soybean monograph (Boerma and Specht 2004).

3.2 Origin and Domestication

The domesticated soybean [*Glycine max* (L.) Merrill] is a member of the Fabaceae family, subfamily Papilionoideae, tribe Phaseoleae (Hymowitz 2004) (Fig. 3.1). Phaseoleae is the most important tribe for species used as

Family	**Fabacae**
Subfamily	**Papilionoideae**
Tribe	**Phaseoleae**
Genus	***Glycine***
Subgenus	***Soja***
***Glycine max* (2*n* = 40)**	
A true domesticated species	

Fig. 3.1 Taxonomic classification of *Glycine max*

food and feed sources, such as soybean, pigeon pea [*Cajanus cajan* (L.) Merr.], and common bean, lima bean and tepary bean (*Phaseolus* spp.) among many others.

The genus *Glycine* is composed of two subgenera, *Glycine* (perennials) and *Soja* (annuals). The subgenus *Soja* includes the cultivated soybean, *G. max,* and *G. soja,* the wild annual soybean relative. *G. max* is a true domesticate, the species would not exist in the absence of human intervention.

Soybean originated in China, domestication of soybean took place ~1500–1100 BC, or perhaps earlier (Palmer and Hymowitz 2004). It was probably grown in the Korean peninsula as well as central China by the first century. From that time up to the 15th to the 16th century, soybeans were introduced in Southeast Asia, and from there to Europe before 1713. Soybean was introduced to North America in 1765.

Goldblatt (1981) indicated that 'the basic chromosome number for Phaseoleae is almost certainly $x = 11$, probably basic in all tribes, emphasizing that aneuploid reduction to $x = 10$ is prevalent through the Papilionoideae'. Hymowitz (2004) and his collaborators hypothesized that a putative ancestor of the genus *Glycine* with $2n = 20$ arose in Southeast Asia. This progenitor, however, is either extinct or has yet to be collected and identified. Tetraploidization through auto- or allopolyploidy of the progenitor species occurred either prior or after dissemination from the ancestral region. Singh et al. (2001) proposed a more complete path of migration from the ancestral region to China (Figs. 3.2 and 3.3). The common progenitor is assumed to be a wild perennial ($2n = 4x = 40$, unknown or extinct) that later evolved to a wild annual ($2n = 4x = 40$, *G. soja*) and finally to the domesticated soybean ($2n = 4x = 40$, *G. max*, cultigens). All described species of the genus *Glycine* exhibit normal diploid meiosis, and are primarily inbreeders (Singh and Hymowitz 1985).

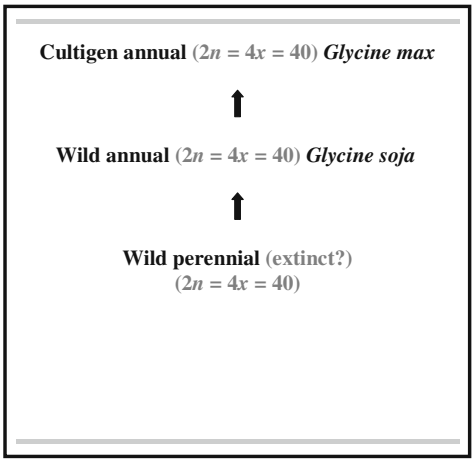

Fig. 3.2 Ancestors of the cultivated soybean

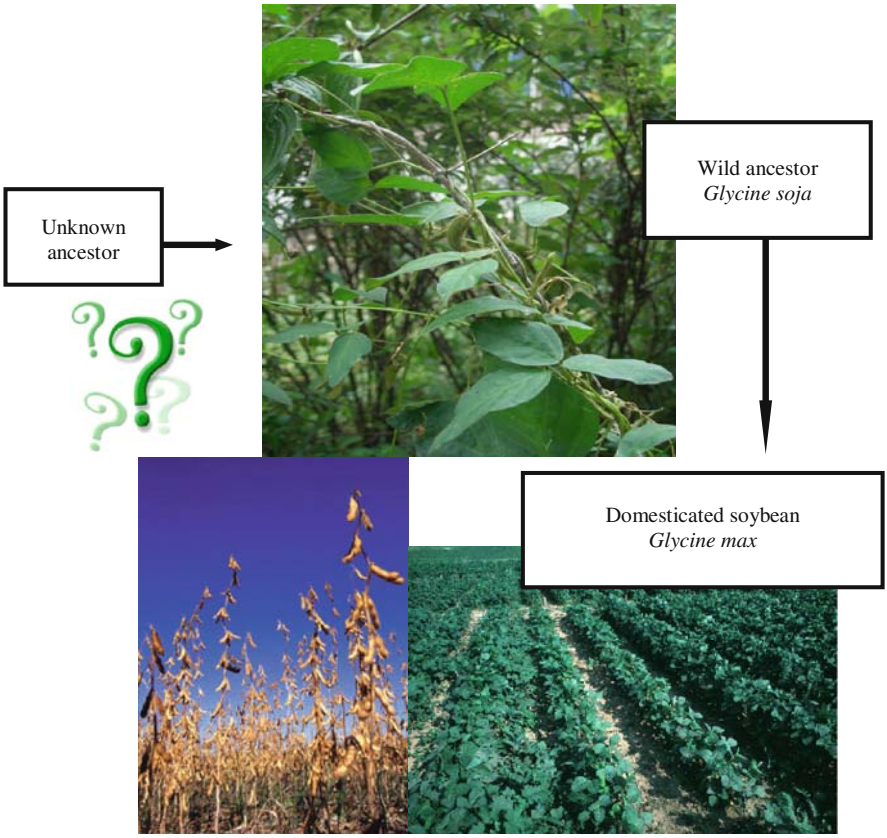

Fig. 3.3 A pictorial of the ancestry of the domesticated soybean and the profound changes in plant phenlogy and morphology by domestication

Genomic relationships among diploid species of the subgenus *Glycine* have been extensively studied and established by cytogenetic analyses, biochemical techniques and molecular methods. Different genomes have been differentiated among several of the *Glycine* spp., and identified as AA, A_1A_1, BB, B_1B_1, and B_2B_2, depending on the species. For a scholarly review of these and related topics the reader is referred to the chapter by Hymowitz (2004).

In the cultivated soybean, the gene pool concept developed by Harlan and de Wet (1971), identifies a primary gene pool constituted by soybean cultivars, landraces and *Glycine soja*. Hybridization among them creates vigorous plants, with normal meiosis and complete seed fertility. Gene segregation is also normal. The secondary gene pool is formed of yet unknown species, and to date the cultivated soybean does not possess one. The tertiary gene pool is formed by the wild perennial species. Production of hybrids between members of the primary gene pool and those of the tertiary gene pool is difficult, where survival of the F_1 seed requires use of special embryo rescue techniques.

In spite of its economic importance, the basic study of the soybean species has lagged behind many others (Hymowitz 2004). Soybean chromosomes are smaller than chromosomes of most crop plants, making difficult the conduct of basic studies. Additionally, it is not considered a model plant for cytogenetic studies because its chromosomes are high in number ($2n = 40$), small in size, and morphological similar lacking distinguishing landmarks. Molecular research has established 20 linkage groups which are not yet associated with their respective chromosomes (Singh et al. 2007). However, newly developed stocks have been formed and it is expected that these, together with new technologies, will provide the tools to conduct genetic studies that will benefit soybean breeding, production and physiology research.

3.3 Varietal Groups

As mentioned earlier, it is believed that the cultivated soybean [*Glycine max* (L.) Merr.] originated in China by domestication of the wild progenitor species, *Glycine soja*. In order to understand the varietal groups of soybean, it is important to look at the genetic diversity within different gene pools worldwide.

Evaluation of genetic diversity has traditionally been conducted using differences in morphological and agronomic traits and/or pedigree (Nelson et al. 1987; Sneller 1994; Gizlice et al. 1994). A limitation of evaluating genetic distances based on agronomic traits is their often high dependence on the testing environment, which greatly reduces the range of soybean genotypes that could be compared directly (Li and Nelson 2001). To overcome this issue, a core set of randomly amplified DNA polymorphism (RAPD) markers was developed to study a broad spectrum of genetic diversity among soybean accessions (Thompson and Nelson 1998).

Perhaps not surprisingly, studies have shown that genetic distance within the progenitor *G. soja* was much greater than within *G. max* but smaller than between groups of *G. soja* and *G. max* (Li and Nelson 2002). The authors also observed that much greater variation was found within relatively small geographic regions in China in *G. soja* accessions than for *G. max* from the same province (Li and Nelson 2002). Molecular genetic analysis of U.S. and Chinese soybean ancestral lines indicated that cluster analysis clearly separated the ancestral gene pools of China and USA (Li et al. 2001). Clusters reflected the geographic origin of the lines tested and showed that large differences existed between northern U.S. and Chinese ancestral lines on the one hand, and between the central and southern Chinese ancestral lines on the other (Li et al. 2001).

Within Chinese germplasm, the coefficient of parentage was used to determine genetic diversity in soybean cultivars released from 1923 to 1995 (Cui et al. 2000). Cultivar pools from three different growing regions in China (Northeast, Northern and Southern China) were completely unrelated and were also influenced by crossing systems and release eras (Cui et al. 2000). Another research

group reported that the mean genetic distance in soybean cultivars within China was much larger than the distance within Japan or South Korea but smaller than that between China and S. Korea or Japan (Li and Nelson 2001).

Attempts have been made to use the diversity of varietal groups from different regions and/or alleles from *G. soja* to improve germplasm diversity and seed yield in the cultivated soybean. Yield quantitative trait loci (QTL) were identified in crosses involving an exotic and adapted parent (Mansur et al. 1996; Orf et al. 1999a; Orf et al. 1999b; Smalley et al. 2005). In 2004, Kabelka et al. reported 15 QTL significantly associated with yield in a cross between 'BSR 101', which has nine of the 10 ancestral lines contributing to North American gene pool, and the experimental line LG82-8379, developed from a cross between two plant introductions (PIs). For nine of the QTL, the exotic parent contributed the high yielding allele (Kabelka et al. 2004) suggesting the genetic potential that exotic germplasm sources may have to further improve seed yield. Using three backcross derived populations, another group reported 13 yield QTL, of which 8 carried a high yielding allele from PI, that mapped to previously reported yield QTL (Guzman et al. 2007). The same group also found significant QTL x environment interaction, probably due to undetectable or weak QTL effects in some environments (Guzman et al. 2007). It is expected that further attempts by different research groups world-wide will result in the finding of more yield QTL coming from exotic sources and further enhancement of genetic diversity in soybean breeding gene pools for yield and other traits' improvement.

Soybean is an autogamous species and all former and current cultivars are inbred lines. However, heterosis has been reported in soybean. A summary of 14 experiments conducted since 1930, reported average mid-parent heterosis (MPH) for yield grain ranging from + 14 to + 46%, whereas high-parent heterosis (HPH) ranged from + 4 to + 34% (Palmer et al. 2001). Despite the existence of genetic male sterility and heterosis expression in soybean, no soybean hybrids are used in commercial production. In China, Prof. Sun Huan from the Jilin Academy of Agricultural Sciences recently released hybrids 'HybSoy 1' and 'HybSoy 2', grown in 200 ha demonstration plots, however, the continuing pollination efficiency problems for seed production have hampered their full release (Sun Huan, Jilin Academy of Agricultural Science, China; pers. comm.). Unless better or more efficient pollinator systems can be found, the genetic male sterility used to develop hybrids will not suffice for the commercial release of hybrid soybean.

3.4 Genetic Resources

Success of the crop is credited to thousands of growers from antiquity to today, and to countless number of researchers in many disciplines who have contributed with plant selections, knowledge and work to improve the species. Soybean

was domesticated from the wild soybean, which is an annual weedy-form climber (Fig. 3.3), whose pods contain black seeds that shatter at maturity (Singh et al. 2007).

An estimated 45,000 unique Asian landraces have been collected and are maintained in *G. max* collections around the world (Carter et al. 2004). These large collections are becoming actively searched, studied and characterized, as new challenges related to plant health and seed production acquire economic importance. The studies will determine the value of the genetic diversity and possible future use.

China possesses the largest collection of *G. max* accessions, 23,578, followed by the US with slightly over 18,000, and the Asian Vegetable Research and Development Center (AVRDC) with 12,508 accessions (Carter et al. 2004). Japan, Russia, Ukraine, India and Brazil, also hold sizable collections, although the actual number of accessions in each is smaller. Many studies on genetic diversity and on the importance of collection and preservation of soybean land races have been done and still are in process. And as new technologies are developed, there is no doubt that even old concerns will be revisited. In the interest of space, however, only some information will be discussed in this section, as an attempt to open up interest areas to the readers. For an extensive review of the subject up to 2004, the reader is referred to the publication by Carter et al. (2004).

In China where the center of origin of the species is located, the collections are actively characterized. Dong et al. (2004) used the database of the National Germplasm Evaluation Program of China (NGEPC) to study geographical distribution of accessions, genetic diversity of 15 qualitative and quantitative traits, and genetic diversity of centers of the cultivated soybeans. Of the 22,637 known accessions studied 20,570 had been collected between latitudes 18° and 53° N and longitudes 80° and 136° E. The authors reported that broader genetic diversity was found in accessions collected between 34° to 41° N and 110° to 115° E, which prompted them to propose one genetic diversity center located downstream of the Yellow River Valley. Northeast China is also thought to be one of the regions in which soybean originated, particularly due to the existence of a number of valuable soybean varieties in the region (Yamanaka et al. 2004). To determine genetic diversity in this region, Yamanaka et al. (2004) studied a group of 3,000 accessions measuring an array of quantitative and qualitative traits, along with DNA determinations. Large variations were observed in traits such as plant height (7–227 cm), protein (11.5–59.4%), and oil contents (10.3–23.6%). These results indicate the potential value of genotypes in the collection for use as breeding materials. The authors also used SSR markers in 253 accessions to determine genetic diversity at the molecular level. Of the 253 accessions, 194 varieties were from Northeast China, and 59 from Japan. SSR markers indicated the two groups are genetically distantly related, and that Chinese accessions are clearly rich in diversity at the DNA level. A study conducted by Chen and Nelson (2005) using RAPD markers, identified six major clusters of varieties that were genetically different although varieties

within each cluster were similar probably due to the fact they were obtained from the same geographical location. Results provided evidence that primitive cultivars of soybeans in China were genetically isolated in relatively small geographical areas. Similar results have also been reported by other researchers (Carter et al. 2004).

Estimates of genetic diversity in the annual wild soybean collection in China have also been obtained (Dong et al. 2001; Ru et al. 2006). Both studies identified genetic diversity in the *G. soja* collections using various diversity indices, applied to morphological and chemical traits (Dong et al. 2001) and to molecular markers, such as AFLP, ISSR, and SSR (Ru et al. 2006). The results obtained by Dong et al. (2001) also suggested the possibility of three centers of origin for *G. soja* in China: the Northeast, the Yellow River Valley, and the Southeast Coast of China. On the basis of the genetic diversity estimates, geographical isolation was and still is an effective mean to conserve diversity. The research groups coincide on the importance of conserving genetic diversity within the cultivated and wild annual soybean collections. These observations reinforce the potential use of the collections for gene-mining, and the need to preserve genetic information and genetic types.

In the US, the search for genetic diversity has also become a concern of researchers and growers, after the realization of how limited the genetic base of the soybean is in the country. In 1994, it was reported that for the northern and southern North America breeding pools, there were only 19 ancestors with 17 of them common to both regions of the US (Gizlice et al. 1994). The 19 ancestors contributed 85% of the genes to each region. The narrow genetic base of the crop had also been identified by earlier studies on genetic diversity in the U.S. (Committee on Genetic Vulnerability 1972; Specht and Williams 1984). The information reaffirmed the concept that modern soybean cultivars in the U.S. are exceptionally uniform with the question remaining about how the impact of selection, climate, and geography is shaping up the present-day genetic diversity in soybean (Carter et al. 2005).

The evidence indicates that the greatest variability will be found in the center of origin of the species, as Vavilov (1922, 1927) first established. Carter et al. (2005) examining genetic relatedness concluded that coefficient of parentage will increase within breeding populations derived from relatively few founding members, thus decreasing genetic diversity. There is a definite need for concerted efforts to expand the genetic base of the soybean in the US. Steps in that direction are currently taking place in several public breeding programs, particularly in the area of resistance to diseases and pests, i.e. the search for resistance to brown stem rot caused by *Phialophora gregata* (Cianzio unpublished information) and to soybean cyst nematode caused by *Heterodera glycines* (Lu et al. 2006).

To address the possible loss of genetic diversity and the consequences due to domestication and other factors, Hyten et al. (2006) conducted a study to determine how human activities on the soybean species over the past 5,000 years may have altered the DNA sequence variation in soybean. The authors

established genetic bottlenecks in soybean as due to (1) domestication (when humans exert artificial selection on a wild species), (2) founding (when only a few individuals are used to introduce a crop into a new region), and (3) selection to improve the species for cropping systems. In addition to these factors, the reproductive physiology of the species also needs to be considered. Soybeans are autogamous, and inbreeding in itself is predicted to decrease diversity.

The complete picture demonstrates that none of these factors favors conservation of genetic diversity, unless a conscious effort is done to diminish the effects of the mentioned factors. Hyten et al. (2006) sequenced 111 fragments from 102 genes in four soybean populations, representing gene pools before and after genetic bottlenecks. To represent all possible gene pools prior to and after the genetic bottlenecks, the researchers selected for the work, elite North America soybean cultivars, Asian landrace founders of the elite cultivars, Asian landraces (with no known relationship to the founding stock), and accessions of the wild progenitor species *G. soja*. With these groups, they were able to show that soybean has lost many rare sequence variants and has undergone numerous allele frequency changes throughout its history. Despite the erosion on genetic diversity in soybean by human intervention, the authors note that modern cultivars have remarkably retained 72% of the sequence diversity present in the Asian landraces, although they lost 79% of the rare alleles (frequency ≤ 0.10) found in the Asian landraces. Domestication was the human effect that had the most important impact on genetic diversity, with less effect from artificial selection subsequently imposed by selective breeding. These findings suggest that the current strategy of using plant introductions in breeding with the objective to increase genetic diversity, is justified and recommended.

Similar concerns about genetic diversity have also been shown by countries in which soybean is a relatively new crop, such as Mexico as well as in others where sizable collections are in place, such as India. In Mexico, in a study conducted by Quintero et al. (2005), 24 lines developed in the country and nine plant introductions from Brazil were characterized at the molecular level by AFLP markers. Results indicated that: (1) lines did not group according to geographical origin, (2) a high polymorphism level (60%) was detected, and (3) the Mexican lines shared a high level of genetic similarity with those from Brazil. Although the sample size used in the research did not represent all soybeans from the humid tropics in Mexico, results indicated that the present genetic diversity is useful to generate new cultivars. Three of the Mexican lines were more divergent at the molecular level than foreign genotypes considered phylogenetically different and that were used as control genotypes. In spite of breeding and selection, and of the limited ancestors used in Mexico, there is still genetic diversity and efforts are made to conserve it.

In India, soybean production systems in the central region of Madhya Pradesh, and on the Malwan plateau, require development of early maturing cultivars with a growing season of <90 days (Bharadwaj et al. 2007). Due to the scarcity of early maturing cultivars, Bharadwaj et al. (2007) referred to the National Research Centre for Soybean (NRC Soybean), in Indore to obtain

accessions and germplasm with a short growing season. Detailed data records and subsequent analyses indicated there was considerable variation for the short-season trait in the sub-sample of the collection studied. An interesting observation of the work was that although genotypes obtained from the same country had a tendency to group together, the authors observed that in general, grouping in clusters was random, indicating that geographical origin and genetic diversity were not related. Bharadwaj et al. (2007) concluded that the germplasm identified in the study could be used as parent material in population development to select for early maturing lines, making use of the genetic diversity still in store.

Establishment of geographically different gene pools is an important aspect to consider when genetic diversity of collections is assessed. Some studies have been conducted to establish different geographical areas within countries (Dong et al. 2004; Quintero et al. 2005). Other works have been done to determine the genetic relationship between accessions obtained from different parts of the world. This is the case of the study by Yamanaka et al. (2007) which compared genetic relationships between Chinese, Japanese, and Brazilian soybean gene pools as revealed by SSR markers. The authors investigated a total of 272 cultivars from the three countries and observed that different from previous reports (Abe et al. 2003), the gene pools from China and Japan could not totally be considered as independent from each other. The Brazilian cultivars in turn, were distantly related to the Chinese and Japanese gene pools, forming a separate cluster from the two groups. On this basis it is expected that exchange of material among the three countries would expand the genetic base and increase genetic variability.

In general, it appears that in spite of the enormous pressure exerted by both human and physical factors on the original and natural genetic diversity in soybean, there seems yet to be a wealth of variability that if needed could be searched for and used. There is, however, an undeniable fact related to the amount of effort in time and resources that may be applied to the search for the special genetic types that the industry and consumers demand. Zohrabian et al. (2003) have indicated the difficulties that exist in ascribing productivity gains to specific genes or accessions because of the nature of the research process in genetic enhancement, the relationship between genes within the genome, and the interaction of genes with the environment. Still, the authors have determined that the lower-bound benefits from utilizing a marginal accession are higher than the upper-bound costs of acquiring and conserving it, justifying the expansion of the US soybean collection. And for that matter, the investments made by any country to acquire and conserve the genetic materials of the plant species that relate to their respective production system are worthwhile.

3.5 Major Breeding Accomplishments

The cultivated soybean has been amenable to major changes in its genetic constitution that allowed the species to acquire wide adaptation to different latitudes, environments, and to possess different genetic composition in the

seed. Undoubtedly in every country where soybeans are grown, breeding is underway to increase yield and improve other traits of economic importance. Achievements that will be discussed here, however, pertain mainly to those attained in the US.

When soybeans were first introduced to the US, they were mostly used as a forage crop. Since the early 1930s major breeding efforts began to make the soybean what it is today. Early soybean breeding in the US, was confined to State Agricultural Experiment Stations (SAES) in Land-Grant Colleges and to the United States Department of Agriculture's Agricultural Research Service (USDA-ARS) (Huffman 2004; Sleper and Shannon 2003). Legal events, i.e., Plant Variety Protection (PVP) Act, the subsequent PVP modification to conform to European cultivar protection laws and the affirmative ruling in the US about the patenting of living material, however, stimulated the private industry sector to invest heavily in soybean breeding. According to Frey (1996), most of the effort in developing improved soybean cultivars or varieties presently comes from industry, followed by soybean breeders from SAES. The USDA-ARS spends the least amount of effort in cultivar development.

Wilcox (2001) estimated that public soybean breeders in the northern soybean production region have increased seed yield about 60% over the past 60 years. Specht et al. (1999) estimated that similar progress in breeding has also been made in the private sector, although results have been obtained during the last two or three decades. Approximately 90% of the US soybean hectarage is planted to cultivars developed from private programs (Sleper and Shannon 2003). Intellectual property protection, the ability to earn high returns on research investments, and the reduction in public budgets have shifted the majority of the soybean breeding effort from the public to the private sector.

Conventional breeding strategies have been successful in improving soybean productivity and quality. Currently, the number of public and private cultivars available to growers for planting is enormous, and most of them have been developed by conventional breeding techniques. Perhaps one of the most widely publicized achievements from the US public breeding effort has been the commercial use of soybean lines having low linolenic acid content, developed at Iowa State University (ISU) (Fehr and Hammond 1996, 1998). Research on altered linolenic acid content of soybean began at ISU in 1968 after a visit by E.G. Hammond to Unilever, a food processor industry in the Netherlands. The researcher learned Unilever was interested in obtaining soybean oil with reduced linolenic acid content to avoid use of hydrogenated oil in food products. Although linolenic acid is needed by the human body, when soybean oil is used to manufacture margarines and other food products, it often needs to be hydrogenated, meaning that hydrogen is added to the fatty acid chains. That process produces trans-fatty acids, and its consumption can increase the risk of developing cardiovascular disease when it is included in the human diet.

With these concerns in mind, two researchers from Iowa State, Hammond and Fehr began a collaboration that spanned 30 + years and led to the development of 1% linolenic acid soybean oil. Several patents were issued to ISU on

the account of this work, but most importantly, the new lines bred by conventional breeding methods have been in commercial production since 2004–2005. In 2004, 12,141 hectares of 1% linolenic soybean varieties were planted in Iowa and the projection was that more than 404,686 hectares of the low-linolenic cultivars were needed to meet the anticipated demand for oil. Asoya, an Iowa-based corporation that manufactures oil from the low-linolenic soybeans, plans to produce 20 million tons of oil from low-linolenic soybeans in the near future. In 2007 three new varieties were offered to growers to enhance the production of oil with 1% linolenic acid. Currently two manufacturers in Iowa process 1% low linolenic soybean oil, Asoyia and Innovative Growers, the latter under the Iowa Natural^{R} label.

Soybean provides about 30% of the world oil production (US Department of Agriculture statistics 2006; www.nass.usda.gov/Publications/Ag_Statistics/agr06), and it is mostly composed of triacylglycerols (Cardinal 2008). In the US more than 73 million acres of soybean are grown supplying 81% of the required edible oils and fats in the US. On average, soybean oil is composed of 110 g kg^{-1} palmitic acid (16:0), 40 g kg^{-1} stearic acid (18:0), 240 g kg^{-1} oleic acid (18:1), 540 g kg^{-1} linoleic acid (18:2), and 70 g kg^{-1} linolenic acid (18:3). Saturated fatty acids are not considered healthy. Unsaturated fatty acids are unstable due to their susceptibility to auto-oxidation and production of undesirable flavors and odors. In contrast, monounsaturated fatty acids are desirable for both human health and oil stability (Cardinal 2008).

In 2006, the US Food and Drug Administration (FDA) began to require food manufacturers to report on nutrition labels the amount of trans-fat in their food products, and a healthy alternative was already in place due to the work by the ISU team. The team identified three genes that individually reduce linolenic acid, *fan 1* in germplasm line A5 (Hammond and Fehr 1983), *fan 2,* and *fan3* (Ross et al. 2000). Individually each of the genes would produce genotypes that would possess an average of 2.9–4.9% of linolenic acid. The three genes combined in the same genotype, however, were able to reduce the linolenic acid in the oil to approximately 1%. The original genotypes possessing reduced linolenic acid content were, however, poor looking from the agronomic point of view. Plants were also short in height, had few pods, thick stems difficult to dry for appropriate harvest, were sickly looking, and lodged badly (Cianzio unpublished results).

Research advances to obtain the high-yielding and low-linolenic lines began in earnest when the team at ISU developed the germplasm line A5 (Hammond and Fehr 1983). The line was selected from the progeny of soybean mutagenized with ethyl methanesulfonate (EMS). An intense all year round effort began using mainly the research facilities that ISU has in Puerto Rico (Cianzio unpublished information). In Puerto Rico it is possible to obtain two generations from October to May (Cianzio 1985), with the third generation of the year grown in Iowa. The majority of the crossing for the release of the low linolenic lines was conducted in Puerto Rico during the winter, along with generation advances. Continuous cycles of crossing among mutant lines, oil and fatty acid

analyses of each line, and subsequent selection for low linolenic content both in Puerto Rico and Iowa were carried out in the fall and winter seasons in Puerto Rico. Selection for agronomic traits was always conducted in the plantings at Iowa during summer. Crossing in Puerto Rico was difficult, since A5 and lines low in linolenic acid behaved in unpredictable ways in terms of time to flower; pollen production was also poor (Cianzio unpublished information). To improve high-yielding potential of the lines possessing the low linolenic acid content, the backcross method of breeding was also utilized by conducting cycles of backcrosses to high-yielding genotypes as recurrent parents along with oil and fatty acid analyses of the progeny, to identify the lowest segregants for linolenic acid in the progeny of each generation. The research effort that lasted almost 40 years and was a real team effort, has yielded the low linolenic acid – high yielding lines that are in commercial production today.

Another major accomplishment at the commercial level that has transcended the physical limits of the species was achieved by the Monsanto Company with the development of Roundup Ready® (RR) soybean, which is the product of genetic transformation considered a genetically modified organism (GMO). This is one of the best examples of the use of a recently developed technology and its applicability for product development. The technique and commercialization of the RR soybean has been so successful to Monsanto, that in February 2007, the Monsanto Company announced the availability of a second transformation event, referred as RR event two or Roundup Ready2yield®. The RR soybean from event one was among the first transgenic crops to reach the market (Parrot and Clemente 2004). It was first commercialized in 1996, and by 2000, RR soybean was grown on 54% of the soybean hectarage in the US. RR soybeans are resistant to the herbicide glyphosate, the active ingredient in the commercial herbicide Roundup, also produced by Monsanto. Presently, RR soybeans are grown on 95% + of the hectarage in the US and in other countries, i.e. Brazil and Argentina. RR soybeans brought more effective weed control into the management practices of low income growers, and soybean yields were expected to be equal or higher to conventional cultivars (Huffman 2004).

The yield level of RR soybean compared to conventional cultivars, however, is still a debatable issue. It seems that at least in some conditions and some environments, RR soybeans pay a yield penalty. It is argued, that the RR event two will be free of this limitation. Growers find the technology easy to apply, not timing critical and effective in a 2-year crop rotation. These may be some of the reasons for the commercial success of planting RR soybeans in the US. For a detailed description of the transgenic procedure used by Monsanto, the reader is referred to the publication by Parrot and Clemente (2004).

The commercial use of GM soybeans has brought to the discussion table different groups with opposed views about the use of genetically engineered or GMO soybeans for food production. Some consider the use of GMO as one of the greatest inventions since the beginning of farming (Huffman 2004). Others caution that the technology has not been sufficiently evaluated as far as the

consequences for humans who consume such food products. The growing and almost permanent controversy over GMO food products and consumers' attempts to make better food purchasing decisions have stimulated interest in food labeling and identity preservation. This controversy has precluded wide use of other GMO soybean cultivars, particularly in Europe.

A second product in commercial production is a high oleic (HO) soybean developed by DuPont, Wilmington, DE (Parrot and Clemente 2004). An extra copy of a gene was inserted in the soybean genome resulting in gene silencing. As a consequence, oleic acid accumulates because it is not converted to linoleic acid, and the seed has a higher oleic acid concentration. Other transgenic soybeans have been developed by Aventis in Strasbourg, France. In each of the two cases, however lack of approval by the European market has prevented wide commercial use of the transgenic soybeans. Still, it is important to recognize that these are breeding accomplishments that would not have been possible without development of the transgenic soybeans and transformation techniques. Soybean breeders were also the other important component of this collaborative work, who contributed to develop the final commercial product.

In addition to the individual case-accomplishments discussed, and considering the soybean commodity as a whole, maybe the most important breeding achievement of all has been the development of the soybean cultivated species as one of the most important oil crops in the world. In the US, after the initial introduction of 19 genotypes both in the northern and southern regions (Gizlice et al. 1994), breeding methods were used to develop numerous cultivars, first as products of the public sector, and lately from the combined efforts of both public and private sectors. These advancements can fairly be attributed to the concerted effort of soybean breeders, and to the research they conducted to develop the array of adapted cultivars available to growers.

Other breeding achievements also of similar importance to the soybean commodity may not have had the direct commercial impact of some of the individual cases previously discussed. However, the search for novel genes in the National Soybean Germplasm Collection has identified new sources of resistance to several diseases and pests, i.e. soybean cyst nematode (Lu et al. 2006), Phytophthora root rot (Burnham et al. 2003; Sandhu et al. 2005); and to physiological traits such as drought tolerance (Carter et al. 2004) among many others. The genes are being incorporated in new cultivars, and will produce an impact in soybean yields through the protection and improved ability of the cultivars to withstand biotic and abiotic stress factors. The search for yield genes in the collection is an undergoing effort which will eventually result in new findings. The caveat with these searches is that once the new genes are identified and characterized, they will have to be bred into high-yielding genetic backgrounds, before their impact in commercial production can be finally assessed. Immediate results will not be available; however their contributions to soybean production will be noticed and valued.

Advances in the area of molecular technology are also contributing to breeding achievements. Presently, disease resistant germplasm lines have been

released in which molecular markers confirm the phenotypic reactions of resistant genotypes (Cianzio et al. 2002, 2006a, b, c, 2007a, b). Proprietary issues and the patents of complete regions of some soybean linkage groups (Webb 1999, 2000), have somewhat precluded a more generalized use of marker-assisted-selection as a means to increase efficiency of breeding efforts. These patents (Webb 1999, 2000) have mainly affected the breeding for brown stem rot and soybean cyst nematode resistance.

The future of soybean breeding will undoubtedly be greatly impacted by advances in the area of soybean genomics. These advances will open up new avenues for breeding, increasing efficiency and feasibility of transferring the required genes to further improve the commercial attractiveness of the soybean. Gene discovery stemming from structural and functional genomics research will certainly lead to new products and to cultivars with improved nutritional and agronomic characters (Shoemaker et al. 2003; Stacey et al. 2004). New levels of increased yield will continue to secure the commercial success of the novel soybean types.

3.6 Current Goals of Breeding

Soybean breeding programs that include oil improvement among their objectives focus on two overall goals: (i) increasing total seed oil concentration and (ii) modifying fatty acid composition of the oil.

3.6.1 Seed Oil Concentration

Improving seed oil concentration has been an intentional or unintentional goal of soybean breeders for centuries. The domesticated crop arose from ancestors with lower oil content and higher protein content, as small, black, hard seeds with low yield. Over many generations of breeding, selections for yield, agronomic characteristics, and seed quality led to large yellow seeds with typical averages of 20% oil and 40% protein making the valued global commodity of modern day.

An intrinsic positive correlation between seed oil concentration and yield is well known (Burton 1987), and gains in oil are typically realized at the expense of loss in total protein concentration. Modern breeders understand that the soybean commodity is valued for its composite components of high protein soy meal and versatile vegetable oil. Thus, a balanced approach for modest gains in oil and yield are often targeted without substantial loss in protein concentration.

3.6.2 Fatty Acid Modification

In the US, the United Soybean Board (USB) has supported a program in recent years known as the Better Bean Initiative (BBI), with aggressive goals to

improve soybean oil and meal quality. Oil quality improvement is targeted with reduced saturates and elimination of *trans*-fatty acids produced by hydrogenation. A third goal is to improve oleic acid concentration for improved oxidative stability of the oil.

3.6.3 Reduced Saturates

Health conscious consumers currently favor a diet lower in saturated fats. Saturated fatty acids are major dietary components responsible for elevating cholesterol. Soybean contains two saturated fatty acids, 16:0, and 18:0. Palmitic acid is the primary saturated fatty acid that is a health concern as it has been correlated to cardiovascular disease. Dupont et al. (1991) suggested keeping saturated fatty acids in the human diet below 10% on a daily basis. The US Food and Drug Administration (FDA) advises keeping total saturated fat less than 7% in the daily diet. Breeders working with the BBI are seeking to develop high yielding soybeans with $<7\%$ total saturates.

3.6.4 Increased Saturates

Although reduction in saturates is a key oil quality breeding target, some breeders pursue projects to increase saturates for the solid fat industry for tub margarine production. Soybeans with increased 16:0 and 18:0 have favorable processing applications to produce low trans-fat margarines (List et al. 1995; Neff et al. 1999). An important consideration in future breeding efforts is that unlike 16:0, 18:0 has been show to either reduce or to have no effect on serum cholesterol levels in humans (Kris-Etherton and Yu 1997).

3.6.5 Increased Monounsaturates

Soybean oil contains the monounsaturated fatty acid 18:1. Major gains in oxidative stability of the oil can be achieved if 18:1 concentration can be enhanced to greater than three times that of normal soybean. The BBI target for soybean 18:1 concentration is 65–75% of total lipid. Levels as high at 80% 18:1, further enhancing oxidative stability, have been achieved by DuPont Co. through genetic engineering, as previously mentioned (Hitz et al. 1995).

3.6.6 Trans-fat Reduction

Global concerns with trans-fat health issues have prompted major processors to improve engineering processes and find oilseed feedstocks capable of producing reduced or zero trans-fat food products. In January 2006, the FDA required US

food labels to list the levels of trans-fat, prompting intense competition and formulation changes in the food industry. Major metropolitan areas, such as New York City recently instituted trans-fat bans in restaurants. Soybean oil is readily available and has been an affordable mainstay for food processors. Reductions in soybean 18:3 concentration provide opportunities for reduced trans-fat food products.

3.6.7 Increased Polyunsaturates

Industrial applications for inks and drying oils would benefit from increased 18:3. This would require development of a specialty soybean, as the majority of the oil is processed for vegetable oil food applications favoring reductions in 18:3, aimed to very specific commercial markets.

3.6.8 Increasing Nutraceuticals in Seed

Soybeans have been consumed in Asian countries for centuries, and more recently in the West, as an important source of protein. During the past few years, a significant body of medical research has accumulated, which recognizes soybean as having a role in the prevention and treatment of chronic diseases (cancer, heart disease, kidney disease, osteoporosis). These effects have been attributed to a large extent to the phytochemicals called isoflavones, which occur naturally in soybean seeds and soy products. Recent studies have uncovered substantial differences in isoflavone levels among Canadian soybean varieties (Primomo et al. 2005a; Al-Tawaha and Seguin 2006).

The elucidation of the mode of inheritance of total as well as specific isoflavone contents is necessary to design an efficient and cost-effective breeding strategy for developing high isoflavone soybean varieties. The existence of 12 isomers, the complexity of the phenylpropanoid pathway by which they are synthesized and the high, often prohibitive cost of analysis using high performance liquid chromatography (HPLC) analysis have hampered such studies. Recent studies conducted at the Southern Illinois University (Njiti et al. 1999; Meksem et al. 2001) have reported the finding of several quantitative trait loci (QTL) on three molecular linkage groups associated with different isoflavones in the Southern soybean germplasm. In a number of studies, the Southern, later maturing, soybean gene pool has been found to be substantially genetically different from the Northern, short-season one, which is used in Northern US and Canada. Recently, the first report on the mapping of QTL in Northern soybean germplasm demonstrated that the loci and alleles in this germplasm pool may be different from those in the Southern one (Primomo et al. 2005b) This provides an opportunity for breeders to develop novel isoflavone profiles by combining the Southern and Northern germplasm.

Tocopherols have been associated with several health benefits, including a decrease in the incidence of prostate cancer among the subjects receiving α-tocopherol (vitamin E) compared to those not receiving it; gamma-tocopherol, found mostly in soy-based foods, appeared to promote prostate health by enhancing the effects of α-tocopherol and selenium (Fischer et al. 2001). In fact, it was suggested that gamma-tocopherol, which is the most prominent in soybean seed may have greater antioxidant benefits than its cousin, α-tocopherol (Rajcan et al. 2005).

Unlike maize tocopherols, which have recently been studied genetically (Wong et al. 2003), there is virtually no information on the genetic control of tocopherols in soybean at all. We have recently completed a study using a RIL population from a cross between parents with different tocopherol levels and profile to study the inheritance and map QTL for the trait in soybean seed (Wohleser 2006; Rajcan unpublished information). Considering the high market value of tocopherols, it is conceivable that breeding of lines with elevated levels of tocopherols may open new market opportunities for the soybean industry. Soybean breeding programs addressing this objectives will need to be developed.

3.7 Breeding Methods and Techniques

3.7.1 Gain from Selection

Soybean seed yield continues to increase in many of the world's soybean production areas. Soybean breeders have periodically evaluated genetic gains by growing soybean cultivars released over a period of time in common trials to quantify annual genetic improvements. Generally linear regression is used to estimate annual rates of gain. From a number of studies of northern US and Canadian cultivars, annual gains were estimated to be from about 10 to 30 kg ha^{-1} (Boerma 1979; Luedders 1977; Specht and Williams 1984; Voldeng et al. 1997; Wilcox et al. 1979). In examining sixty years of public cultivar development using cooperative test results (Uniform Soybean Tests, Northern Region), annual improvement rates were found to range from about 22 to 30 kg ha^{-1} (Wilcox 2001). A study of genetic progress using southern US soybean cultivars reported an annual improvement rate of 14 kg ha^{-1} (Ustun et al. 2001). Similar studies in India (Karmakar and Bhatnagar 1996) estimated an annual improvement rate of 22 kg ha^{-1}. Specht et al. (1999) also collected data from multinational private soybean breeding companies for maturity group II and III privately developed cultivars and reported an annual improvement rate of about 30 kg ha^{-1} which was triple the rate from their previous report using publicly developed cultivars (Specht and Williams 1984). Long term yield trends provide another avenue to explore soybean improvement. Specht et al. (1999) reported that US soybean yields increased at approximately 23 kg ha^{-1} annually over the period from 1928 to 1998. In the Midwest USA, during the period 1972 to 2003, county yield increases ranged from about 15 to 38 kg ha^{-1} annually (Egli 2008). Significant yield

increases were not seen in a number of countries growing non-irrigated soybeans nor in counties where high levels of double crop soybean were grown (Egli 2008).

When long term yield trends are examined, the yield improvements seen are a result of improved agronomy and production practices, environmental changes and improved genetics. Similarly to maize (Kucharik 2008), earlier planting of soybean is also likely responsible for some of the increased yield seen over time. The rising concentration of atmospheric CO_2 also plays a role in annual increasing yield. During the period from 1972 to 1997, it has been estimated that about 15% of on-farm soybean yield increase is due to increased atmospheric CO_2 (Specht et al. 1999). To determine the role that genetics plays in improved yield, examination of old and new cultivars in the same trials may be the most useful methodology.

3.7.2 Sources of Gains from Selection

From studies of short-season soybean in Canada, newer cultivars had smaller leaf area indices accompanied by increased leaf photosynthetic and stomatal conductance rates compared to older cultivars (Morrison et al. 1999). Yield improvements in newer cultivars have been the result of higher harvest index manifested in a greater number of seeds per plant (Morrison et al. 2000). Yield improvements in China were also found to be primarily due to increases in harvest index (Cui and Yu 2005). In a comparison of two older and two newer cultivars both an increase in harvest index and an increase in dry matter accumulation explained the yield improvement; however in this comparison, harvest index was less important than dry matter accumulation (Kumudini et al. 2001). Decreased lodging in newer cultivars has been reported in a number of studies (Specht et al. 1999; Ustun et al. 2001; Voldeng et al. 1997; Wilcox 2001), which may play a role in improved yield through maintenance of an upright, photosynthetically optimally-oriented canopy, ease of harvesting and reducing harvest losses.

Stress tolerance in maize has increased in more modern cultivars (Tollenaar and Wu 1999). In soybean, as plant populations increased from 33 to 100 plants m^{-2}, differences between older and new cultivars became more apparent (Specht et al. 1999). Similarly, genetic improvement rates were low at low populations and were maximized at plant stands of three to four times current commercial plant stands (Cober et al. 2005). Selection for higher yield may have indirectly contributed to selection for tolerance to stress resulting from higher plant populations.

3.7.3 Parent and Population Structure

Soybean breeders need to choose the type of cross, or the parental and population structure to use in hybridization for population development. Choices range from the simple biparental cross to complex crosses or even the case where strict pedigree information is lost to favor recombination as in recurrent

selection programs. To provide a snapshot of current practices of public soybean breeders, an analysis of cultivar and germplasm registrations in *Crop Science*, and cultivar descriptions in the *Canadian Journal of Plant Science* was undertaken with parent and population structure being summarized (Table 3.1). Publicly developed cultivars were overwhelmingly derived from biparental crosses. A very limited number of cultivars were developed from backcross-derived populations. In this case, the two introgressed traits were modified oil composition, and disease resistance. In the development of germplasm lines, more complex parental structures were used, although biparental crosses were still used about 80% of the time. Three way crosses were used to develop germplasm with modified oil composition, pest resistance and dense pubescence. Backcrosses were used to introgress pest resistance, and growth habit traits into established cultivars.

Table 3.1 Summary of soybean cultivar and germplasm descriptions published from 2004 to 2006 in *Crop Science* and *Canadian Journal of Plant Science*

	Cross type			Breeding method[1]			
	Bi-parental	Three way	Back-cross	SSD[2]	Pedigree	Bulk/Mass	EGT[3]
Cultivars							
Crop Sci.	30	0	2	24	2	0	5
CJPS	10	0	0	8	1	1	0
Total	40	0	2	32	3	1	5
Germplasm							
Crop Sci.	33	3	6	8	6	0	14

[1] It was not possible to determine the breeding method from all descriptions.
[2] Single seed descent or a modification of single seed descent.
[3] Early generation testing.

Parents may be selected from publicly or privately developed cultivars, released germplasm lines, plant introductions or unreleased breeding lines. In the US, it is increasingly common to find soybean cultivars with patent protection (Table 3.2). In the period from 1991 to 1995 only four cultivar patents were

Table 3.2 Number of patents for different crops issued by the United States Patent and Trademark Office resulting from a search for cultivar, variety, or hybrid, or inbred in patent titles

Crop	1986–1990	1991–1995	1996–2000	2001–2005
Wheat	0	0	0	7
Canola/rapeseed	0	0	0	10
Canola/rapeseed inbred	0	0	0	0
Corn/maize	0	5	103	93
Corn/maize inbred	6	63	308	263
Soybean	0	4	287	388

issued for soybean by the US Patent and Trademark Office, while ten years later 388 patents were issued during the period 2001–2005. The number of patents issued in the later period approximately equals the number issued for maize, and far exceeds the number issued for rapeseed/canola or wheat. A concern regarding variety patenting is the limit imposed on use of patented cultivars as parents. This limitation in use is commonly included in the patents issued. Soybean breeders in public and small or medium private institutions may have fewer unencumbered choices for parents in the future.

3.7.4 Advancing Toward Homozygosity

Following a successful cross and development of heterozygous plants, the soybean breeder needs to return the breeding material to the homozygous, or more accurately the near homozygous state. Breeders have two basic choices, rapid fixing of genes in soybean lines usually without any selection as it is done in the single-seed descent method, or practicing selection during the selfing generations. From an analysis of recent cultivar and germplasm registrations in *Crop Science*, and of cultivar descriptions in the *Canadian Journal of Plant Science*, single seed descent was the preferred breeding methods of public soybean breeders (Table 3.1). Pedigree breeding methods, with or without early generation testing, was a distant second choice of these breeders. The bulk method, with or without mass selection, was rarely used. When examining germplasm releases, a greater range of breeding methods were reported.

Single seed descent can be rigorously applied, where each pure line is developed from a different F_2 plant, since only a single seed is selected and advanced to the next generation. To save labour, the single seed descent method can be modified; single pods may be harvested and all of the seeds in the pod are advanced to the next generation. This modification is identified as single pod descent or modified single seed descent. To determine the effect of multiple seed descent resulting from generation advance of a pod rather than a single seed in grain legumes, a simulation study predicted that the practice should still result in every third F_6 plant being derived from a different F_2 plant (Macchiavelli and Beaver 2001). A further modification introduces selection against inferior genotypes during single pod descent. Single pod descent with selection, in this case for maturity, was found desirable since it reduced the size of the population advanced and produced superior lines (Destro et al. 2003). In a similar study pedigree selection, single seed descent, and single seed descent with selection for early maturity were compared, and single seed descent with selection was found to be the most cost-effective (Byron and Orf 1991). This modification of single seed descent would only be useful when generation advances are grown in environments where selection can be practiced and where the trait is highly heritable on a single plant basis.

Early generation testing is a procedure which tests heterogeneous families and then selects homozygous lines from superior families. The procedure gains

efficiency since it results in yield tests of fewer homozygous lines. In an evaluation of possible variants of early generation testing, F_2 derived families were suggested as the most efficient testing generation compared to F_1 or F_3 derived families (St. Martin and Geraldi 2002). Single rows can be used to yield test in early generations since they were good predictors of performance in multiple environment advanced yield trials (Hegstad et al. 1999). In an evaluation of optimum selection pressure for early generation families versus preliminary pure line testing, historical data from the Ohio State soybean breeding program was examined. An equal selection intensity was recommended across these stages of a breeding program (St. Martin and Futi 2000).

3.7.5 Participatory Plant Breeding

Participatory plant breeding is a new model for plant breeding which, while primarily focused on increasing the productivity of resource-poor farmers in the developing world, could be used throughout the agricultural sector. Most of this research is conducted in farmers' fields with farmers and researchers working side by side. Much information on participatory plant breeding has been reviewed and synthesized in a monograph by Weltzien et al. (2003). Beside the social benefits of participatory plant breeding, such as empowering citizens in rural communities, benefits in breeding may include increased productivity for niche producers in niche environments. Farmer participation is included to better understand farmer preferences and to meet end-user needs. Participatory plant breeding could be most beneficial where major crop commodity research is unavailable (orphan crops), where marginal production areas, or high genotype by environment interactions preclude development of a few widely adapted cultivars, or where crops have diverse end uses.

Most participatory plant breeding programs involve farmers testing genetically fixed, i.e. pure line materials. Many programs also involve farmers in setting plant breeding objectives while fewer programs involve farmers in selection within segregating generations. With the exception of on-farm county strip trials, which may include public or private experimental lines, to our knowledge there are no reports of participatory soybean breeding programs. On the basis of results from other crops (Kornegay et al. 1996), soybean breeders may find occasions where it would be advantageous to adopt participatory plant breeding strategies.

3.7.6 Selection Among Pure Lines

Selection among breeding lines approaching 'pure line status' or homozygosity begins with discarding inferior lines and ends with identification of a few superior lines that are moved to commercial production. Breeders need to

allocate resources among and within populations as well as within years of testing from preliminary to advanced trials.

From an evaluation of 30 lines in each of ten populations, it was recommended that a maximum of 15 lines per population should be tested to allow sampling of as many populations as possible (Helms et al. 2002). To evaluate resource allocation for preliminary trials, five test locations were used to verify selection based on either one or two locations in preliminary tests, and the use of a single replicated test at a single location (Helms et al. 2002) was recommended.

To deal with large preliminary trials and the spatial variation that can be present within these trials, a number of statistical approaches are available. Nearest neighbor analysis or lattice designs were found more efficient, compared to randomized complete block designs, in reducing error variance in soybean experiments (Vollmann et al. 1996). Helms et al. (1995), however, did not find nearest neighbour adjustments useful compared to unadjusted means for yield selection in two populations.

3.7.7 Intra-cultivar Variation

While cultivars of self-pollinating crops are regarded as pure lines or highly homogeneous, there is some suggestion that variation within cultivars could be exploited as suggested by Rasmusson and Philips (1997) in barley and by Tokatlidis et al. (2004) in wheat. In soybean, three cultivars were studied for intra cultivar variation for seed composition, seed oil and protein levels, and significant differences were found among lines within each cultivar for seed composition (Fasoula and Boerma 2005). A further examination of the three cultivars found intra cultivar variation for seed weight and time to maturity (Fasoula and Boerma 2007). The authors suggest that intra cultivar selection could be carried out to retain uniformity within cultivars or that the newly uncovered variation could be used for further improvements.

3.7.8 New Technology in Plant Breeding Operations

Technological advances continue to be made in areas that affect plant breeding operations. Gains in efficiency allow more plots to be grown with the same amount of personnel or diversion of personnel to new areas of research.

Software programs, either commercially developed or written by the individual breeder, are used throughout breeding programs. Tasks managed by software include seed inventory, experimental design, field planning, generation of field books, data logging, experimental analysis, and multiple location and year analysis. A major improvement involves the use of relational databases compared to flat file systems. Flat file systems involve separate files for each experiment and a simple task such as a name change is required to be made in

each separate file; while in a relational database, a single name change results in the name being changed in all locations throughout the system. Laboratory information management systems (LIMS) are software programs which manage samples, instruments and dataflow. Near-infrared reflectance spectroscopy instruments, balances, and other data collection devices can be interconnected which reduces chances for errors in data transcription and aids in data collection efficiency.

Data management has been improved through less direct entry of identifiers or data observations. Postal equipment can be used for direct printing on seed envelopes or bags. Bar code readers can be used to directly enter plot numbers from bar codes on bags or tags. As well bar code readers can be used to enter data when data options are preprinted on summary sheets, for example, hilum colours are entered through an operator 'shooting' the appropriate hilum colour bar code. Radio frequency identification (RFID) tags can replace barcodes and barcode readers. RFID pot labels are available and used in the horticultural industry. RFID tags are being tested on individual seed packets, as a replacement for barcodes, where all seed envelopes in a shipping box could be read by placing the box near a RFID reader.

Improvements in harvesting technology allow faster harvesting and reduced post-harvest processing. Combine harvesters are available which can harvest and yield test individual progeny rows and make a selection or rejection decision based on predetermined parameters. Small plot combines can be equipped with on board weighing systems, as well as on board near-infrared equipment to determine moisture, protein and oil content. Plot combines can also be equipped with samplers which will sub-sample the plot harvest, then package and label the sample. Developments in in-crop canopy scanning technology may allow prediction of yield before harvest because of the significant relationship between normalized difference vegetation index (NDVI) at pod development and early seed filling and seed yield (Ma et al. 2001). This technology could allow rejection of lines before harvest and reduce use of resources for harvesting.

3.8 Integration of New Biotechnologies into Breeding Programs

The accelerated production of biodiesel in North America has prompted greater attention by breeders to target increases in total crude oil per hectare of soybean production. Pantalone et al. (2004) outlined a series of quantitative trait loci (QTL) that govern seed oil content. Some of those QTL are environmentally stable, while others are sensitive to particular types of environmental growing conditions. However, few of the oil QTLs have been confirmed. Opportunities exist to confirm oil QTL to facilitate use of this biotechnology for soybean breeders for increasing total oil. Moreover, examining the impact that accumulation of environmentally stable oil QTLs have on seed oil

concentration would make further contributions to existing knowledge. This information, together with measurements of soy meal characteristics will lead to opportunities to develop new varieties more suitable to biodiesel production in major soybean producing regions, while maintaining the quality of the soy meal for livestock and human nutrition industries.

Recent discoveries in molecular markers will help direct strategies for improving the quality of soy oil with respect to altered fatty acid profiles. Those discoveries are inherently based upon germplasm developed for modified fatty acid composition.

3.8.1 Reduced Saturates – Germplasm and Biotechnologies

The germplasm line N79-2077-12, developed by USDA-ARS, Raleigh, NC was the first low 16:0 soybean (Wilson 2004; Burton et al. 1994). It has the recessive genotype $fap_{nc}fap_{nc}$ which reduces the C16:0 content to about half that of normal soybean. The same level of 16:0 reduction was found in the germplasm ELLP2 which has the *fap*fap** genotype. A different allele is found in germplasm C1726 which has the genotype fap_1fap_1 which does not reduce the 16:0 as effectively as the other two alleles. However if fap_1fap_1 is coupled with $fap_{nc}fap_{nc}$ as in the case of both N94-2575 and C1943 germplasm lines, then the collective reduction of 16:0 is about one-third that of normal soybean. This low level of palmitate can alternatively be realized by a different single allele fap_3fap_3 as occurs in the germplasm A22 (Wilson 2004). Breeders have assembled various combinations of these alleles to more modern adapted germplasm and elite lines.

Cardinal et al. (2007) demonstrated that the low 16:0 phenotype fap_{nc} was due to a deletion in the GmFATB-1A gene. The authors developed allele-specific PCR primers for this gene, and they also showed that on average, there is a yield reduction when a line is homozygous for the fap_{nc} allele. Allele-specific primers are perfect matches between the DNA marker and the existence of the trait. Such markers are highly valuable to breeders who can develop larger populations with the intent to find high yielding types among the group of individuals that carry the specific DNA marker. Moreover, breeders who are successful in combining high seed yield with low 16:0 have unique genetic resources that can be further utilized for oil improvement (see major breeding achievements section, above).

3.8.2 Increased Saturates – Germplasm and Biotechnologies

Wilson (2004) reported that germplasm homozygous recessive for fap_2 (C1727), fap_{2b} (A21), fap_4 (A24) or fap_5 (A27) produce oil with 150% or greater 16:0 than normal soybean. Stearic acid concentration in soybean may be genetically

increased by mutations at the *Fas* gene locus (Spencer et al. 2003). Most of these variants have been induced by chemical or X-ray mutagenesis. An exception is the USDA germplasm line, FAM94-41 (9% C18:0) which carries a natural mutation, temporarily designated as the recessive fas_{nc} allele (Pantalone et al. 2002). Spencer et al. (2003) detected a major QTL on linkage group B2, near the *Fas* locus governing increased 18:0 concentration from FAM94-41. Five additional germplasm lines were reported to carry homozygous recessive alleles: fas^a [A6, (Hammond and Fehr 1983)], fas^b [FA41545, (Graef et al. 1985)], *fas* [A81-606085, (Graef et al. 1985)], st_1 [KK-2 (Rahman et al. 1997)] or st_2 [M25, (Rahman et al. 1997)] that influence 18:0 concentration in soybean oil. Increases in 16:0 and 18:0 would be of benefit for the margarine industry in order to produce low trans-fat tub margarine.

3.8.3 Increased Monounsaturates – Germplasm and Biotechnologies

The germplasm line N78-2245 was the first soybean developed with elevated C18:1 (approximately 42%), nearly twice that of normal soybean (Wilson 2004). This line contains a natural mutation in the FAD2-1 gene encoding the predominant ω-6 desaturase. The FAD2 gene was clearly shown to govern soybean 18:1 concentration. Hitz et al. (1995) expressed it in antisense orientation, producing up to 80% 18:1. Also, a soybean line with natural gene combinations was achieved with >70% 18:1 (Burton pers. comm.). Recently Burton et al. (2006) registered the germplasm line N98-4445A (60% 18:1) in the USDA active germplasm collection, making it freely available to breeders targeting improvement in 18:1 concentration. Breeders working with the BBI embarked on a challenging mission over the last few years to rapidly develop new cultivars with elevated 18:1 content. The goal is for breeders to utilize the new USDA germplasm line, N98-4445A (Burton et al. 2006) and introgress six QTL loci that collectively govern mid-18:1 (50–65%). QTL are genomic regions genetically linked to a trait, and typically several QTL (several genes) need to be accumulated together in a single individual for full trait expression. In the mid-oleic soybean project, the goal is to assemble three QTL in a backcross line and three complementary QTL in the same backcross line and make a convergent cross after the BC5 stage. These six QTL are represented by simple sequence repeat (SSR) markers in the plant DNA. Additional work led to the discovery of single nucleotide polymorphisms (SNP) associated with the mid-18:1 trait. These SNPs are single base-pair changes in the DNA, and as SNP maps are developed and refined (Choi et al. 2007), this new technology will serve as an excellent selection mechanism to improve oil quality.

3.8.4 Reduced 18:3 – Germplasm and Biotechnologies

A major commercial thrust targets low C18:3 soybean development to meet the demands of reduced trans-fat vegetable oils and food products. The first reduced 18:3 germplasm line was N78-2245 with 6% 18:3 (Burton et al. 1989) which was produced through recurrent selection for increased 18:1 concentration. Wilcox et al. (1984) used EMS to create a recessive *fanfan* C1640 germplasm line with approx 3.5% 18:3. Germplasm line A5 was also created by chemical mutagenesis producing the fan_1fan_1 genotype, conferring approximately 4% 18:3 (Hawkins et al. 1983). Fehr et al. (1992) were able to combine two additional mutations forming germplasm line A29 with three recessive alleles $fan_1fan_1fan_2fan_2fan_3fan_3$. This genetic material fostered a recent discovery (Bilyeu et al. 2003) of three different isoforms of omega-3 desaturase genes. This lead the authors to develop 'perfect' molecular markers designed from the actual DNA sequence, which allows molecular breeders to use DNA to select individuals carrying one or more of the low 18:3 genes with complete accuracy. Accumulation of the three recessive genes collectively will reduce 18:3 concentration in soybean oil to 1%. Plans are currently in progress for a USDA release of a germplasm line containing two publicly available recessive alleles (Bilyeu pers. comm.). New commercial soybean cultivars containing all three recessive alleles are currently in production (Fehr pers. comm.).

3.8.5 Increased Polyunsaturated Fatty Acids – Germplasm and Biotechnologies

Soy oil components are routinely used in inks, coatings, and resins. Increasing the C18:3 concentration would enhance these industrial products. Currently no improved germplasm have been reported with elevated 18:3. However the USDA germplasm collection contains more than one hundred accessions of *Glycine soja*, the wild relative of soybean, that exhibit 18:3 concentrations more than twice that of cultivated soybean (*Glycine max*). The wild species is fully sexually compatible with soybean, allowing gene transfer for enhanced polyunsaturated fatty acids to be realized. Pantalone et al. (1997a) made interspecific hybridizations between N87-2120-3 (*G. max*) x PI342434 (*G. soja*) and PI424031 (*G. soja*), identifying a wide range in ω-6 desaturation and ω-3 desaturation among progeny, suggestive that the wild species carries alternative forms of *Fad* and *Fan* alleles. The recombination of alternative desaturases with *G. max* alleles acted in an additive genetic manner, producing higher 18:3 concentration in the oil (Pantalone et al. 1997b; Rebetzke et al. 1997). Opportunities exist to create mapping populations targeting the discovery of QTL for unique *G. soja* alleles, and use marker-assisted selection specifically to target increased 18:3 for industrial uses.

3.8.6 Oil Constituents with High Value

3.8.6.1 Sterols

The modern soybean oil refining process allows capture of stigmasterol, a compound that is well valued for its commercial synthesis of steroidal hormones and pharmaceutical products (Wilson 2004). Stigmasterol, along with campesterol and β-sitosterol are three secondary alcohol phytosterols that are present in soybean oil. Stigmasterol occurs in highest concentration. Although sterol concentration in the oil of soybean seeds is positively correlated with growth temperature, there is little variation for breeders to take advantage of. Little is known of alleles that influence the genetic regulation of soybean sterols and these compounds typically are not traits targeted for improvement by breeders. Nevertheless, Wilson (2004) showed a significant negative correlation between stigmasterol concentration and 18:3. Indeed when 18:3 dropped from 10 to 4% of lipid concentration, stigmasterol more than doubled. Thus breeders working on developing low 18:3 lines to cultivar status may have opportunities to verify stigmasterol concentration to document enhancement of this valuable oil constituent for the commercial oleochemical industry.

3.8.6.2 Tocopherols

Soybean oil contains delta, gamma, and alpha forms of tocopherol. The α-tocopherol form is natural vitamin E, and soybean is the leading commercial source of this vitamin. Tocopherol compounds protect polyunsaturated fatty acids from oxidation. As antioxidants, the Δ form is more effective than the γ form, followed by the α form of tocopherol (Wilson 2004). Despite the presence of ~1,000–2,000 mg kg^{-1} of tocopherols in soybean oil following the refining process, the oil still requires hydrogenation to maintain adequate oxidative stability (Wilson 2004). This suggests that genetic improvement of this oil constituent would augment breeders targeted oil quality goals. For example, Wilson (2004) indicated that soybeans with genetically reduced 18:3 showed greater α-tocopherol, yet lower γ-tocopherol. The decline in oxidative stability from the lowered γ-tocopherol could be offset if the increased 18:1 trait was coupled with reduced 18:3, and improved processing efficiency of vitamin E would be a favorable constituent product.

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Chapter 4
Oilseed Rape

Wolfgang Friedt and Rod Snowdon

4.1 Introduction

Oilseed rape, or canola (*Brassica napus* ssp. *napus*; genome AACC, $2n = 38$) is today the world's third-leading source of both vegetable oil and oil extraction meal. Total world consumption of vegetable oil amounts to approx. 97 million metric tons (2003), of which 27.9 Mt is soybean oil, 27.8 Mt palm oil, 12.1 Mt rapeseed oil, 8.0 Mt sunflower oil, 5.8 Mt peanut oil, and 4.9 Mt cottonseed oil. Due to its favourable seed oil composition, low-erucic acid rapeseed or canola oil is a valuable source of nutritional oils and fats (salad oil, margarine). For example, from the total rapeseed oil produced and processed in Germany (approx. 2.5 Mio. t) about 0.5 Mio. t are used for nutritional purposes. The majority, however, is used for producing transportation fuel; around 1.5 Mio. t are processed into biodiesel (rapeseed oil methyl ester, RME) while some 0.5 Mio. t of processed oil are directly used in diesel engines of tractors or lorries (Thywissen, personal communication). The demand and use of vegetable oil for non-food purposes is currently rising world-wide. A rough overview on the major procedures for the production of biofuels from vegetable oils is presented in Fig. 4.1 (source: http://www.biofuelstp.eu/fuelproduction.html; see also Demirbas 2007).

4.2 Origin and Domestication

The species *Brassica napus* L. originated through spontaneous interspecific hybridisation between turnip rape (*Brassica rapa* L., syn. *campestris*; genome AA, $2n = 20$) and cabbage (*Brassica oleracea* L.; genome CC, $2n = 18$), resulting in an amphidiploid genome comprising the full chromosome complements of its two progenitors. Because no wild *B. napus* forms are known, it is assumed that

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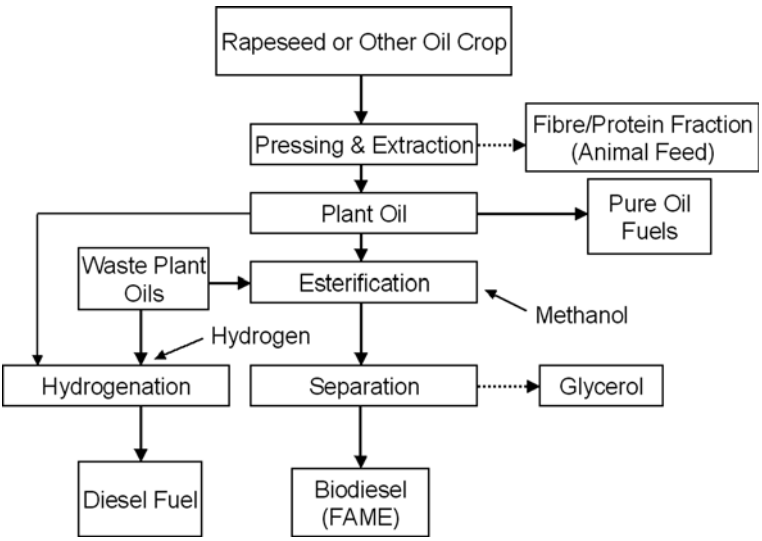

Fig. 4.1 Schematic overview on the production of biofuels from vegetable oils (image re-drawn from source: http://www.biofuelstp.eu/fuelproduction.html)

the species appeared relatively recently when the parental species began being cultivated in geographical proximity. The occurrence of spontaneous chromosome doubling in crosses among closely-related *Brassica* diploid species is well documented; the related amphidiploids Indian or brown mustard (*Brassica juncea*; genome AABB, $2n = 36$) and Abyssinian or Ethiopian mustard (*Brassica carinata*; genome BBCC, $2n = 34$) arose in the same manner after crosses of black mustard (*Brassica nigra*; genome BB, $2n = 16$) with *B. rapa* and *B. oleracea*, respectively.

Brassica vegetables and oilseeds were among the earliest plants to be systematically cropped by mankind. There are indications that a vegetable crucifer was widely cultivated as early as 10,000 years ago. In India records have been identified which suggest that oilseed brassicas (probably *B. rapa*) were being used as early as 4000 BC, and 2000 years ago their use had spread into China and Japan. Swedes (*B. napus* ssp. *napobrassica*) were known in Europe at the time of the Romans, and utilization (probably of *B. rapa*) for oil purposes in northern Europe is thought to have begun around the 13th century. By the 16th century, rapeseed was the major source of lamp oil in Europe, although it was not until the 18th century that significant cultivation areas of the crop were recorded (Kroll 1994; Kimber and McGregor 1995). For winter oilseed rape only three distinct local landraces are known. These evolved in different European climate zones and hence display variation in vegetative growth and winter hardiness. The first released cultivar 'Lembkes', selected in Germany from a Mecklenburg landrace in the early 20th century, was extensively exploited in French, Swedish, German and Polish breeding

programs. Spring-sown oilseed rape was first grown in Canada in the mid 20th century. Large-scale worldwide production of oilseed rape did not begin until the mid 1970s, however, when the value of rapeseed oil and seed meal was significantly improved as a result of intensive breeding efforts (see below).

4.3 Varietal Groups

The primary gene pool of oilseed rape is separated into two distinct *B. napus* subspecies, comprising on the one hand the swedes (*B. napus* ssp. *napobrassica*) and on the other hand *B. napus* ssp. *napus*. The latter includes winter and spring oilseed and fodder rape forms, along with distinct leaf rape forms (*B. napus* ssp. *napus* var. *pabularia*) that used to be common as a winter-annual vegetable in many parts of the world (Siberian kale, Hanover salad; German: Schnittkohl; French: chou à faucher; Chinese: xi yang you cai). Oilseed forms of *B. napus* are cultivated in Europe and Asia predominantly as winter rapeseed, whereby in Australia, Canada, and northern Europe only spring forms are suitable. The differentiation into winter and spring forms is governed by a genetic mechanism controlling the requirement for vernalisation to promote the onset of flowering. Spring oilseed rape does not require vernalisation and is not winter-hardy, hence the crop is sown in spring and stem development begins immediately after germination. Winter oilseed rape on the other hand is sown in autumn and survives the winter in a leaf rosette form on the soil surface. In the following spring a long vertical stem develops, and shortly before the floral development lateral branches are formed. Flowering generally occurs in late spring, with pod development and ripening taking place over a period of around 6–8 weeks until mid-summer. In contrast to *B. rapa* and *B. oleracea*, its diploid progenitors which often display self-incompatibility, *B. napus* is a facultative outcrossing species with a high degree of self-pollination. When insect pollinators are abundant a greater proportion of cross-pollination can occur.

4.4 Genetic Resources

4.4.1 Genetic Diversity in the Primary Gene Pool

Modern oilseed rape breeding material has a relatively narrow genetic diversity (Hasan et al. 2006). This is attributable to a combination of geographical constraints and selection bottlenecks during the origin of the species and its subsequent domestication. In particular, the zero erucic acid and low glucosinolate seed quality traits (00-quality) carried by the vast majority of modern varieties (canola varieties) originate from single sources, namely the spring cultivars ‘Liho’ and ‘Bronowski’, respectively. Consequently, there is a need

to introduce new genetic variation to breeding material, since most early 00-quality cultivars shared a more or less common parentage (Thompson 1983; Downey and Rakow 1987).

Owing to their generally unsuitable seed characters, however, in particular high contents of seed erucic acid (C22:1), glucosinolates, and other anti-nutritive substances, fodder and vegetable rape forms have been generally overlooked for breeding of oilseed cultivars in recent decades. On the other hand, such material represents a potentially valuable source for improved pathogen and pest resistance, and introduction of untapped germplasm into breeding lines also has the potential to improve heterosis in hybrid breeding. However, the construction of distinct genetic pools, as used for example in maize hybrid breeding, is still in progress in commercial breeding efforts.

Due to the large number of closely related cruciferous crop species an enormous variety of germplasm resources are available in international gene bank collections for evaluation and introgression of traits of agronomical interest into oilseed *Brassica* breeding material. Such resources have often been successfully used in particular for the identification and interspecific transfer of novel disease resistances to oilseed rape breeding lines. The number and diversity of available *Brassica* gene bank accessions can make it difficult to identify relevant germplasm, however, even within the primary and secondary gene pools. Well-characterised core collections, that represent as much as possible of the available diversity within a manageable number of genotypes, are therefore a valuable resource for utilization of novel germplasm in breeding efforts.

Compared to the narrow gene pool of present 00-quality oilseed rape breeding material, erucic acid- and glucosinolate-containing plant material represents a comparatively genetically divergent source for the development of heterotic rapeseed forms (see Röbbelen 1975; Thompson 1983; Schuster 1987). Because of the emphasis on oil quality, such material has found only limited use in practical rapeseed breeding in the past few decades. However, strong heterotic effects are observed in experimental crosses between material of distant geographical and genetic origin (Lefort-Buson et al. 1987; Brandle and McVetty 1990), and efforts are increasing to develop new cytoplasmic-genetic male-sterile and restorer lines as the most promising system for the production of new hybrid cultivars. Following appropriate quality conversion, inbred lines and DH lines with a high genetic distance to existing 00-quality varieties have the potential to become an important resource for the development of high-performance pools with improved combining ability compared to existing 00-rapeseed material.

Within the framework of a recently completed European project '*Brassica* collections for broadening agricultural use', extensive collections of *Brassica* accessions from European gene banks were evaluated for morphological diversity and selected agronomic traits (van Soest et al. 2004). Within four international sub-groups, the available gene bank material for *B. oleracea*, *B. rapa*, *B. napus* and *B. carinata* was documented, characterised, evaluated and rationalised to generate core collections representing as much as possible the range

of available diversity useful for *Brassica* crop breeding. Passport data for over 3,500 *B. napus* accessions are available in the ECP/GR Central Crop Database (BrasEDB: http://www.cgn.wur.nl/pgr/collections/brasedb/). A preliminary core collection of 200 accessions was selected, covering the different systematic groups, using various evaluation criteria (Poulsen et al. 2004). Particular emphasis was placed on the geographic origin of the material, with the collection being selected to represent as much as possible the diversity present in all of the countries for which accessions were available. Minimum descriptors for numerous morphological characters were characterised, and together with data from agronomical evaluations this information has been made available on the BrasEDB website. In order to facilitate the use of the core collection for oilseed rape breeding purposes, a number of relevant resistance and seed quality traits were evaluated in the preliminary core collection in field and greenhouse trials. Around 1,100 accessions were analysed for various seed quality characters, and together the morphological, resistance and quality data were used to reduce the core collection to around 150 accessions that cover the broad variation in agriculturally important traits (Lühs et al. 2003; Poulsen et al. 2004). A subset of the preliminary core collection was also genotyped using SSR markers to evaluate the extent of genetic diversity in the oilseed-type accessions (Hasan et al. 2006). Collectively the final core collection comprises a genetically diverse set of genotypes with wide variation for numerous traits of agronomic interest. Because commercial oilseed rape breeders were directly involved in the evaluation and selection of the material, these accessions represent a valuable resource for more detailed screening of further traits of interest with regard to introgression of novel germplasm into canola and oilseed rape breeding material (Poulsen et al. 2004).

Whilst such collections are valuable in identifying 'hotspots' of variation within the relevant gene-pools, they usually consist of heterogeneous and heterozygous breeding material. This limits their long-term use for correlating detailed genetic studies. To overcome these problems British researchers and breeders are currently developing a homozygous *B. napus* 'Diversity Fixed Foundation Set' (DFFS) for long-term molecular and trait exploration in the oilseed rape gene-pool. The DFFS lines, fixed from founder lines by haploid techniques or inbreeding by single-seed descent, are defined as 'an informative set of genetically fixed lines representing a structured sampling of diversity across a genepool'. The fixed lines will be multiplied and archived and will be available for distribution (for more information see http://www.brassica.info/resource/plants/diversity_sets.php/).

4.4.2 Expanding Genetic Variability by Interspecific Hybridisation

One strategy to broaden the genetic basis of oilseed rape breeding material is the production of resynthesised (RS) rapeseed by crossing the original ancestors,

B. oleracea and *B. rapa*. This has the potential not only to increase genetic variability with a view to hybrid breeding, but also to broaden the genetic base in *B. napus* with respect to pest and disease resistances. For such interspecific hybridizations a variety of biotechnological tools, for example embryo rescue techniques or protoplast fusion, are used to circumvent incompatibility barriers. In some cases RS rapeseed forms have resulted in successful release of cultivars carrying novel resistance genes from the diploid species. For example, Diederichsen and Sacristan (1996) successfully used protoplast fusion to transfer resistance to clubroot (*Plasmodiophora brassicae*) from *B. oleracea* to *B. napus*. Through advanced backcrossing a race-specific resistance was subsequently transferred from RS rapeseed progeny to elite winter oilseed rape, and the winter oilseed rape varieties 'Mendel' (Table 4.1) and 'Tosca' derived from this material were released in the early 2000s to specifically combat this disease in affected areas of Great Britain and Germany. In another example, Mithen and Magrath (1992) generated synthetic lines of *B. napus* carrying resistance to blackleg disease (*Leptosphaeria maculans*, anamorph: *Phoma lingam*) derived from *B. rapa* via embryo culture. The resistance was then integrated successfully into spring canola, resulting in the release of the cv. 'Surpass' in the late 1990s and subsequent efforts to introgress this resistance into winter oilseed rape material. This *Phoma* resistance from *B. rapa* has in the meantime been overcome by virulent *L. maculans* isolates in Australia, and the clubroot resistance from *B. oleracea* is also race-specific and hence not durable without careful agronomic management (e.g. crop rotation). Nevertheless, these examples demonstrate the potential utility of *B. oleracea* and *B. rapa* for the identification and combination of novel resistance genes to important oilseed rape pathogens.

Table 4.1 Breeding oilseed rape cv. 'Mendel' resistant to clubroot (*Plasmodiophora brassicae*) through introgression of clubroot resistance from *Brassica rapa*

Breeding stage	Breeding material/activity
Sources	Resynthetic rapeseed '1543' (ECD-04 × ECD-15)
	ECD-04 = *B. rapa* ssp. *rapifera* (resistent)
	ECD-15 = *B. oleracea* var. *acephala* cv. Verheul
Backcross to modern oilseed rape	(Falcon × '1543') × Falcon
Production of DH-lines	3,437 lines (NPZ-lab + SU-lab)
	Selection for resistance, 00-quality, and seed yield
Male parent	Selection of Bl. 6431/96 as male parent
New F_1 hybrids	with females MSL004C and MSL007C, e.g. cv. 'Mendel'

This strategy has the potential to prove particularly valuable for development of resistance to *Verticillium* wilt. This disease, caused by the host-adapted pathogen *V. longisporum*, causes serious yield losses in affected areas of Sweden, Denmark, Great Britain and the north of Germany. The fungus forms microsclerotia which can persist in the soil for more than a decade, and because

accredited fungicides are not available the only current alternative for effective control of the disease in short crop rotations is the breeding of resistant cultivars. Very little resistance is available in either winter or spring rapeseed, thus necessitating a search for resistance sources in related species. Transfer of resistance from *B. oleracea* to *B. napus* was reported by Happstadius et al. (2003), while Rygulla et al. (2007a,b) reported the combination of resistances from *B. oleracea* and *B. rapa* in novel RS *B. napus* genotypes by interspecific hybridization, assisted by embryo rescue. After characterizing the resistance by genetic mapping (Rygulla et al. 2008) it should be possible using marker-assisted backcrossing to simultaneously transfer A- and C-genome resistance genes into elite rapeseed lines, as a starting point for the development of new cultivars with combined resistance from the diploid progenitors.

Interspecific crosses are also an important source of seed colour variants for breeding of light-seeded *B. napus*. Brown or yellow seeds are of particular interest for breeding of oilseed rape because of their association with a thinner seed coat resulting in reduced dietary fibre content. This considerably improves the feed quality of rapeseed meal after oil extraction (Shirzagedan and Röbbelen 1985; Slominski et al. 1994, 1999). Light seed colour and low fibre content are considered to coincide because the biochemical pathways leading to lignin (fibre) and pigment synthesis have common precursors such as pcoumarate (Theander et al. 1977; Whetten et al. 1998). Furthermore, the reduction in testa thickness in yellow-seeded oilseed rape has also been found to be associated with increased seed oil and/or protein content per dry weight (Xiao and Liu 1982; Piotrowska et al. 2003). A variety of different yellow-seeded rapeseed materials has been generated by interspecific crosses between yellow-seeded *B. rapa* and brown-seeded *B. oleracea* (Schwetka 1981) or *B. alboglabra* (Chen et al. 1988; Rahman 2001, 2003). The yellow-seed trait has also been introduced to *B. napus* from *B. chinensis* (Liu 1983), *B. juncea* (Rashid et al. 1994) and *B. carinata* (Rashid et al. 1994; Meng et al. 1998; Rahman 2001, 2003).

Other *Brassica* species and even less closely-related genera are also important as potential sources of disease resistance for oilseed rape breeding. A prime example for this is the use of interspecific and intergeneric hybrids as a source for new resistance against blackleg disease. The genetic basis of blackleg resistance in *B. napus* in European cultivars originates for the most part from the French cultivar 'Jet Neuf', characterized by a polygenically controlled adult plant resistance not expressed at the seedling stage (Cargeeg and Thurling 1980). In contrast, all *Brassica* species containing the B genome exhibit an absolute and stable resistance to most of the aggressive pathogen isolates studied to date; B genome resistance is mono- or oligo-genically controlled (see Rimmer and van den Berg 1992; Dixelius 1999) and efficient from the seedling stage onwards. Thus, B genome donors like *B. nigra* (L.) Koch (BB, $2n = 16$) and *B. juncea* (L.) Czern (AABB, $2n = 36$) have been extensively used as genetic pool in an attempt to develop resistant oilseed rape (e.g. Roy 1978; Sacristán and Gerdemann 1986; Sjödin and Glimelius 1989; Chèvre et al. 1996; Plieske et al. 1998; Dixelius 1999). On the other hand,

some aggressive isolates of the pathogen have been shown to overcome the resistance of *B. juncea* (Purwantara et al. 1998). *Leptosphaeria maculans* exhibits a broad variation in virulence, giving it the potential to adapt quickly to a given resistance (Kuswinanti et al. 1999). Generation of durable resistance therefore necessitates the application of a broad spectrum of resistance sources in oilseed rape breeding. For this reason, interspecific and intergeneric transfer of blackleg resistance from wild crucifers is an interesting alternative, and in recent years progress has been made to introgress resistance into oilseed rape from different sources, including *Sinapis arvensis* (Snowdon et al. 2000; Winter et al. 2003) and *Coincya monensis* (Winter et al. 2003). Other examples of intergeneric hybridisation for resistance gene transfer into *B. napus* include resistance to beet cyst nematodes on *Raphanus sativus* addition chromosomes (Thierfelder and Friedt 1995; Voss et al. 2000), whereas Klewer et al. (2003) used sexual and somatic hybridisation in an attempt to transfer resistance to *Alternaria* blackspot into *B. napus* from *B. elongata, Sinapis alba, Diplotaxis tenuifolia* and *D. erucoides*. In such broad intergeneric hybrids ovary culture techniques are absolutely necessary to overcome incompatibility barriers, however a successful transfer of the desired trait is sometimes achieved. The prerequisite for this is that intergenomic chromosome recombination takes place in early backcross generations before the loss of non-homologous donor chromosomes.

4.5 Major Breeding Achievements

Oilseed rape has become a major international crop only over the course of the past three decades. This rapid advance to one of the major arable crops is a result of spectacular breeding success. The oil from rapeseed and most other brassicas naturally contains a high quantity of erucic acid (C22:1, *cis* 13-docosenoic acid), which has a bitter taste and in high doses has been implicated in cardiac health problems. This serious limitation to rapeseed as a foodstuff was overcome only by the development of '0' and '00' rapeseed varieties in the 1970s (Stefansson 1983; Downey and Röbbelen 1989; Downey 1990). The first major breakthrough came with the initial 0-quality cultivars with erucic acid levels of less than 1% (Stefansson and Hougen 1964). Earlier rapeseed cultivars contained up to 50% erucic acid in the seed oil (Table 4.2). The identification of the fatty acid mutants from which the first 0-rapeseed derived was made possible by major improvements in high-throughput seed analysis techniques, in particular gas chromatography. The first erucic acid-free variety, derived from a spontaneous mutant of the German spring rapeseed cultivar 'Liho', was released in Canada in the early 1970s. The value of the crop was still suppressed by the presence of high quantities of glucosinolates in the seed, however, which made rapeseed meal unsuitable as a livestock feed. In monogastric animals the digestion of glucosinolates results in the release of toxic by-products that can cause liver and kidney damage

Table 4.2 Variation of seed lipid composition in different types of oilseed rape (*B. napus*)

		Fatty acid[1] composition (%)									
Oil type	Breeding method	12:0	14:0	16:0	18:0	18:1	18:2	18:3	20:1	22:1	others
Canola (00-quality rapeseed oil)	Mutation and breeding	–	–	4	2	60	21	10	–	–	3
High lauric	Genetic engineering	37	4	3	1	33	12	7	–	–	3
High myristic	Genetic engineering	–	18	23	2	34	15	4	–	–	4
High stearic	Genetic engineering	–	–	4	29	15	19	22	1	–	10
High oleic (HO)	Mutagenesis	–	–	4	2	80	5	5	2	–	2
High oleic (HO)	Genetic engineering	–	–	4	1	84	5	3	1	–	1
Low linolenic	Mutagenesis	–	–	4	2	61	28	3	1	–	1
Low linolenic	Genetic engineering	–	–	4	2	68	22	1	1	–	2

[1]Major fatty acids: C12:0 lauric, C14:0 myristic, C16:0 palmitic, C18:0 stearic, C18:1 oleic, C18:2 linoleic, C18:3 linolenic, C20:1 eicosenic, C22:1 erucic acid

along with lymph dysfunction. In 1969 the Polish spring rape variety 'Bronowski' was identified as a low-glucosinolate form, and this cultivar provided the basis for an international backcrossing program to introduce this polygenic trait ('Bronowski' was found to possess at least three recessive genes for low glucosinolate content) into high-yielding erucic acid-free material. The result was the release in 1974 of the first 00-quality spring rapeseed variety, 'Tower', with zero erucic acid and low glucosinolate content, and thus marking the begin of the advance of oilseed rape (canola) to one of the most important oil crops in temperate regions in the following decades.

Another important achievement regarding seed oil quality is the development of high oleic acid, low linolenic acid (so-called HOLL or HOLLi) types. The oil of varieties such as 'Splendor' or 'Nexera' is characterized by oleic acid contents of more than 75% and linolenic acid contents of less than 3% (Table 4.2). This gives the oil a substantially higher oxidative stability, which is particularly beneficial when used as a frying oil because the formation of deleterious *trans*-fatty acids is strongly reduced. These new varieties are the result of experimental mutagensis and conventional selection, where at least three major genes had to be modified to achieve the HOLLi phenotype. Therefore, it is not surprising that this phenotype is associated with a yield penality due to linkage drag. However, intensive breeding activities using molecular breeding tools are expected to provide new high yielding HOLLi oilseed rape cultivars in the near future.

With regard to enhancing the seed yield potential of rapeseed, the development of functional male sterility systems for the production of true hybrid seed has definitely been an enormous achievement. Present-day rapeseed hybrids are single crosses based on two parental inbreds. For developing male sterile females, both systems of cytoplasmic male sterility (e.g. the Ogura CMS introduced from radish) and genic male sterility (GMS, controlled by nuclear genes only) are commercially used, particularly in Europe. The first winter oilseed rape hybrid varieties were registered in 1995 (Frauen and Paulmann 1999) and are now covering a large part of commercial (winter) rapeseed production in Europe.

The Male Sterility Lembke (MSL) GMS system is based on a spontanous mutant selected in the nursery of the German breeding company Norddeutsche Pflanzenzucht HG Lembke in the early 1980s. The MSL system allows the production of fully restored rapeseed hybrids without any yield or quality penalty, and all *B. napus* genotypes function as restorers. The INRA-Ogura CMS system from radish (Ogura 1968), introduced to *B. napus* by INRA, France, relies on an introgression from the radish genome including the Rf gene for fertility restoration. By recombination and pedigree breeding, Pioneer Hi-Bred scientists identified an Rf line with very low and stable glucosinolate content, allowing the production of fully restored hybrids with canola quality. In more recent lines the length of the original radish introgression sequence is significantly reduced, leading to improved agronomic and quality characters (Delourme et al. 1998).

4.6 Current Goals of Breeding

The general goals of oilseed rape breeding are summarized in Table 4.3. Major goals with high or very high priority are: tolerance to late planting and winter hardiness, plant height and lodging resistance, resistance to blackleg disease, Verticillium wilt and (if possible) sclerotinia, very low contents of erucic acid and glucosinolates, high oil content and marketable seed yield (M. Frauen and others, personal communication).

Table 4.3 Four major fields and associated detail traits of oilseed rape breeding

Agronomic traits	*Disease and pest resistance*
– Tolerance to late planting	– Phoma and Vericillium
– Winter hardiness	– Clubroot and Cylindorsporium
– Plant height and lodging resistance	– Sclerotinia (resistance to be identified)
– Ripening time (early maturity)	– Virus resistance (TuYV)
– Nutrient efficiency and drought tolerance	– Various insects pests (pant resistances remain to be identified)
– Shattering resistance	
– Herbicide tolerance	
Yield potential	*Seed quality*
– Oil content	– Very low erucic acid content (C22:1 <0.2%)
– Seed yield components	– Low glucosinolate content (<18 mmol/kg seed)
– Harvest index	– Reduced fibre (lignin) content and improved digestibility (monogastric animals)
– Total and marketable seed yield	

Source: NPZ-Lembke, see Christen and Friedt (2007).

4.6.1 Seed and Oil Yield Potential and Stability

After the widespread adoption of 00-quality as the accepted standard for the use of the crop in human and animal nutrition, the major focus in breeding efforts returned to improving the seed and oil yield along with yield stability (Fig. 4.2). With respect to morphological and agronomical characteristics, the yield of oilseed rape is composed of the number of siliques per unit area, the number of seeds per silique and the 1000-seed weight (Diepenbrock 2000). Improvement of productivity encompasses several agronomic parameters such as early maturity, resistance to lodging and shattering as well as resistance to weeds, insects and particularly to the major diseases.

The most significant diseases of oilseed rape in most major growing regions are sclerotinia stem rot (*Sclerotinia sclerotiorum*) and stem canker (*Leptosphaeria maculans*, anamorph: *Phoma lingam*), also known as blackleg disease (Fig. 4.3); however, there are also a number of additional diseases of local importance (Table 4.4). Verticillium wilt caused by *Verticillium longisporum* is a particular problem in Sweden and Germany, light leaf spot (*Pyrenopeziza brassicae*) in northern parts of Europe and clubroot (*Plasmodiophora brassicae*) in Scandinavian countries and the northern United Kingdom.

Fig. 4.2 Rapeseed performance trials for identification of candidate varieties at a commercial breeding station (photo: U. Baer, NPZ-Lembke, Germany)

Fig. 4.3 Incidence of phoma leaf spots (blackleg disease) caused by *Leptosphaeria maculans* (anamorph: *Phoma lingam*) on a young leaf of oilseed rape in autumn (photo: U. Baer, NPZ-Lembke, Germany)

Table 4.4 Agronomical relevance, potential damage, possibility of control by chemicals or genetic resistance, and priorities for further research regarding major pathogens and pests of oilseed rape

Pathogen or pest	Agronomical relevance	Potential risk of damage	Chemical prophylactic control	Genetic resistance available	Need for research
TuYV (virus)	+++	++	–	+	+
Plasmodiophora brassicae	+	+	++	+ monogenic	+/–
Peronospora parasitica	+	+/–	++	?	–
Leptosphaeria maculans	+++	+++	++	+	++
Sclerotinia sclerotiorum	+++	+++	++	(?) GMO	+++
Verticillium longisporum	+++	++	–	+	++
Alternaria brassicae	(+)	+/–	(+)	+	–
Cylindrosporium concentricum	–	–	(+)	?	+
Fusarium oxysporum	+/–	+	–	+	–
Nematodes	?	?	–	+ (?)	?
Insect pests	+++	+++	++ resistance	(?) GMO	++

Breeding for increased disease resistance in oilseed rape involves a range of strategies. For a number of diseases, including light leaf spot and blackleg disease, a broad degree of natural variation is present in the species and has been successfully used to breed varieties with more or less sustainable, quantitative resistance. For other diseases, particularly sclerotinia stem rot, Verticillium wilt and clubroot disease, very little resistance or tolerance is present in modern varieties. Cultural control methods, particularly rotation, are therefore particularly important for control of clubroot and sclerotinia, however in the case of sclerotinia the use of chemical plant protection is still vital for efficient disease control. For clubroot and Verticillium, on the other hand, effective fungicides are not available. Due to the long persistence of these fungi in the soil the use of resistant varieties is therefore the only way for effective long-term control of these diseases in affected regions where the production of oilseed rape in crop rotations is steadily increasing. In some cases transfer of resistant germplasm to *B. napus* from other *Brassica* species and related crucifers has been successfully applied.

4.6.2 Improvement of Seed Components

Seeds of oilseed rape contain both valuable (nutritional) and anti-nutritional compounds (Table 4.5). The gross seed composition can vary widely depending

Table 4.5 Major rapeseed compounds determining the feeding value for farm animals

Compounds determining feed value	Antinutritive or undesired compounds
Seed oil (45%): Fatty acids	Glucosinolates (isothiocyanates) Sinapine ("stinking eggs" of brown laying hens)
Storage protein (23%): Amino acids	Lignin (restricting energy content) Phytic acid (availability of P by animals)

Source: K.H. Südekum, personal communication.

on both genetic and environmental factors, with a large influence of temperature, water and nutrient supply. The oil content ranges from around 36 to 50% (on a dry matter basis), while the oil-free meal contains 33–48% protein (Canvin 1965; Appelqvist and Ohlson 1972; Arnholdt and Schuster 1981; Marquard and Schuster 1981; Salunkhe et al. 1992). The improvement of oil content is an important breeding goal due to the primary economic value of the oil component and its relatively high heritability (Grami et al. 1977). Increasing oil content has to date been quite successful due to the ease and speed with which oil content can be measured by non-destructive NMR (nuclear magnetic resonance) techniques. Although oil and protein content are negatively correlated, improvements can be achieved through selecting for the sum of the two seed components (Grami et al. 1977; Arnholdt and Schuster 1981; Stefansson 1983). However, low glucosinolate rapeseed meal still presents several problems for use in livestock feeds. Besides the presence of undesirable compounds like sinapic acid esters, phytic acid and phytates, phenolic acids and tannins, the comparatively high crude fibre content (approx. 15% of dry oil-free meal) is disadvantageous (Shahidi 1990; Thies 1991; Salunkhe et al. 1992). Due to the small size of the seeds the hull, accounting for about 10–20% of the seed weight, imparts most of the fibre content to the meal (Appelqvist and Ohlson 1972; Anjou et al. 1977). Currently, the most promising route to reducing fibre and hull content genetically, is to breed cultivars with a yellow (light) seed coat, like the pure yellow-seeded cultivars occurring in the sarson subspecies of *B. rapa* or in *B. juncea*. Since yellow seed coats are significantly thinner than brown or black ones, the development of pure yellow-seeded *B. napus* cultivars with agronomically acceptable performance still remains an important goal in quality breeding towards increased oil and protein content.

The value and suitability of rapeseed oil for nutritional or industrial purposes is again determined by its fatty acid composition. The identification of naturally occurring zero-erucic mutants in both *B. napus* and *B. rapa* was indeed the first discovery opening the era of mutant-derived quality improvement in oil crops (Downey 1964; Stefansson and Hougen 1964; Röbbelen 1990). Canola-quality rapeseed low in saturated fatty acids and almost lacking nutritionally undesirable very long-chain fatty acids meets all the requirements of a prime edible oil (Ackman 1990; Downey and Bell 1990; Trautwein 1997). Despite the beneficial nutritional properties of α-linolenic acid (18:3n–3), the oxidative stability of the oil can be improved by decreasing the linolenate content from an

average of 10% to less than 3%, which results in enhanced shelf life (Rakow et al. 1987; Pleines and Friedt 1988; 1989; Downey and Bell 1990) and a reduction of *trans*-fatty acids. The latter is a particularly important nutritional quality characteristic for high-temperature frying oils in the fast-food and food-processing industries (Mensink and Katan 1993). In the last three decades improvements of the C18 fatty acid composition in rapeseed (*B. napus*) were achieved by selecting altered linoleate/linolenate genotypes after chemical mutagenesis. Initially, the fatty acid profiles of these lines indicated that nearly all of the linolenic acid was being directed to linoleic acid and that the level of oleate increased only insignificantly (Rakow 1973; Röbbelen and Nitsch 1975; Röbbelen and Thies 1980; Rakow et al. 1987; Röbbelen 1990). In 1988, the spring rapeseed cultivar 'Stellar', which produces oil containing less than 3% linolenate, was released for commercial production in Canada, although its agronomic performance was less than satisfactory (Scarth et al. 1988). Today HOLL or HOLLi varieties provide an important new quality of rapeseed oil for nutritional purposes (Table 4.2).

Due to strong environmental and marked maternal influences, only low correlations have been found between the contents of polyenoic fatty acids determined in half-seeds and their progenies. The most important factor influencing the biogenesis of the unsaturated fatty acids is the prevailing temperature during seed development (Pleines and Friedt 1988, 1989). Recent reports described mutants with reduced levels of polyunsaturated fatty acids (PUFAs) obtained by blocking oleic acid desaturation. The development of canola cultivars with reduced levels of PUFAs accompanied by higher oleate content would produce a dietary oil with additional markets (Marsic et al. 1992). For industrial applications a very high content of oleic acid (80–90%) is preferred because this is most suitable for consecutive chemical synthesis reactions (Lühs and Friedt 1994).

4.7 Breeding Methods and Techniques

As a facultatively outcrossing but predominantely self-pollinating species, oilseed rape is traditionally bred by classical line-breeding methods, and the majority of oilseed rape cultivars are pure lines derived from breeding schemes designed for self-fertilizing crops, i.e. pedigree selection or modifications thereof. However, since the discovery and development of male sterility systems in *B. napus* hybrid breeding has become an equally important aspect of cultivar development in oilseed rape. Due to the generally high response of *B. napus* genotypes to microspore culture techniques, the use of doubled haploid (DH) production (see Section 4.8.1) has also become common practice in commercial breeding programs and has already resulted in numerous licensed cultivars. Doubled haploids are today also widely used for production of homozygous parental lines for breeding of oilseed rape hybrids.

4.7.1 Traditional Line Breeding

By selection against self-incompatibility alleles *B. napus* can be developed as a predominantly self-pollinating species. In the past, corresponding breeding schemes have been adopted for developing open pollinated (OP) rapeseed varieties with a moderate degree of inbreeding, also called 'line varieties'. In this case breeding is a rather straight-forward process including repeated plant selection (first for highly heritable traits) starting in segregating F_2 populations from cross combinations of suitable parents ("combination breeding"). After achieving adequate homogeneity, field observations (e.g. diseases, agronomic traits) and subsequently replicated yield tests with continuously increasing complexity (locations, years) are carried out. This leads to the identification of suitable variety candidates to be submitted to official tests by the respective plant variety office, e.g. the European Community Plant Variety Office (CPVO, Angers/France) or the German Bundessortenamt (Hannover/Germany). Repeated tests are the basis for variety release and are followed by more practice-oriented yield tests before marketing of recommended varieties can commence (Table 4.6).

This classical scheme was modified and improved by the development of efficient haploid techniques for oilseed rape. Today, most breeding companies will derive microspore cultures from F_1 plants and regenerate doubled haploid (DH) plants thereof. Since DH lines are completely homozygous, the subsequent breeding procedure can be accelerated and run more efficiently: First field trials for yield estimation can be done one year earlier than in the classical scheme, and the whole testing period can be reduced (e.g. 2 instead of 3 years). Therefore, variety candidates can be submitted earlier for registration and the marketing of successful candidates can start a couple of years earlier than with lines developed via pedigree breeding (Table 4.6). In addition, homozygous DH lines are very suitable for use in hybrid breeding, depending on their *per se* performance and combining ability.

4.7.2 Hybrid Breeding and Cytoplasmic Male Sterility Systems

Although for many years the emphasis in oilseed rape breeding was strongly focused on open pollinating varieties, up to 30% heterosis for seed yield has been reported for *B. napus* (e.g. Schuster 1969; Grant and Beversdorf 1985; Lefort-Buson et al. 1987; Brandle and McVetty 1989), and for both winter rapeseed and spring canola hybrid varieties have rapidly gained in importance over the past decade as effective systems for controlled pollination were developed. In current European winter rapeseed material yield improvements of up to 15% have been reported for F_1 hybrids compared to non-hybrid open-pollinated varieties. This has led to a major increase in production of hybrid rapeseed in the leading producing countries. For example, although only 21

Table 4.6 Generalized scheme for breeding of OP (line) varieties of oilseed rape – classical pedigree selection (left) versus haploid method (right)

Year	Pedigree breeding generation	Material	Procedure	Haploid method step	Material	Procedure
1	F_1 (S_0)	Cross progeny	Propagation	F_1	Cross progeny	Microspore culture (androgenesis)
2	F_2 (S_1)	Segregating population	Plant selection	A_1	Segregating DH plants	Selection and propagation
3	F_3 (S_2)	Selfed plant progenies	Field observations	A_2	DH lines	Replicated field test of progenies (1 location)
4	F_4 (S_3)	Inbred lines	Replicated field test of progenies (1 location)	A_3	DH lines	Replicated yield test of progenies (>2 locations)
5	F_5 (S_4)	Inbred populations	Replicated yield test of progenies (2 locations)		DH lines	Testing of variety candidates (1)
6		Inbred populations	Replicated yield test of progenies (>2 locations)		DH lines	Testing of variety candidates (2)
7		Inbred populations	Testing of variety candidates (1)		Certified seed production, variety registration	Testing of variety candidates (3)
8		Basic seed production	Testing of variety candidates (2)			Official variety trials
9		Certified seed production, variety registration	Testing of variety candidates (3)		Start of marketing	Official variety trials
10			Official variety trials			
11		Start of marketing	Official variety trials			

F_1–F_5 = Filial generations; S_1–S_4 = Selfing generations; A_1–A_3 = Androgenetic generations.

(28%) of the 76 German winter rapeseed cultivars listed by the German Plant Variety Office in 2008 were hybrids (Bundessortenamt 2008), more than 50% of the 1.5 million hectares of German winter rape in 2007/2008 were planted with hybrid varieties. Already in 2003/2004 the hybrid cultivar 'Talent' had replaced the open-pollinating 'Express' as the most widely-cultivated winter oilseed rape variety in Germany, the first time a hybrid cultivar had achieved the top position. One of the most important reasons for the upsurge in interest in hybrid varieties is that they tend to have higher yield stability and better adaptation to low-input cropping systems than conventional cultivars (Budewig and Léon 2003; Friedt et al. 2003).

Numerous cytoplasmic male sterility (CMS) systems have been discovered and are used in brassica crops. Because CMS arises from specific interactions between the mitochondrial and nuclear genomes, the combination of cytoplasm and nucleus from different species often results in complete or partial male sterility and in many cases functional mutations of floral structure. Two spontaneous male sterile cytoplasms, *nap* and *pol*, are found in *B. napus*. The *nap* system was the first to be identified, originating from intraspecific crosses using 'Bronowski' (Thompson 1972) or 'Hokuriku 23' (Shiga and Baba 1973) as the male parent. Most other *B. napus* CMS systems also result from interspecific or intergeneric crosses, often using known sterility-inducing systems from other species. The best example for this is the widely used 'INRA-Ogura' CMS originating from *Raphanus sativus* (Ogura 1968), which was transferred to oilseed rape by French scientists some 30 years ago (Bannerot et al. 1974). Although this system was described by Tokumasu (1951) as a genic male sterility, in *B. napus* it is expressed as cytoplasmic male sterility. The 'Ogura' CMS in radish (*Raphanus sativus*) is caused by an aberrant mitochondrial gene, *orf138*, that prevents the production of functional pollen without affecting female fertility. *Rfo*, a nuclear gene from radish that restores male fertility, alters the expression of *orf138* at the post-transcriptional level.

Although numerous CMS systems are available from different sources, their use in oilseed rape breeding is often inhibited by instability, the absence of suitable restorer or maintainer lines, or negative effects of the cytoplasm used to induce the male sterility. Environmental instability of the expression of *nap* male sterility means this system is unsuitable for hybrid production, and the 'Polima' (*pol*) system was only made workable by screening of huge numbers of lines in different environments (Bartkowiak-Broda et al. 1991) in order to identify stable maintainer genotypes. The monogenically inherited restorer genes for *B. napus* 'Polima' CMS can be readily introduced into elite lines, and *pol* is therefore now effectively used to produce registered F_1 hybrid spring canola varieties in numerous countries. Male-sterile inducing cytoplasm can also have negative effects on flower morphology, nectar production, or on yield, and sometimes chlorophyll deficiencies also need to be overcome. In some cases suitable *B. napus* restorer lines have been produced for *B. tournefortii* CMS (Banga et al. 1995; Stiewe et al. 1995a,b). Restored F_1 hybrids based on the 'Ogura' CMS system are under increasing production in France, Germany and

Fig. 4.4 Male fertile (*left*) and genic male sterile (Male Sterility Lembke; *right*) rapeseed flowers (photos: U. Baer, NPZ-Lembke, Germany)

other European countries. Hybrid cultivars based on the commercial 'Male-Sterility Lembke' (MSL) system are also prominent among winter oilseed rape varieties in Germany. The advantage of this genic male sterility system (Fig. 4.4) is that all *B. napus* lines restore fertility.

4.8 Introduction of New Biotechnologies into Breeding Programs

4.8.1 Tissue Culture and Haploid Techniques

Brassica napus is one of the most amenable crop species to improvement through biotechnology. For instance, it is possible to reproducibly obtain haploid and subsequently doubled-haploid (DH) plants through anther and/or microspore culture (e.g. Weber et al. 2005). The principle advantage of haploidy techniques is the rapid fixation of segregating genotypes, occurring in lower frequency, in which recessive genes coding for specific traits are combined in the homozygous condition. Thus, utilization of microspore culture can allow a substantial acceleration of the breeding cycle. Besides haploid techniques, wide hybridizations using embryo rescue techniques or protoplast fusion can also be used to create novel genetic variation. This has been a particularly successful method for integration of new disease resistance genes from related species into oilseed rape.

4.8.2 Genetic Modification

Oilseed rape is particularly amenable to *Agrobacterium tumefaciens* mediated transformation, and during the last two decades considerable progress has been achieved in the development of genetically modified (GM) varieties. Consequently, the global area of transgenic crops has grown continuously. Beside soybean, cotton and corn, canola is one of the four principal crops in which GM technologies are utilized. Herbicide tolerant canola was grown on 3.6 million hectares, equivalent to 5% of the global transgenic area, in 2003. A limited public acceptance and unclear administrative legislation in European Union member states has prevented expansion of GM traits into European oilseed rape varieties. In Canada, on the other hand, herbicide-resistant GM canola currently (2008) comprises about 80% of the canola production in western Canada (see http://www.canola-council.org/). The first glufosinate-ammonium tolerant *B. napus* spring cv. 'Innovator' was registered for production in Canada in 1995 (Oelck et al. 1995). Although weed control in canola is possible with available herbicides, multiple treatments with chemicals of different herbicide families are often required for control of all weeds. Certain cruciferous weeds such as wild mustard (*Sinapis arvensis*) and stinkweed (*Thlaspi arvense*), are difficult to control, and the use of speciality herbicides for cruciferous weed control is sometimes required. In addition, the multiple uses of herbicides increases production costs and the chemical load on soils. The availability of several types of herbicide tolerant plants allows for rotation of herbicides, minimising the risk of weeds becoming resistant to any particular chemical. Several varieties of transgenic herbicide-tolerant oilseed rape are grown and processed in the USA, Canada and China.

Genetic modification of the fatty acid composition is also an option to make rapeseed oil more competitive in various segments of the food and industrial oil markets. One of the central objectives in this context is the genetic modification of the seed storage oil by maximising the proportion of specific or functional fatty acids in order to obtain tailor-made raw materials suited for various industrial purposes (Friedt and Lühs 1998; Biermann et al. 2000). However, the quality of vegetable food products has increased in relevance for human nutrition in recent decades with the advent of so called 'functional foods'. With regard to specific properties of such nutritive substances, genetic engineering offers the possibility to adapt plant storage lipids to meet specific nutritional, industrial and even therapeutic requirements (Leckband et al. 2002; Friedt et al. 2004). Rapeseed oil is unique in having a large spectrum of usability and positive properties for food and non-food applications. Genetic engineering of plant lipid biosynthesis in rapeseed has already led to commercialisation, with transgenic varieties expressing genetically modified fatty acid patterns being available since 1995 (cf. Friedt and Lühs 1998).

4.8.3 Genetic Mapping, Genome Analysis and Marker-Assisted Selection

Molecular markers have been widely used to map agronomically important genes in oilseed rape and frequently play an important role in breeding and selection procedures. The complete sequencing of *Arabidopsis thaliana* (The Arabidopsis Genome Initiative 2000) and the development of comparative genetic and physical maps between *B. napus* and Arabidopsis (e.g. Parkin et al. 2005) has enormous potential for application in gene identification and breeding in oilseed rape. The genome of *B. rapa* is expected to be completely sequenced within the near future (for progress see http://www.brassica.info), and current technological developments in the field of ultra-fast DNA sequencing are beginning to revolutionise the fields of polymorphism discovery, genome analysis and molecular breeding. The number of expressed sequence tag (EST) sequences available for *B. napus* has skyrocketed in the past few years as sequencing costs have diminished, enabling DNA sequence mining to become extremely useful for the identification and development of single nucleotide polymorphisms (SNPs) in oilseed rape. In the near future it can be expected that high-density *B. napus* SNP arrays will play an important role in development of dense genetic maps for oilseed rape. Next-generation sequencing technologies are set to rapidly accelerate SNP discovery, so that ultra-high density SNP maps will probably become commonplace in the near future. High-throughput SNP screening methods will also be a valuable resource for whole-genome allele-trait association studies, which potentially will play a major role in the identification of genes contributing to complex traits.

4.8.3.1 Genetic Maps and QTL Analysis

The first genetic map for *B. napus* was developed by Landry et al. (1991) using restriction fragment length polymorphism (RFLP) markers. In the subsequent two decades a large number of *B. napus* genetic maps were generated worldwide using different rapeseed crosses, and considerable efforts have been invested in the localisation of genes and QTL controlling agronomically relevant traits. The most extensive *B. napus* genetic map published to date was an ultra-dense map of 13,551 sequence-related amplified polymorphism (SRAP) markers that were assembled into an ultra-dense bin map by Sun et al. (2007). A marker density of 8.45 SRAPs per cM was achieved, which according to the authors could correspond to more than one marker per 100 kb. This demonstrates the great potential of ultra-dense mapping for map-based gene cloning; the availability of ultra-dense maps based on sequence-annotated SNP marker techniques derived from next-generation sequencing or EST-SNP arrays will in the near future hugely expand the opportunities for rapid discovery of candidate genes for both simple and complex traits.

A detailed summary of *B. napus* crosses, mapping populations, marker systems, map details and the quantitative traits that were studied until 2006 is given by Snowdon et al. (2006). In the following we will describe some of the more recent genetic mapping studies, including QTL analysis of traits that had previously been less intensively studied, along with some novel approaches for identification of genetic markers and candidate genes closely linked to important traits in oilseed rape. In some cases marker-assisted genome scans were implemented to introgress novel genetic diversity into oilseed rape breeding lines.

As the importance of hybrid cultivars has increased over the past decade, there has been growing interest in identifying the mechanisms and potential genomic loci responsible for the manifestation of heterosis in oilseed rape. Different strategies have been used to analyse the quantitative genetics of heterosis for yield and related traits with the assistance of genetic maps. For example, Radoev et al. (2008) mapped QTL contributing to additive, dominant and overdominant heterosis effects in a mapping population of 250 doubled haploid lines that were tested in two-year, multi-location field trials along with a corresponding set of test hybrids from each of the DH lines with a common male-sterile tester parent. Heterosis levels of up to 30% for grain yield were used to map QTL involved in heterosis for yield and related seed traits. A large number of epistatic interactions were found to interact with dominance and overdominance effects to control expression of heterosis.

Use of genome-wide marker screens can also be useful for the introduction of novel genetic diversity for the exploitation of heterosis in hybrid breeding. For example Li et al. (2006) described a marker-assisted approach to develop new types of *B. napus* with introgressions of A genome chromosomes from *B. rapa* and C genome chromosome segments from *B. carinata*. When crossed with conventional *B. napus* these new types demonstrated elevated levels of so-called 'intersubgenomic heterosis' for seed yield and related traits. Basunanda et al. (2007) utilised dense whole-genome marker scanning to identify DH lines in which the genes for zero erucic acid along with QTL for low glucosinolate content were introgressed from a 00-quality variety into a novel genetic background of semi-synthetic $++$ quality rapeseed. Test hybrids generated using these genetically diverse introgression lines as pollinators showed high mid-parent heterosis for seed yield (Basunanda et al. 2007; Gehringer et al. 2007).

Dissection of yield and yield component traits is another important aspect that has been analysed extensively in oilseed rape by QTL analysis. For example, Chen et al. (2007) recently reported on the detection of numerous QTL for yield and yield-related traits in DH and immortalised F_2 populations and found some QTL that contributed significantly to numerous yield-related traits and could be interesting targets for yield improvement. As the technologies for highly-dense genetic mapping improve and it becomes possible to more accurately integrate and compare map and QTL data from different populations, it will be of great interest to see whether important QTL related to yield co-localise in different materials, and whether such QTL may interact with

epistatic loci involved in yield heterosis. Meta-analyses with multiple mapping populations and large, common marker sets will hopefully enable such determinations in the future.

4.8.3.2 Male Sterility

Considerable advances have been made in recent years in mapping and marker development for genes controlling genic and cytoplasmic male sterility systems in oilseed rape. For example, Yi et al. (2006), Lei et al. (2007) and Huang et al. (2007) described the fine-mapping of three recessive genic male-sterility genes using amplified fragment length polymorphism (AFLP®: Keygene, Wageningen, The Netherlands) and amplified consensus genetic markers (AGGM) in large segregating populations, and the anchoring of the linked markers to previous *B. napus* genetic maps. In each case flanking marker sequences covering a region of well under 1 cM in *B. napus* were used to delineate syntenic chromosome regions in Arabidopsis that may contain the orthologs to the respective sterility genes. Hong et al. (2008), Xie et al. (2008) and Xiao (2008) described the development of sequence-based markers with tight linkage to an epistatic genic male sterility suppressor gene, while He et al. (2008) generated sequence-characterised markers linked to a cytoplasmic male sterility fertility restoration gene. In each of these cases AFLP markers and bulked-segregant analysis played an important role in whole-genome marker saturation to identify sequences with very lose linkage to the responsible genes. Sequence annotations to Arabidopsis and an often well-conserved synteny can assist greatly to identify potential candidates in corresponding chromosome regions, and bulked-segregant analyses have proved a valuable method to fine-map and clone genes involved in male sterility and fertility restoration. In a different approach based on differential gene expression, Wu et al. (2007) used suppressive subtractive cDNA techniques and cDNA microarray hybridisation to try to identify candidate genes for a dominant genic male sterility in *B. napus*. A number of genes involved in male gametogenesis pathways were among the differentially expressed genes between fertile and sterile near-isogenic lines.

4.8.3.3 Oil Content and Quality

Identification and utilization of important genes contributing to oil content is one of the major aims of seed quality breeding in oilseed rape. Fu et al. (2007) mapped loci with major contributions to oleic, linoleic and linolenic acid content, and developed markers tightly linked to the responsible fatty acid desaturase genes which can be used in effective selection of HOLLi genotypes. A recent publication compared oil content QTL in different mapping populations and revealed that some major gene loci appear to influence this complex trait in different genetic backgrounds. Delourme et al. (2006) localised oil content QTL in two large, genetically divergent mapping populations and compared their locations to previously mapped QTL from earlier studies. In some cases the

QTL were found to be consistently revealed across different genetic backgrounds. In particular, a QTL on N3 was revealed in all the studies and other QTL on N1, N8 and N13 were found in three out of five different studies. Other QTL were located in homeologous genome regions, while some were specific to a particular genetic background and potentially carry novel alleles. These results show the potential for combination of favourable alleles at different QTL to increase seed oil content. Furthermore, examples were given of how Arabidopsis genomic data could be used to derive markers and identify candidate genes for oilseed rape QTL. The study was also a good example demonstrating the added value of consolidated information from different segregating populations in order to identify meta-QTL involved in a specific trait in different genetic backgrounds.

4.8.3.4 Yellow Seed Character

Much interest has developed recently with respect to the breeding of yellow-seeded oilseed rape and canola with improved seed meal quality. The yellow-seed trait in *B. napus* is generally associated with a reduced seed coat thickness; this leads to a reduced contribution of the seed coat to the seed meal after oil extraction and a consequent lowering of anti-nutritive crude fibre and phenolic compounds. Unfortunately seed colour itself is difficult to use as a morphological marker for improved meal quality because the accumulation of seed coat tannins is highly sensitive to temperature, light intensity and other abiotic factors. Therefore there is a considerable effort to identify major genes contributing to reduced seed coat in different yellow-seed materials and develop markers for effective breeding of high-performing light-seeded varieties. Badani et al. (2006) localised a major QTL with a large contribution to seed colour and acid detergent fibre content in two different yellow-seeded winter rapeseed sources. The gene was flanked by markers originating from *B. napus* chromosome N18, although later work showed that the chromosome segment containing the major QTL derived from chromosome N9 and the responsible gene may be present on a non-reciprocal translocation. In Chinese oilseed rape with a completely different genetic background as compared to the previous study, Fu et al. (2007) also found a major dominant QTL that appeared to be localised on chromosome N9. Liu et al. (2005) and Xiao et al. (2007) also developed closely linked markers to a major gene for yellow seed colour in Chinese oilseed rape. It will be of great interest to determine whether the same major genes are influencing seed colour-related traits in these genetically diverse materials, and also to use the markers and candidate genes to identify new allelic diversity for these traits among other oilseed rape materials.

4.8.3.5 Resistance to Biotic and Abiotic Stress

Mapping and marker development for resistance genes to biotic stress factors are important goals in oilseed rape breeding. In recent years continued progress

has been made in the map-based cloning of genes contributing to resistance against blackleg disease, the major disease of oilseed rape worldwide (see Rimmer 2006). Mayerhofer et al. (2005) described the fine mapping of loci involved in seedling resistance to blackleg in two different canola cultivars. Both loci localised to the same position on *B. napus* chromosome N7, and a collinear chromosome region could be identified in Arabidopsis. A complex pattern of tandem duplications was identified in the *B. napus* genome region containing these loci. Apparently, duplication and sequence divergence during the polyploidisation events that led to the *Brassica* species may also have played a major role in the evolution of resistance to major pathogens. Candidate genes for blackleg resistance were also identified in Arabidopsis by Staal et al. (2006); fine-mapping was performed in recombinant inbred lines to identify two genes that were associated with resistance and contained typical resistance gene sequence motifs. The contribution to resistance was confirmed by reverse genetics.

For a number of other diseases of oilseed rape breeding efforts have been hindered by a lack of resistance sources. In some cases this can be overcome by introduction of resistance genes from exotic *B. napus* materials, resynthesised rapeseed or other interspecific crosses, however the availability of useful selection markers is a prerequisite for effective combination of quantitative resistances in elite germplasm. For example, Rygulla et al. (2007a) identified QTL-linked markers associated with resistance against *Verticillium longisporum* introduced from *B. oleracea*, while Werner et al. (2008) described QTL involved in resistance against clubroot disease (*Plasmodiophora brassicae*). Resistance to *Sclerotinia sclerotiorum* is a major breeding aim in most of the major oilseed rape growing areas of the world, however little resistance has yet been identified against this disease in *B. napus*. QTL analysis of a partial resistance in Chinese rapeseed lines was characterised by Zhao and Meng (2003), and gene expression profiles produced by resistant and susceptible genotypes in response to *S. sclerotiorum* infection were analysed by Zhao et al. (2007) using microarray analysis. Early response genes to pathogen inoculation were integrated into the QTL map, leading to the identification of a number of candidate genes for the defense reaction. Among the genes that co-localised with interesting resistance QTL, some plant cell wall-related proteins and WRKY transcription factors were identified as potential contributors to defence against sclerotinia rot.

Abiotic stress resistance is also gaining increasing attention in oilseed rape breeding, although the regulatory mechanisms involved in whole-plant reactions to drought conditions, nutrient deprivation or cold stress can be extremely complex. Considerable efforts have been made to investigate genes involved in vernalisation requirement and flowering time in *B. napus* and related species, and in recent years it has become clear that some of the major genes controlling flowering traits may play an important global role in regulation of gene expression in general. In recent years the role of FLOWERING LOCUS C (FLC) homologs and associated genes in the regulation of flowering time and related

traits, and their involvement in relevant QTL for these traits, has been confirmed in *B. napus* and its diploid progenitors (e.g. Pires et al. 2004; Kim et al. 2007; Lou et al. 2007; Okazaki et al. 2007; Razi et al. 2008).

4.8.3.6 Novel Genomic Tools

The astonishing current developments in next-generation sequencing technologies offer unprecedented opportunities for new genomics-based breeding and selection strategies. In particular, as soon as the sequencing of the reference A genome of *B. rapa* is completed it will be possible to resequence large portions or the *B. oleracea* C genome and consequently the A and C genomes of *B. napus*. Even without a reference sequence, the next-generation sequencing technologies enable large-scale comparative sequencing of BAC libraries from elite breeding lines for a relatively low and continuously decreasing cost, so that whole-genome selection in oilseed rape and other major crops is likely to become a reality in the foreseeable future. This new sequence-based genomics era will probably completely change the way that genetic mapping, genome analysis and marker-assisted selection are performed in crop plants. Presumably, high-throughput chip-based screening of large segregating progenies will play a major role in breeding and selection. Such techniques will also be a pivotal technology in the application of whole-genome association genetics methods for the identification and utilisation of genes involved in important complex traits. Already the use of genome-wide transcriptome analysis has enabled the identification of potential global gene expression regulators that could be useful for significant yield increases. Ultra-deep expression profiling with the help of next-generation sequencing has the potential to take this kind of gene discovery to a new level. Although oilseed rape genome research has long profited from the close relationship to Arabidopsis, one of the most intensively studied plant species, in the near future it is likely that a vast array of genomic tools will also be available for brassica crops. One major limitation to genome-assisted breeding is the current lack of associations between genomic data and detailed, reliable phenotypic data for factors contributing to major traits. Intensive phenotyping, including high-throughput physiological and metabolite profiling, may be the most important key to understanding important complex traits such as oil content, seed developmental characters, biotic and abiotic stress tolerance and the manifestation of yield characters in oilseed rape. Novel and high-throughput phenotpying technologies should therefore be an important priority in coming years in order to facilitate the identification of genomic and transcriptomic variation associated with economically important characters.

4.8.3.7 Utilization of Synteny to Arabidopsis

Synteny to Arabidopsis and increasing quantities of aligned genomic sequence data from *Brassica* species are also helpful to identify candidate genes and

potentially gene-linked markers for important traits in oilseed rape. To demonstrate the power of synteny-based marker development, Hasan et al. (2008) developed potentially gene-linked markers for seed glucosinolate loci via structure-based allele-trait association studies in genetically diverse *B. napus* genotypes. Association analyses were performed in a core set of gene bank accessions, and included simple-sequence repeat (SSR) markers whose orthologs in *A. thaliana* were expected to be physically closely linked to promising candidate genes for glucosinolate biosynthesis. Using this approach, four genes involved in the biosynthesis of indole, aliphatic and aromatic glucosinolates were tested for associations to total seed glucosinolate content in *B. napus*. Markers linked to homoeologous loci of all four genes in *B. napus* were found to be associated with a significant effect on the seed glucosinolate content. This example shows the potential of *Arabidopsis-Brassica* comparative genome analysis for synteny-based identification of gene-linked SSR markers that may be used in marker-assisted selection for important traits in oilseed rape.

4.9 Seed Production

The appropriate procedure of seed production depends on the variety type, i.e. open-pollinated (OP) cultivars versus hybrid cultivars. Since the former represent essentially homozygous inbred lines, they can easily be reproduced and increased by cultivation in isolation, i.e. with sufficient distance to other oilseed rape crops in order to exclude cross pollination.

In comparison, the procedure of hybrid seed production is much more complicated. Current rapeseed hybrid cultivars are single cross F_1 hybrids based on two parental (female and male) inbred lines. For developing male sterile females, both systems of cytoplasmic male sterility and genic male sterility (controlled by nuclear genes only) are commercially used (see above). Initially, the parental inbreds have to be propagated under strict isolation. In the next step, F_1 seed production is carried out in alternate stripe cultivation of female seed parent and male pollinator lines (Fig. 4.5). For hybrid seed production, attention has to be paid to specific requirements: minimum male sterility of female lines must be 98% and the minimum hybridity of hybrid seed harvested must be 90%. Hybridity is determined by counting typical male fertile (restored) plants in field-grown seed progeny.

General requirements for seed characteristics and production include: (1) Planting requirements for seed production (determined via field inspections), (2) seed characters including purity, germination rate, and moisture level, (3) contamination with seeds from all other species, and (4) phytosanitary requirements (live insect pests or mites, contamination with sclerotia of *Sclerotinia*). In general, the requirements are more stringent for certified seed than for basic seed (Table 4.7).

Fig. 4.5 F_1 hybrid seed production through alternate strip cultivation of male sterile (seed parent) and male fertile (pollinator parent) rows at different developmental stages: before flowering (*top*), full bloom (*center*), and after the end of flowering with male parent removed (*bottom*) (photos: U. Baer, NPZ-Lembke, Germany)

Table 4.7 Minimum requirements for the production and purity of basic and certified oilseed rape seed according to the German federal seed trade regulations

Requirements for seed characteristics and production	Basic seed	Certified seed
Plant requirements for seed production (determined via field inspections)		
No. of non-typical rapeseed plants (off-types) which may cause cross pollination (max. number of plants per 150 m^2)	5	10
Species whose seeds are difficult to eliminate by cleaning (max. number of plants per 150 m^2)	10	15
Minimum distance (m) to other species whose pollen can cause cross-fertilization	200	100

Table 4.7 (continued)

Requirements for seed characteristics and production	Basic seed	Certified seed
Seed characters		
Purity (%)	98	98
Germination rate (%)	85	85
Residual moisture (%)	9	9
Contamination levels		
Maximum contamination with seeds from all other species (% total weight)	0.3	0.3
Wild mustard/charlock (maximum number of seeds)	10	10
Dock/sorrel, except common and golden dock (maximum number of seeds)	2	5
Wild oats, oatgrass (maximum number of seeds)	0	0
Phytosanitary requirements		
Live insect pests or mites	0	0
Sclerotinia sclerotiorum (number of intact or partial sclerotia)	10	10

Source: Adapted from Rutz (2004).

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Chapter 5
Other Brassicas

Leonardo Velasco and José M. Fernández-Martínez

5.1 Introduction

The genus *Brassica* is one of the most important genera contributing to agriculture. It includes a total of 41 species, six of them of economic importance: *B. juncea* (L.) Czern., *B. napus* L., *B. nigra* (L.) Koch, *B. oleracea* L., and *B. rapa* L., worldwide distributed, and *B. carinata* A. Braun, restricted to Ethiopia and surrounding countries. Other species have been cultivated in the past, are nowadays cultivated on a small scale, and/or wild types are locally used (Gladis 1989). The genus encompasses very diverse types of plants, grown as vegetables, fodder, and sources of oils and condiments (Prakash and Hinata 1980).

Vegetable oil from *Brassica* spp. represents nowadays the third source of vegetable oil in the world market. Most of the oil is obtained from double-zero or canola cultivars, which produce seed oils with less than 2% erucic acid and seed meals with less than 30 μmol of aliphatic glucosinolates per gram of oil free meal (Downey and Rakow 1987). Double-zero germplasm has been developed for *B. napus* (Downey et al. 1969), *B. rapa* (Kondra and Stefansson 1970), and *B. juncea* (Love et al. 1990), whereas the development of double-zero germplasm of *B. carinata* is underway (Márquez-Lema et al. 2006).

Brassica napus is the major oilseed crop of the genus and it is cultivated worldwide. Other *Brassica* oilseed crops are cultivated at smaller scale, although some of them are locally important (e.g. *B. juncea* and *B. rapa* in India and Canada and *B. carinata* in Ethiopia) and in some cases their cultivation is spreading in recent years. Winter forms of *B. rapa* are mainly grown in areas with severe cold climates such as parts of Sweden and Finland. The spring cultivars of *B. rapa* are extensively cultivated in western Canada, parts of Sweden and Finland, northwest China, and the Indian subcontinent. The latter is also the major area of cultivation of *B. juncea*, although the crop is becoming relevant in other areas such as Canada and Australia due to the recent availability of double-zero cultivars. *B. carinata* cultivation is still restricted to

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Ethiopia and surrounding countries. The following discussion deals with oilseed brassicas other than *B. napus*, which is reviewed in another chapter of this volume.

5.2 Origin and Domestication

Crop brassicas comprise three elementary species having homeologous genomes named A (*B. rapa*; $n = 10$), B (*B. nigra*; $n = 8$), and C (*B. oleracea*; $n = 9$), and three amphidiploids that originated from natural hybridizations between two of the elementary species: *B. carinata* (BC; $n = 17$), *B. juncea* (AB; $n = 18$), and *B. napus* (AC; $n = 19$) (U 1935). They have been cultivated since ancient times as vegetables, fodder, and sources of oils and condiments (Prakash and Hinata 1980). Current oilseed types mainly belong to *B. napus*, *B. rapa*, *B. juncea*, and *B. carinata*. *Brassica* crops include very distinct forms that are cultivated for different uses. Storage organs (turnips), leaves, and seeds are utilized in the varying forms of *B. napus*, *B. rapa*, and *B. juncea*. *B. carinata* is used in the Ethiopian plateau as a vegetable and as a source of oil. The first records on the use of the seeds as spices date back to Sanskrit literature around 3000 BC (Prakash and Hinata 1980).

5.2.1 Brassica rapa

The species includes a vast number of morphologically divergent forms that are cultivated as green vegetables or for their storage organs (turnips) and seed oils. Two main centres of origin have been suggested: the Mediterranean area, which is considered as the primary centre of origin of European forms, and eastern Afghanistan and the adjacent region of Pakistan (McNaughton 1979). The species is divided into seven subspecies, some of them considered for a long time as separated species. Oilseed forms are included into subsp. *oleifera* (oilseed turnip or turnip rape), subsp. *trilocularis* (yellow sarson), and subsp. *dichotoma* (brown sarson, toria) (Hanelt 1986). The subsp. *oleifera* has been suggested to be the basic cultivated form nearest to the wild type (McNaughton 1979).

5.2.2 Brassica juncea

Brassica juncea is a mustard species, known as Indian or oriental mustard, with great economic importance. It is used as an oil, vegetable, and condiment crop (Vaughan and Hemingway 1959). It is generally accepted that *B. juncea* originated from *B. nigra* and *B. rapa* somewhere in the Middle East or Central Asia (Prakash and Hinata 1980).

After *B. oleracea*, *B. juncea* is the species of the genus that exhibits the greatest intraspecific variability, although it has been studied to a smaller extent than *B. oleracea* (Hanelt 1986). The greatest diversity of forms occurs in India and China. Cultivation has spread the species worldwide, especially to southern- and south-eastern Asia (mainly Malaysia and Indonesia), the regions from south-western Asia to northern Africa and south-eastern Europe, western Europe (especially England), western Africa, and America (Hanelt 1986). The species is divided into four subspecies, some of them mainly used as root vegetables (subsp. *napiformis*) or as leaf vegetables (subsp. *tsatsai*, subsp. *integrifolia*). The oilseed forms mainly belong to subsp. *juncea* (Gladis 1989).

5.2.3 Brassica carinata

Brassica carinata, commonly known as Ethiopian mustard, arose as a natural cross between *B. nigra* and *B. oleracea* in north-eastern Africa, probably in the Ethiopian plateau, where wild forms of *B. nigra* co-exist with cultivated forms of *B. oleracea* since ancient times (Tsunoda 1980). The species is only found under cultivation, mainly in Ethiopia and surrounding countries (Hanelt 1986). The crop is used both as a leaf vegetable as well as an oil crop. Although some authors have reported little differentiation into crop types (Labana and Gupta 1993), others have identified distinct leaf cabbage types of *B. carinata* (Tcacenco et al. 1985). The species has been traditionally considered to contain low intraspecific variability (Hanelt 1986), although recent morphological and molecular studies have shown large genetic diversity within germplasm of this species (Alemayehu and Becker 2002; Teklewold and Becker 2006; Warwick et al. 2006).

5.3 Varietal Groups

5.3.1 Open Pollinated, Synthetic, and Hybrid Cultivars

Brassica rapa is an allogamous species with a high degree of self-incompatibility. Conversely, the amphidiploid species *B. juncea* and *B. carinata* are partially allogamous, with a high degree of self-pollination. Experiments in *B. carinata* have shown an average outcrossing rate of 30% (A. Teklewold, personal communication). Unlike *B. napus*, in which hybrid cultivars are replacing open pollinated varieties, most cultivated varieties of the other oilseed brassicas are open pollinated, mainly developed through pedigree selection (*B. juncea*, *B. carinata*) or recurrent selection (*B. rapa*). Synthetic cultivars composed of two or three parental lines are also used to exploit heterosis in *B. rapa*. Hybridization systems based on both nuclear and cytoplasmic male sterility have been developed for *B. juncea* (Pandey et al. 1999; Sodhi et al. 2006).

5.3.2 Winter and Spring Cultivars

Brassica rapa includes annual and biennial forms. The latter have vernalization requirements to flower and they can be cultivated as winter crops only in areas with cold winters. The former have practically no vernalization requirements and they are cultivated as spring crops in areas with cold winters or as fall-sown crops in areas with mild winters. Winter cultivars of *B. rapa* have been important in the past, but nowadays they have been largely replaced in most areas by more productive winter cultivars of *B. napus*. Only spring types exist of *B. juncea* and *B. carinata*.

5.3.3 Wild-Type, Single-Zero, and Double-Zero Cultivars

The seed oil extracted from wild-type *Brassica* seeds contains high erucic acid content, usually between 35 and 45% of the total seed oil fatty acids. Additionally, the seed meal contains high glucosinolate content, commonly above 100 μmol g^{-1} seed. The toxic and antinutritive nature of both compounds has represented a serious obstacle for the commercialization of seeds from wild-type cultivars. The development of germplasm with seed oil free of erucic acid led to the development of single-zero cultivars of *B. rapa* (Downey 1964), *B. juncea* (Kirk and Oram 1981) and *B. carinata* (Alonso et al. 1991), whereas an additional drastic reduction of glucosinolate content produced double-zero cultivars of *B. rapa* (Downey et al. 1969; Jönsson et al. 1975) and *B. juncea* (Love et al. 1990). Double-zero cultivars are also known as canola.

5.4 Genetic Resources

Brassica genetic resources are very rich in variability due to the large number of closely related species. In addition to the three amphidiploid species (*B. napus, B. juncea, and B. carinata*), which originated from spontaneous interspecific hybridization between the diploid species (*B. oleracea, B. rapa*, and *B. nigra*), there are other species of the genus *Brassica* and other related genera within the tribe Brassiceae from which it is possible to identify useful variability that can be transferred to turnip rape and mustard oilseed crops. A complete list of wild germplasm of *Brassica* and allied crops has been published (Warwick et al. 2000). Germplasm resources are available for *Brassica* breeders through international bank collections. Important *Brassica* germplasm collections are maintained in Europe by the European Cooperative Programme for Plant Genetic Resources (ECPGR) members. The database of these collections from 22 countries and 36 institutions include 19,600 accessions available in the ECPGR database (http://www.cgn.wur.nl/pgr/collections/brasedb/). Other important collections are maintained in the North Central Regional Plant Introduction

Station (NCRPIS) of USA (database available at http://www.ars-grin.gov/npgs/searchgrin.html), the National Bureau of Plant Genetic Resources (NBPGR) of India, and the Institute of Biodiversity Conservation and Research (IBCR) of Ethiopia.

As described below in this chapter, these resources have been successfully used in the identification and transfer of traits of interest, such as oil and meal quality, disease resistance, and cytoplasmic male sterility to the oilseed species *B. rapa, B. juncea* and *B. carinata*. However, due to the number and diversity of germplasm collections it is difficult to select plant materials for specific objectives. In order to provide efficient means of identifying useful traits, extensive collections from European gene banks were documented, evaluated and characterised within an EU research project (van Soest et al. 2004), and core collections representing useful variability for *Brassica* oilseed breeding were generated. These core collections constitute useful resources for identifying valuable agronomic and quality traits that can be transferred to cultivated brassicas. For example, the resulting core collection of *B. rapa* contains a broad variation for disease resistance (Pinnegar et al. 2004). The evaluation of the *B. carinata* material showed a wide variation for seed quality traits (Font et al. 2004). Other germplasm evaluations in *B. carinata* collections identified a high level of variability for agriculturally important traits (Alemayehu and Becker 2002; Teklewold and Becker 2006; Warwick et al. 2006). Similarly, evaluations of large germplasm collections of *B. rapa* and *B. juncea* have identified a high level of variation for agronomic and quality traits in these species (Knowles et al. 1981).

5.5 Major Breeding Achievements

The most relevant breeding achievement in *Brassica* oilseed crops other than *B. napus* have been related to the improvement of oil and meal quality, especially the development of double-zero or canola cultivars of *B. rapa* and *B. juncea*.

5.5.1 Oil Quality

Germplasm free of erucic acid was first identified in *B. rapa* through single-seed selection within the cultivar 'Polish' (Downey 1964). Zero-erucic acid germplasm was developed in *B. juncea* through single seed selection in entries with reduced levels of this fatty acid (Kirk and Oram 1981). In *B. carinata*, zero erucic acid germplasm was developed through intraspecific selection (Alonso et al. 1991) as well as through interspecific introgression of genes from *B. juncea* (Getinet et al. 1994) or both *B. juncea* and *B. napus* (Fernández-Martínez et al. 2001).

Additionally to the elimination of erucic acid, seed oil quality of *Brassica* oilseed crops has been improved through the development of germplasm with low linolenic acid and/or high oleic acid content. Significant reductions of linolenic acid levels have been achieved in *B. rapa* (Auld et al. 1992; Laakso et al. 1999), *B. juncea* (Sivaraman et al. 2004), and *B. carinata* (Velasco et al. 2004). Mid and high oleic acid types have been also developed in *B. rapa* (Auld et al. 1992; Tanhuanpää et al. 1996b), *B. juncea* (Stoutjesdijk et al. 1999; Sivaraman et al. 2004), and *B. carinata* (Velasco et al. 2003). Additionally, germplasm with increased erucic acid content, advantageous for non-food applications, has been developed in *B. carinata* (Velasco et al. 1998).

5.5.2 Meal Quality

Seed glucosinolates are the major factor limiting the value of the meal for animal feed. Germplasm with reduced levels of glucosinolates was developed in summer (Downey et al. 1969) and winter types of *B. rapa* (Jönsson et al. 1975) as well as in *B. juncea* (Love et al. 1990), which in combination with previously developed zero-erucic acid types led to the development of double-zero cultivars of both species. The first double zero cultivar of *B. rapa*, Candle, was registered in Canada in 1997. The first double zero cultivars of *B. juncea*, Arid and Amulet, were registered in Canada in 2002. Double zero cultivars of *B. carinata* have not been developed yet, even though some progress is being made in the reduction of seed glucosinolate content in this species (Márquez-Lema et al. 2006).

5.6 Current Goals of Breeding

5.6.1 Seed Yield and Adaptation

Adaptation to growing conditions is an important objective in breeding programmes, especially in areas under risk of drought or frost. Despite the great yielding potential of current cultivars of *B. napus*, especially the novel hybrid cultivars, crop yields are surpassed by other *Brassica* crops under certain areas due to a better adaptation to local environments. In many cases, comparative trials have involved *B. napus* commercial cultivars versus landraces or germplasm accessions of the other species not subject to modern breeding techniques, which emphasizes the great potential for alternative *Brassica* spp. under certain areas.

Spring-type cultivars of *B. rapa* are better adapted than *B. napus* cultivars to short season growing areas (for example due to early spring or fall frosts), because of their early maturity. Early maturity is also an advantage when the growing season is limited by the occurrence of drought. *B. carinata* and *B. juncea* are more resistant to drought than *B. rapa* and *B. napus*. A comparative evaluation of the

performance of *B. carinata* and *B. napus* in southern Spain concluded that the better performance of *B. carinata* was produced by a faster canopy development early in the season, combined with a longer flowering and grain filling period in the presence of drought and high air temperatures (Fereres et al. 1983). *B. carinata* has also been found to be better adapted than *B. napus* under warm and droughty conditions during grain development in regions of the United States (Knowles et al. 1981), Canada (Getinet et al. 1996; Warwick et al. 2006), India (Malik 1990), and Italy (Mazzoncini et al. 1993). *B. juncea* is also better adapted than *B. napus* to low rainfall areas, with the additional advantage over *B. carinata* of earlier maturing (Getinet et al. 1996; Burton et al. 2007).

5.6.2 Vernalization Requirements and Flowering Time

Brassica rapa includes annual and biennial forms, the latter requiring vernalization, i.e. the promotion of flowering in response to a prolonged period of growth at low temperatures. High vernalization requirements are needed for fall planting in cold climates, but they are undesirable for the development of early-maturing, spring sown cultivars. Studies in this species have identified three loci controlling vernalization-responsive flowering time and three additional loci controlling flowering time that are not responsive to vernalization (Osborn et al. 1997).

5.6.3 Male Sterility

Heterosis breeding has played a major role in recent years in increasing crop yields in *B. napus*. Similarly, exploitation of heterosis is probably the best approach to increase grain yield in the other *Brassica* oilseed crops. Accordingly, extensive research has been conducted to develop male-sterility systems suitable for commercial hybrid production. A number of sources of genetic or nuclear male sterility (NMS) have been identified in *B. rapa* and *B. juncea* (Pandey et al. 1999). Similarly, several cytoplasmic male sterility (CMS) and fertility restoration systems have been developed in *B. juncea* (Pandey et al. 1999), some of them being further transferred to *B. rapa* and *B. carinata*. However, in most cases the CMS systems were not suitable for commercial production. Sodhi et al. (2006) have reported a new CMS system initially identified in *B. napus* and transferred to *B. juncea*, with the particularity that restoration was provided by any line different to the maintainer. The CMS system, designated 126-1, is expected to be present in commercial hybrids in the short term (Sodhi et al. 2007).

5.6.4 Self-Compatibility

The diploid species of *Brassica* are in general self-incompatible. The self-incompatibility system is mainly controlled by a single locus *S*, which includes three highly polymorphic genes that are transmitted as one segregational unit

(Sakamoto and Nishio 2001). There are several self-compatible lines in *B. rapa*. In the cultivar Yellow Sarson, self-compatibility is controlled by two loci, *S* (non-functional haplotype) and *M*, the latter being independent but epistatic to *S* (Fujimoto et al. 2006).

5.6.5 Seed Colour, Oil, Protein and Fibre Content

Seed colour variations are common in *B. rapa, B. juncea* and *B. carinata*, with genotypes ranging from dark brown to reddish-brown and from yellow-brown to yellow. Yellow-coated seeds have higher oil and protein contents and lower fibre content, which is attributable to a lower proportion of seed coat (Daun and DeClercq 1988). Selection for yellow seed coat is therefore an important breeding objective. In the three species, the trait has been found to be maternally inherited. Most studies on *B. rapa* and *B. juncea* have concluded that yellow seeds are the result of recessive alleles at two loci (Padmaja et al. 2005; Rahman et al. 2007). In *B. carinata*, Getinet and Rakow (1997) proposed that yellow seed coat was produced by a dominant repressor gene which inhibits the expression of seed coat pigment synthesis genes.

5.6.6 Oil Quality

Oil quality is largely determined by the fatty acid composition and the presence of minor compounds such as tocopherols and phytosterols affecting the nutritional and/or technological properties of the oil. Different breeding strategies have conducted to the development of modified fatty acid profiles, which have been summarised above in the section of major breeding achievements. Erucic acid content is controlled by multiple additive alleles at one locus in *B. rapa* (Dorrell and Downey 1964) and at two loci in *B. juncea* (Kirk and Hurlstone 1983) and *B. carinata* (Getinet et al. 1997). High oleic acid content (>80%) is under monogenic control in *B. rapa* (Tanhuanpää et al. 1996b) and digenic control in *B. carinata* (Nabloussi et al. 2006). Mid oleic acid levels (>70%) in *B. juncea* have been obtained by antisense suppression of the *fad2* gene (Sivaraman et al. 2004).

Research on minor oil components has been scarce in *Brassica* species other than *B. napus*. Goffman et al. (1998) evaluated tocopherol content and profile in several *Brassica* spp., concluding significantly higher concentrations of alpha-tocopherol in *B. carinata* accessions. The tocopherol profile has been modified in transgenic lines of *B. juncea*, which exhibited a concentration of 62% alpha-tocopherol and 36% gamma-tocopherol, compared to 10% alpha-tocopherol and 87% gamma-tocopherol in the untreated control (Yusuf and Sarin 2007).

5.6.7 Seed Meal Quality

Breeding for low glucosinolate content has been the principal objective in relation to seed meal quality in *Brassica* species. Progress in this field has been

mentioned above in the section of major breeding achievements. Genetic studies on low glucosinolate content in *B. rapa* concluded monogenic inheritance for glucobrassicin and progoitrin contents, which were independently inherited (Kondra and Stefansson 1970). In *B. juncea*, Sodhi et al. (2002) concluded the involvement of seven genes in the genetic control of total glucosinolate content.

Other important breeding objectives such as the reduction of phytates and phenolic compounds have been scarcely addressed in *Brassica* crops, except in *B. napus*. Zhao (2007) has recently identified variation for low phytate content in *B. rapa*, which opens up the possibility of breeding for this trait.

5.6.8 Disease Resistance

Some diseases cause problems in most growing areas, while others are restricted to certain areas. The most important fungal diseases affecting *Brassica* crops are Sclerotinia (*Sclerotinia sclerotiorum*), blackleg (*Leptosphaeria maculans*), white rust (*Albugo candida*), Alternaria blight (*Alternaria* spp.), and downy mildew (*Peronospora parasitica*). *Brassica rapa* is very susceptible to these diseases. Additionally a root rot complex, known as brown girdling root rot complex, caused primarily by *Rhizoctonia solani*, causes problems to this crop in Canada (Soon et al. 2005). *B. juncea* is susceptible to most of the diseases, although it shows a high degree of resistance to blackleg (Sacristan and Gerdemann 1996). White rust, downy mildew, and *Alternaria* are the more severe diseases of this crop in India (Sangeetha and Siddaramaiah 2007). *B. carinata* has a broad resistance to most of the diseases affecting the other *Brassica* crops (Anand et al. 1985; Sacristan and Gerdemann 1996; Choudhary et al. 2000).

Breeding for resistance to major diseases is one important objective in *B. rapa* and *B. juncea*. In *B. rapa*, sources of resistance to blackleg (Falak et al. 1999; Leflon et al. 2007), *Sclerotinia* (Falak et al. 2006), white rust (Tanhuanpää and Vilkki 1999a), *Alternaria* blight (Choudhary et al. 2000), and brown girdling root rot (Woods et al. 2000) have been developed. In *B. juncea*, white rust resistance has been introgressed from *B. napus* (Somers et al. 2002). Some Australian germplasm of this species has been found to show some degree of tolerance to *Sclerotinia* (Singh et al. 2007). Development of *Alternaria* blight resistance in *B. juncea* has not been achieved through conventional breeding, but there is promising ongoing research to develop resistant sources through transgenic research (Mondal et al. 2007). Resistance to downy mildew is available in Indian germplasm (Yadava and Singh 1999).

5.6.9 Insect Resistance

The mustard aphid (*Lipaphis erysimi*) is one of the most important pests of oilseed brassicas, causing severe yield losses particularly in India (Patel et al.

2004). *B. carinata* has less susceptibility to aphid infestations than *B. rapa* and *B. juncea* (Malik 1990; Rana 2005). Resistance to the mustard aphid in *B. juncea* was achieved by transformation with a cDNA encoding different types of agglutinins (Kanrar et al. 2002; Hossain et al. 2006).

5.7 Breeding Methods and Techniques

Breeding strategies are strongly determined by the mode of reproduction of the different species. In the partially allogamous species *B. juncea* and *B. carinata*, pedigree selection and backcrossing have been widely used to develop line cultivars, although breeding of improved populations as 'synthetic cultivars' has also been considered (Becker et al. 1999). In the self-incompatible species *B. rapa*, breeding procedures suitable for partially allogamous species are not appropriate, with the exception of backcrossing. For this species, various types of recurrent selection have been efficient to develop high yielding cultivars (Downey and Rakow 1987). The development of hybrid cultivars as a means of full exploitation of heterosis can be used in the three species.

5.7.1 Developing New Sources of Variation

Breeding programs deal with the manipulation of naturally existing genetic variation available through germplasm collections or newly generated variation. The amount of natural genetic variation available to the *Brassica* oilseed breeder, considering all the species of the genus, is impressive. The first step in searching for novel traits is the evaluation of intraspecific variation existing in local varieties and landraces. Although the within-species variability is often insufficient for many traits, there are examples of identification of variation through the evaluation of genetic resources within the same species, such the isolation of erucic acid-free lines of *B. rapa* (Downey 1964), *B. juncea* (Kirk and Oram 1981) and *B. carinata* (Alonso et al. 1991), or lines with high oil content (De Haro et al. 1998) or low linolenic acid content in *B. carinata* (Velasco et al. 1997a).

A further approach for incorporating desired traits that are not present in the crop species is the hybridization between related species. Interspecific hybridization with other species within the triangle of U has been used to eliminate erucic acid in *B. carinata* (Fernández-Martínez et al. 2001; Getinet et al. 1994) or to develop low glucosinolate types in *B. juncea* (Love et al. 1990). Intergeneric hybridisation has been widely used to develop CMS systems (Pandey et al. 1999; Sodhi et al. 2006). Genetic engineering is being increasingly used to develop novel variation for a number of traits, which is reviewed below.

Mutagenesis has been successfully used to generate genetic variation for useful traits, particularly seed oil quality traits, in *Brassica* spp. In *B. carinata*,

mutants with reduced (Velasco et al. 1995) and increased (Velasco et al. 1998) levels of erucic acid, as well as mutants with altered levels of unsaturated fatty acids (Velasco et al. 1997b) were obtained using chemical mutagenesis. In Indian mustard, Oram and Kirk (1993) also reported induced mutants with reduced linolenic acid content, whereas Bhat et al. (2001) developed CMS-induced mutants.

5.7.2 Breeding of Line and Population Cultivars

For the amphidiploid partially allogamous species *B. juncea* and *B. carinata*, although they always undergo some outcrossing, the most commonly used breeding method has been pedigree breeding to develop line cultivars. The first step involves the selection of parents to be crossed after a careful evaluation of the traits to be combined, followed by artificial hybridization. After crossing, selfing is continued during seven to eight successive generations. Selection is made each generation to combine the desired traits such as low erucic acid and low glucosinolate content, high yield, and disease resistance, in stable uniform lines. The best homogeneous F_6 or F_7 plots are used to obtain breeder´s seed of new candidate cultivars (Downey and Rakow 1987; Becker et al. 1999).

Alternatives to the pedigree method are the single seed descent (SSD) and the development of doubled haploids (DH) lines. The latter abbreviates the time required to achieve homozygosity. Haploids can be induced in large amounts by culturing microspores on appropriate nutrient media to produce plantlets, and then the seedlings are induced to chromosome doubling with colchicine treatment. A simplified alternative method is based on microspore colchicine treatment in vitro. This technique has been widely adopted by *B. juncea* breeders (Sodhi et al. 2002), but it has been more sporadically used in *B. carinata* (Chuong and Beversdorf 1985) and *B. rapa* (Seguin-Swartz et al. 1983).

Backcrossing is a breeding method that has been also successfully used in cultivated *Brassica* species to transfer simply inherited traits such as low erucic acid or low glucosinolate content to adapted high-yielding lines. The procedures used to transfer the zero erucic trait to *B. juncea* and *B. carinata* using this method are the same described for *B. napus* (Downey and Rakow 1987), since the genetic control of zero erucic content in the three species is similar: embryonic control and two loci with multiple alleles having an additive effect (Kirk and Hurlstone 1983; Getinet et al. 1997). In the diploid *B. rapa*, erucic acid is controlled at a single locus (Dorrell and Downey 1964). The heterozygous seeds *E1e1E2e2* (*E1e1* in the case of *B. rapa*) are selected in the F_1 or BC_1F_1 generations, which enables to make a backcross per generation, thus speeding up the transfer of the trait. In the case of recessive traits that do not show up in the F_1 generation, for example the high oleic acid trait of *B. carinata* controlled by two recessive genes (Nabloussi et al. 2006), the use of marker-assisted selection strategies allowing an efficient identification of heterzygotes facilitates

the introgression. An interesting discussion on the value of the backcross method in combination with marker-assisted selection for traits with complex inheritance such as total glucosinolate content can be found in Ramchiary et al. (2007b). Depending on the objectives, a combination of backcrossing and pedigree selection is frequently used (Downey and Rakow 1987). This is particularly useful when the breeder wishes to combine simply inherited traits such as low erucic acid with more complex traits such as high oil content and high seed yield.

In the cross-pollinated self-incompatible forms of *B. rapa*, the standard breeding method is recurrent selection. This method, described in detail by Downey and Rakow (1987) and Dhillon et al. (1995), starts with the planting of spaced plants from source populations (landraces, progenies from crosses, etc.). These plants are harvested individually and a portion of the seeds from each plant is used for progeny row evaluation. The seed of selected rows are further evaluated for quality traits and the reserve seed from selected single plants is composited and planted under isolation to allow random mating and form a new population that will enter a new cycle. The selection is continued for at least three cycles. Another procedure used in *B. rapa* has been the production of synthetics by mixing two or three parental components (Becker et al. 1999). This method was even suggested for the partially allogamous species *B. juncea* and *B. carinata* to make a partial use of the heterosis. However, at present most of the efforts on utilization of heterosis are directed to produce F_1 hybrid cultivars.

5.7.3 Breeding of Hybrid Cultivars

As in *B. napus*, the exploitation of the available heterosis in the other oilseed brassicas can be used for enhancing productivity. Many studies have shown significant levels of heterosis in these species, for example in *B. juncea* (Pradhan et al. 1993), *B. rapa* (Falk et al. 1994), and *B. carinata* (Teklewold and Becker 2005). The interest in developing hybrid cultivars has gained importance in recent years, as effective systems of male sterility to control pollination have been developed. The utilization of genetic or nuclear male sterility (NMS) available in *B. juncea* and *B. rapa* presents economical limitations due to the high labour costs involved to rouge out the male fertile plants in the seed production fields before flowering. These difficulties have been overcome through the development of cytoplasmic male sterility (CMS) and fertility restoration systems. These systems permit the full exploitation of heterosis in F_1 hybrids by the relatively inexpensive means of producing male-sterile female (A), maintainer (B) and restorer (R) lines. In *B. juncea*, a number of CMS sources have been obtained (reviewed in Pandey et al. 1999 and Sodhi et al. 2006), but the lack of appropriate restorer lines has hampered their exploitation for producing commercial hybrid seed (Sodhi et al. 2006). However, a new CMS

named 126-1 was recently reported to be suitable for hybrid production in *B. juncea* (Sodhi et al. 2006), and a hybrid cultivar based on this CMS source has been produced and tested with success over a large area in the mustard growing region of India (Sodhi et al. 2007). In *B. rapa*, although the potential of CMS-based hybrids has been experimentally demonstrated (McVetty 1995), commercial developments have been less successful. No reports on hybrid cultivars have been published for *B. carinata*.

After effective systems for controlled pollination are available, the most important task is the optimization of all steps of the method to identify the best hybrid combinations. The selection of the best cross combinations can be made in three steps (Becker et al. 1999): selection among the components for their *per se* performance, selection among the test crosses for general combining ability, and selection among the hybrids for both general and specific combining ability.

5.7.4 Breeding Techniques

The following techniques, used for greenhouse and field nurseries as well as for seed quality evaluation, are usually considered in oilseed mustard and turnip rape breeding programs.

5.7.4.1 Selfing and Artificial Hybridization

In the partially allogamous species *B. juncea* and *B. carinata*, self-pollinated seed is obtained by enclosing one or more racemes free of open flowers with paper or microperforated plastic bags. In all the species, intraspecific crosses are performed by emasculating flower buds that are about to open or will open within the following day, by removing with tweezers the six undehisced anthers. Pollination is accomplished by picking selected anthers with forceps and applying them to the stigma of emasculated flowers. In the field nurseries, pollen is often applied by dusting emasculated stigmas with racemes of male flowers gathered in the early morning and stored with their stems in water. Fertilization occurs normally within 24 h of pollination, but protective bags should remain in place for at least 3 days. For interspecific hybridization, the success rate depends on the direction of the cross. In crosses involving diploid and amphidiploid species, more seed set is observed when the amphidiploid species is used as the female parent (Downey and Rakow 1987).

5.7.4.2 Techniques Used for Agronomic Evaluation

Newly developed cultivars have to be carefully evaluated before they can be released. For early-generation testing, each genotype is usually sown in single-row plots with check cultivars intercalated every five to ten rows. For breeding

material undergoing precise evaluation, four to six-row plots are used. Phenological and morphological data are recorded in the field. At harvest, seed yields of each plot are recorded and a subsample is taken for analysis of seed quality traits.

5.7.4.3 Laboratory Techniques for Seed Quality Evaluation

Success in the improvement of seed quality traits in oilseed brassicas has been facilitated by an important previous research on novel analytical methods and techniques suitable for screening purposes, but still reliable enough. The half-seed technique was developed for the analysis of the fatty acid profile in part of the cotyledonary tissue without affecting the germination ability of the rest of the seed (Downey and Harvey 1963). The use of near-infrared reflectance spectroscopy (NIRS) has contributed to the success of breeding programs focusing on seed quality. The technique is non-destructive and used for the simultaneous analysis of a wide range of seed quality traits in *Brassica* seeds, including oil, protein, glucosinolates, fibre, fatty acids, bulk density, thousand seed weight, and others (Velasco and Fernández-Martínez 2002). An example of the value of NIRS in breeding programs was its application in a mutagenesis program of *B. carinata* that contributed to broaden the genetic variation of this species for different traits, particularly for seed oil fatty acid profile (Velasco et al. 1995, 1997b, 1998). The technique has also been adapted to single-seed analysis for fatty acid profile in *B. carinata*. Velasco et al. (2003) reported its application in a breeding program aimed at developing high oleic acid content in this species.

5.8 Integration of New Biotechnologies into Breeding Programs

The development of molecular tools to assist in selection and to develop novel genetic variation has represented a major landmark in *Brassica* oilseed breeding. *Brassica* crops benefit from the close phylogenetic relationship with *Arabidopsis*, the model plant for forefront research on plant molecular genetics. Because of its diploid genome shared by *B. napus*, research on *B. rapa* was particularly intensive in the first stages of molecular breeding research, both on vegetable and oilseed types. Important efforts on *B. juncea* molecular research have been also made, especially in recent years. The situation of *B. carinata* is completely different, since there are few research groups with molecular and biotechnological facilities conducting research on this species. Because of the high degree of sequence conservation within the genus, research on other *Brassica* species, especially *B. nigra* and *B. oleracea*, is also of great utility for the oilseed *Brassica* species.

5.8.1 Genetic Markers and Genetic Linkage Maps

Like in other species, the first genetic linkage maps on *Brassica* species were produced using restriction fragment length polymorphism (RFLP) markers. The first genetic map of *B. rapa* was developed for vegetable types (Song et al.

1991). The first RFLP map of oilseed *B. rapa* was based on 360 marker loci distributed in 10 linkage groups, which is the haploid chromosome number of the species (Chyi et al. 1992). In *B. juncea*, the first RFLP map with good genome coverage was produced by Cheung et al. (1997), consisting of 343 loci arranged in 18 major linkage groups, which is the haploid chromosome number of the species.

A second generation of genetic linkage maps was produced by using PCR-based markers, mainly random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) markers. Tanhuanpää et al. (1996a) developed a genetic map of *B. rapa* using 22 RFLP and 144 RAPD markers, distributed over the 10 linkage groups. In *B. juncea*, Sharma et al. (2002) developed a genetic map with 130 RAPD markers arranged in 21 linkage groups, whereas Lionneton et al. (2002) constructed a map based on 264 AFLP and 9 RAPD markers distributed in 18 linkage groups. RAPD, AFLP, and inter simple sequence repeat (ISSR) bands can be converted into more reproducible sequence-characterized amplified region (SCAR) markers, which have been developed for *B. rapa* (Tanhuanpää and Vilkki 1999b) and *B. juncea* (Ripley and Roslinsky 2005; Ashutosh et al. 2007).

The use of microsatellites or simple sequence repeats (SSRs) represented a further advance in molecular breeding due to their extraordinary level of informative polymorphism, repeatability, and amenability to automation. Microsatellites have been mainly developed from the diploid species of *Brassica*, including *B. rapa*, and from *B. napus* (Suwabe et al. 2002; Lowe et al. 2004). Because of the high transferability of SSR markers across related *Brassica* species, SSR markers developed from *B. rapa*, *B. oleracea*, *B. nigra* and *B. napus* have been used for molecular research and breeding in *B. juncea* (Ramchiary et al. 2007a) and *B. carinata* (A. Márquez-Lema, personal communication).

Expressed sequence tags (ESTs) constitute a novel source of DNA-based markers that are physically associated with coding regions of the genome. Several types of markers based on ESTs have been developed for *Brassica* species, for example EST polymorphisms (ESTP) (Choi et al. 2007) and EST-based RFLPs (Kim et al. 2006) in *B. rapa*, and EST-SSRs in *B. juncea* (Hopkins et al. 2007).

High-density linkage maps have been developed for both *B. rapa* and *B. juncea*. In *B. rapa*, a map comprising a total of 556 markers, including 278 AFLP, 235 SSR, 25 RAPD, and 18 sequence-based markers, covering ten linkage groups, was developed. The total length of the linkage map was 1,182 cM, with an average marker interval of 2.8 cM (Choi et al. 2007). Another linkage map of this species was constructed using 545 sequence-tagged loci, mainly EST-based RFLPs. The map covered 1,287 cM, with an average mapping interval of 2.4 cM (Kim et al. 2006). In *B. juncea*, a high density linkage map included 996 AFLP and 33 RFLP markers, aligned in 18 linkage groups. The total map length was 1,629 cM, with an average interval of 3.5 cM between adjacent loci (Pradhan et al. 2003).

5.8.2 Molecular Breeding

5.8.2.1 Germplasm Characterization

In the genus *Brassica*, molecular markers have been used for determination of phylogenetic relationships (Song et al. 1988) as well as for identification of genetic variation present in germplasm collections. Studies on genetic diversity among germplasm accessions based on molecular markers have been conducted in *B. rapa* (Zhao et al. 2005), *B. juncea* (Burton et al. 2004), and *B. carinata* (Teklewold and Becker 2006; Warwick et al. 2006).

5.8.2.2 Molecular Mapping

Seed Colour

Molecular markers linked to loci controlling seed coat colour have been developed in *B. rapa* and *B. juncea*. In *B. rapa*, Teutonico and Osborn (1994) mapped the recessive gene *Yls* controlling yellow seed colour using RFLP markers. In *B. juncea*, Sabharwal et al. (2004) and Padmaja et al. (2005) identified AFLP and SSR markers, respectively linked to two independent loci controlling seed coat colour, whereas Mahmood et al. (2005) detected three QTL that individually explained 43%, 21%, and 16%, respectively, of the phenotypic variation for this trait.

Oil Content and Quality

Several studies have identified QTL affecting seed oil content in *B. juncea*. Lionneton et al. (2002) identified two QTL for this trait in an evaluation under a single environment. Ramchiary et al. (2007a) evaluated a doubled haploid mapping population under three environments, identifying two QTL affecting oil content in all the three environments, one QTL affecting oil content in one environment, whereas other four QTL were identified in single environments.

Extensive research has been conducted to develop molecular markers linked to genes controlling fatty acid biosynthesis and to cloning and sequencing such genes. In *B. rapa*, an SCAR marker linked to a locus for high oleic acid content was developed (Tanhuanpää et al. 1996b). The locus was identified as the omega-6 fatty acid desaturase (*fad2*) gene, which enabled the development of *fad2* allele-specific markers (Tanhuanpää et al. 1998). One major QTL for increased oleic acid content was identified in high erucic acid *B. juncea* (Sharma et al. 2002). In *B. rapa*, Tanhuanpää and Schulman (2002) identified three QTL affecting linolenic acid content in a population segregating for low linolenic acid levels. One of the QTL was identified as the omega-3 fatty acid desaturase (*fad3*) gene through a candidate gene approach. Gupta et al. (2004) identified two QTL underlying the variation for erucic acid content in a population

developed from a zero and a high erucic acid line. The QTL were associated with two fatty acid elongase 1 (*FAE1*) genes. The authors identified seven single nucleotide polymorphisms (SNPs) between the sequences of the genes in high and low erucic acid lines. In *B. rapa*, Teutonico and Osborn (1994) mapped the gene controlling erucic acid content on an RFLP linkage map.

Glucosinolate Content

Molecular markers associated with seed glucosinolate content have been identified in crosses involving low and high glucosinolate cultivars of *B. juncea*. Mahmood et al. (2003) identified two QTL associated with gluconapin (3-butenyl) content, three QTL affecting sinigrin (2-propenyl), and five QTL associated with total glucosinolate content. Interestingly, the major QTL associated with individual glucosinolates were not significant for total glucosinolate content. Ripley and Roslinsky (2005) developed an SCAR marker linked to high sinigrin content after conducting bulked segregant analysis with ISSR markers. Lionneton et al. (2004) used a population developed from crosses of two lines with high glucosinolate content but different glucosinolate profiles, identifying two consistent QTL for both sinigrin and gluconapin content.

Morphological and Agronomic Traits

Several studies have identified QTL consistently associated with morphological and agronomic traits. Teutonico and Osborn (1994) mapped one locus controlling presence or absence of leaf hairs in *B. rapa*. Lionneton et al. (2004) found two distinct QTL for plant height in *B. juncea*. In the same crop, Mahmood et al. (2007) identified three QTL for plant height that were significant, at least, in two or three environments. Ramchiary et al. (2007a) conducted QTL analysis in a *B. juncea* population segregating for 12 agronomic traits such as plant height, number of primary and secondary branches, siliquae per plant, seeds per siliqua, etc. The authors identified 65 QTL for these traits using data from three environments, nine of them detected in all the three environments.

Male Sterility

A stable CMS line of *B. juncea* was developed through somatic hybridization with *Moricandia arvensis*, which was also the genetic source for fertility restoration (Prakash et al. 1998). Ashutosh et al. (2007) identified two AFLP and one SCAR markers linked to the fertility restoration locus *Rf*.

Vernalization Requirements and Flowering Time

Studies in *B. rapa* have identified three QTL controlling vernalization-responsive flowering time and three additional QTL controlling flowering time that are not responsive to vernalization (Osborn et al. 1997). In *B. juncea*, which has no

vernalization requirements, Ramchiary et al. (2007a) identified four QTL affecting flowering time, two of them expressed over three contrasting environments and the other two QTL expressed in two environments. Mahmood et al. (2007) identified five QTL affecting days to first flowering considering the average of four environments. One of the QTL was also significant in all the four individual environments, and it was tightly linked to QTL affecting days to end of flowering, days to maturity, and also plant height.

Disease Resistance

White rust caused by *Albugo candida* is one of the most important diseases of *B. rapa* and *B. juncea*. *A. candida* is classified into ten races based on host specificity. *B. juncea* is infected by race 2, whereas *B. rapa* is infected by race 7, although there is variation for virulence between biotypes of the same race. Most studies have shown that resistance to both races is governed by single dominant genes (Tanhuanpää 2004). Several independent loci controlling resistance to white rust have been mapped in *B. rapa* (Kole et al. 2002; Tanhuanpää 2004) and *B. juncea* (Cheung et al. 1998; Somers et al. 2002; Varshney et al. 2004).

Blackleg is the most important disease affecting *B. rapa* worldwide. At least five resistance genes have been identified in *B. rapa* germplasm. Two of them, *Rlm1* and *Rlm7* have been mapped to the R7 linkage group of this species (Leflon et al. 2007). Two resistance genes identified in *B. juncea*, *LMJR1* (dominant) and *LMJR2* (recessive), have been mapped in the B genome of this species (Christianson et al. 2006).

5.8.3 Marker Assisted Selection

The availability of molecular markers tightly linked or based on the sequences of genes of interest is a powerful tool for selection based on the genotype rather than the phenotype of the plants. The accuracy of marker assisted selection largely depends on the number of genes involved and the proximity of the markers to the gene. For erucic acid content, which is a trait controlled by additive alleles at two loci, allele-specific SNP markers have been developed in *B. juncea*, which ensures an accurate tagging of the trait in marker assisted backcross programs (Gupta et al. 2004). Similarly, Tanhuanpää et al. (1998) developed an allele-specific marker in *B. rapa* based on the sequence of the *fad2* locus, which encodes the enzyme responsible for the desaturation of oleic acid to linoleic acid.

The opposite situation is the selection for complex genetic traits, such as glucosinolate content, based on molecular markers identified through QTL analysis and not based on the sequences of the genes. In this case, the existence of epistatic and context dependent interactions may considerably reduce the selection efficiency (Ramchiary et al. 2007b).

5.8.4 Transgenic Breeding

Transgenic technologies are increasingly being used in *Brassica* oilseed breeding. Much of the research is being conducted in *B. juncea*. One of the main objectives has been to modify the fatty acid profile of the seed oil. In this research area, transgenic *B. juncea* plants with increased levels of oleic acid and reduced levels of linolenic acid (Stoutjesdijk et al. 1999; Sivaraman et al. 2004; Jha et al. 2007), or with reduced levels of total saturated fatty acids in the seeds (Yao et al. 2003) have been developed. Genetic manipulation of this crop has also resulted in the production of transgenic plants with ability to accumulate gamma-linolenic acid in the seeds (Hong et al. 2002; Das et al. 2006). The tocopherol fraction of the seeds has been also modified through a transgenic approach. Yusuf and Sarin (2007) produced transgenic lines with enhanced accumulation of alpha-tocopherol by overexpressing a gamma-tocopherol methyl transferase cDNA.

Transgenic research has been applied to the fields of disease and insect resistance. Mondal et al. (2007) enhanced resistance to *Alternaria* blight in *B. juncea* by transformation with a glucanase protein from tomato. Resistance to the mustard aphid has been achieved through transgenic approaches (Kanrar et al. 2002; Hossain et al. 2006). Herbicide resistance is another trait for which transgenic *B. juncea* lines have been developed (Green and Salisbury 1999; Mehra et al. 2000).

In recent years, there is a considerable interest in using *B. juncea* for phytoremediation of contaminated soils. There are a number of transgenic lines of *B. juncea* with improved ability to accumulate toxic metals and metalloids (Gasic and Korban 2007) or organic pollutants (Flocco et al. 2004).

5.9 Seed Production

The rapeseed and mustard breeder identifies pure lines or populations that perform better than the currently used cultivars. The seed of this material, referred to as breeder seed or prebasic seed, is maintained and increased by the breeder and it is genetically the purest source of a cultivar. When applied to hybrid cultivars, it refers to the seed of male-sterile (A), maintainer (B) and restorer (R) lines. The initial increase of breeder seed, referred to as foundation seed, is also grown, directly or indirectly, under the supervision of the plant breeder. Registered seed is the progeny of breeder and foundation seed handled under procedures acceptable to the certifying agency to maintain satisfactory genetic purity and identity. Certified seed is the progeny of breeder and foundation seed.

The increase and production of breeder, foundation, and certified seed is critical and requires highly qualified seed producers. The standards of purity, identity and certification procedures vary from country to country (Downey

and Rakow 1987). For example, the levels of uniformity required in Europe are greater than for other producing regions of the world. The requirements for cultivar uniformity vary also with the species, being less stringent for cross-pollinated *B. rapa* populations.

The increase of breeder/foundation seed of pure line cultivars, as well as A, B and R lines in the case of hybrid seed production, must be accomplished under spatial isolation or using screened cages. For spatial isolation, McVetty (1995) recommended in Canada an isolation distance of 800 m and the R line planted 2 m around the hybrid seed production field. In China, Dianrong (1999) recommended an isolation distance of over 2,000 m. The planting ratio (female:male) is also a crucial aspect for an economical hybrid seed production. Depending on the CMS source, Yadav and Chakrabarty (2007) suggested female:male ratios varying from 8:2 to 16:2 in *B. juncea*. Two hives of honeybees per hectare are placed within the maintainer and hybrid seed production field during the pollinating period (Dianrong 1999).

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Chapter 6
Sunflower

José M. Fernández-Martínez, Begoña Pérez-Vich, and Leonardo Velasco

6.1 Introduction

Sunflower (*Helianthus annuus* L.) oil is the fourth most important vegetable oil in world trade at present with an annual production of around 9 million tonnes and a cultivated acreage of over 22 million hectares, mainly concentrated in the Russian Federation, Ukraine, India, and Argentina, which totalize more than 50% of sunflower world acreage. Although of North American origin, where it was domesticated by the Native American Indians for its edible seeds (Heiser et al. 1969), the transformation of sunflower into a major oilseed crop only took place in the second half of the 20th century due to two major breeding achievements: the drastic increase of oil percentage in sunflower achenes achieved in the Former Soviet Union from 1920 to 1960 (Gundaev 1971), and the development of a cytoplasmic male sterility system (Leclercq 1969) combined with fertility restoration by nuclear genes (Kinman 1970) that enabled the commercial production of hybrid seed. The subsequent development of short-stemmed, high yielding hybrid cultivars with high oil content well adapted to mechanised cropping represented the transformation of sunflower into a cash crop and sunflower oil into a major commodity in world trade.

Conventional sunflower produces a healthful oil with great consumer acceptance because of its high content of monounsaturated and polyunsaturated fatty acids as well as high vitamin E content. In recent years, new sunflower oil types for specific applications, mainly in the food industry, have been developed through conventional breeding approaches. Such specialty oils are called to play an important role in a further development of the sunflower crop.

Unlike other oilseed crops such as soybean and canola, commercial sunflower has not been subject to transgenic breeding so far. However, sunflower breeders have been very successful in attaining a wide diversity of breeding objectives, from developing novel seed oil quality types to incorporating genetic

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resistance to most of the pests and diseases that threaten the crop. The history, current status, and future prospects of breeding advances in sunflower are reviewed in this chapter.

6.2 Origin and Domestication

The cultivated sunflower (*Helianthus annuus* L.) is a member of the family Compositae (Asteraceae). The basic chromosome number is n = 17. The genus includes diploid, tetraploid and hexaploid species. The closest relatives appear to be *Tithonia*, *Viguiera* and *Phoebanthus* (Heiser et al. 1969). The common sunflower (*H. annuus*) is the most important species grown commercially, although other species are also cultivated, e.g. *H. tuberosus*, which is grown for production of edible tubers, and several other species grown as ornamentals. The name *Helianthus* is derived from the Greek words "helios," meaning sun, and "anthus," meaning flower. The Spanish name for sunflower, "girasol," and the French name "tournesol" literally mean "turn with the sun," a trait exhibited by sunflower until anthesis, after which the capitula (heads) face east.

Heiser et al. (1969) proposed a species classification of the genus *Helianthus* including 14 annual and 36 perennial species from North America (in three sections and seven series) and 17 species from South America. More recent classifications (Schilling and Heiser 1981; Jan and Seiler 2007) have introduced some modifications. The new classification brings the number of species to 51, with 14 annual and 37 perennial species (Tables 6.1 and 6.2).

Prior to the arrival of the European explorers to the New World, the progenitor of cultivated sunflower, the wild *H. annuus* was restricted to the southern U.S. (Heiser 1978). Wild *H. annuus* was used for food by the Native American Indians and, due to its association with humans, it became a camp-following weed that was introduced into the central part of the U.S., where it was domesticated and carried to the east and southwest (Heiser et al. 1969). The earliest evidence of domesticated sunflower has been dated at 4,625 B.P. (Crites 1993).

The origin of cultivated sunflower has been also investigated using molecular techniques. The use of randomly amplified polymorphic DNA (RAPD) markers supported the hypothesis that wild *H. annuus* was the progenitor of cultivated sunflower (Arias and Rieseberg 1995). Further studies using allozyme variation (Cronn et al. 1997) concluded that wild *H. annuus* from the Great Plains include the most likely progenitor of domesticated sunflower. More recent results based on the genetic relation between wild and extant domesticates (Harter et al. 2004) support the hypothesis that extant domesticated sunflowers arose from wild populations in the central part of the U.S. Other investigations using QTL analysis have studied the identity of traits that were the primary targets of strong selection during domestication (Burke et al. 2002). They concluded that strong directional selection for increased achene size appears to have played a central role in sunflower domestication.

Table 6.1 Infrageneric classification of annual *Helianthus* species (n = 17)

Section[a]	Species
Helianthus	*H. annuus* L.
	H. anomalus S.F. Blake
	H. argophyllus Torr.& A. Gray
	H. bolanderi A. Gray
	H. debilis Nutt.
	subsp. *debilis*
	subsp. *cucumerifolius* (Torr. & A. Gray) Heiser
	subsp. *silvestris* Heiser
	subsp. *tardiflorus* Heiser
	subsp. *vestitus* (E. Watson) Heiser
	H. deserticola Heiser
	H. exilis A. Gray
	H. neglectus Heiser
	H. niveus (Benth.) Brandegee
	subsp. *canescens* (A. Gray) Heiser
	subsp. *niveus*
	subsp. *tephrodes* (A. Gray) Heiser
	H. paradoxus Heiser
	H. petiolaris Nutt.
	subsp. *fallax* Heiser
	subsp. *petiolaris*
	H. praecox Engelm. & A. Gray
	subsp. *hirtus* (Heiser) Heiser
	subsp. *praecox*
	subsp. *runyonii* (Heiser) Heiser
Agrestes	*H. agrestis* Pollard
Porteri	*H. porteri* (A. Gray) Pruski

[a] Schilling and Heiser (1981); Jan and Seiler (2007)

The domesticated sunflower was introduced from North America into Europe by the early Spanish explorers in 1510 (Putt 1997), where they initially gained popularity as a garden ornamental. The agronomic development of sunflower as an oilseed crop and for use as edible achenes (confectionery types) took place in Russia, where a number of landraces had been developed by the late 1800s. Initial selection emphasis was given to early maturity, disease and pest resistance, and high seed oil content. Sunflower was reintroduced from Russia to North America in the latter part of the 19th century (Putt 1997).

6.3 Varietal Groups

There are three major groups of varieties of cultivated sunflower (*H. annuus*): those used for the extracted seed oil (oilseed types), those for the direct consumption of the seeds (confectionery types), and those used as ornamentals.

Table 6.2 Infrageneric classification of perennial *Helianthus* species

Section[a]	Series	Species	Chromosome number (n)
Ciliares	*Ciliares*	*H. arizonensis* R.C. Jacks.	17
		H. ciliaris DC.	34,51
		H. laciniatus A. Gray	17
Ciliares	*Pumili*	*H. cusickii* A. Gray	17
		H. gracilentus A. Gray	17
		H. pumilus Nutt.	17
Atrorubens	*Coronasolis*	*H. californicus* DC.	51
		H. decapetalus L.	17,34
		H. divaricatus L.	17
		H. eggertii Small	51
		H. giganteus L.	17
		H. grosseserratus M. Martens	17
		H. hirsutus Raf.	34
		H. maximiliani Schrad.	17
		H. mollis Lam.	17
		H. nuttallii Torr. & A. Gray	
		subsp. *nuttallii*	17
		subsp. *parishii* (A. Gray) Heiser	17
		subsp. *rydbergii* (Britton) R. Long	17
		H. resinosus Small	51
		H. salicifolius A. Dietr.	17
		H. schweinitzii Torr. & A. Gray	51
		H. strumosus L.	34,51
		H. tuberosus L.	51
Atrorubens	*Microcephali*	*H. glaucophyllus* D.M. Sm.	17
		H. laevigatus Torr. & A. Gray	34
		H. microcephalus Torr. & A. Gray	17
		H. smithii Heiser	17,34
Atrorubens	*Atrorubentes*	*H. atrorubens* L.	17
		H. occidentalis Riddell	
		subsp. *occidentalis*	17
		subsp. *plantagineus* (Torr. & A. Gray) Heiser	17
		H. pauciflorus Nutt.	
		subsp. *pauciflorus*	51
		subsp. *subrhomboideus* (Rydb.) O. Spring & E.E. Schill.	51
		H. silphioides Nutt.	17
Atrorubens	*Angustifolii*	*H. angustifolius* L.	17
		H. carnosus Small	17
		H. floridanus A. Gray ex Chapm.	17
		H. heterophyllus Nutt.	17
		H. longifolius Pursh	17
		H. radula (Pursh) Torr. & A. Gray	17
		H. simulons E. Watson	17
		H. verticillatus Small	17

[a] Schilling and Heiser (1981); Jan and Seiler (2007)

Hybrid varieties are nowadays predominant for all three groups. By far, the major portion of sunflower production is devoted to oil extraction (Miller and Fick 1997). Sunflower oil has been traditionally viewed as a healthful vegetable oil and it is considered a premium oil for salad, cooking, and margarine production. The seeds of confectionery sunflower varieties are used as snack food as well as for feeding birds and small animals. The main characteristics that differentiate oilseed and confectionery sunflowers are oil content and seed size. The oilseed varieties have small black seeds with low hull content and very high oil content (about 50%). Conversely, confectionery sunflower varieties have larger seeds, which are usually black with white stripes, with lower oil content (about 30%) and a higher hull percentage.

Oilseed sunflower varieties are divided into three groups according to their oleic acid content: linoleic, mid-oleic, and high oleic. Linoleic (traditional) varieties have linoleic acid content between 45 and 75%, depending on the environment. It is considered a healthy vegetable oil suitable for salad and margarine production. The seed oil of mid- and high-oleic varieties has an oleic acid content of 55–75% and 85–90%, respectively. These oils are characterized by a better thermooxidative stability, which makes them more appropriate for frying purposes.

The main criterion of quality of confectionery sunflower is the seed size (Lofgren 1997). The largest size (>7 mm) type goes into the in-shell market to be used as snack. Medium-size seeds are hulled for the kernel market and those with the smallest size go into the bird and pet feeding market.

The last group of sunflower varieties includes those grown for ornamental purposes. There is great diversity for floral colour (yellow, cream, orange, rose, red, burgundy and bicolour) and morphology as well as for plant height (Fick 1976). Head diameter can vary from 10 to more than 30 cm and plant height from 50 to 500 cm (Miller and Fick 1997). Ornamental sunflower cultivars are used in gardens, home landscapes or as cut flowers. Cultivars used in home gardens are usually classified in groups based on plant height, which include giant or very tall (2.5–5 m), semi-dwarf (1–2.5 m), and dwarf types (<1 m). The cultivars used as cut flowers are pollen-free types which incorporate the cytoplasmic male-sterility trait. Ornamental cultivars usually incorporate genes from wild *Helianthus* spp.

6.4 Genetic Resources

Genetic resources are the base of crop improvement. They consist of the total pool of variability that exists in the cultivated species and also in related species that are sexually compatible with the cultivated one. Cultivated sunflower can be crossed with most of the 51 *Helianthus* spp. Sunflower germplasm resources can be categorized as *ex situ* resources (accessions preserved in seed banks) and *in situ* resources (wild populations and land races).

6.4.1 Germplasm Collection and Maintenance

6.4.1.1 *Ex Situ* World Collections

Aggressive collection of wild and cultivated sunflower germplasm for preservation in seed banks is crucial to make it easily available to sunflower breeders. Given the tenuous situation of some wild species in their natural habitats and the replacement of local landraces by outstanding high-yielding improved cultivars, seed banks may provide the only way to preserve these germplasm resources for posterity.

Systematic collection, introduction and conservation of sunflower germplasm were carried out by the N.I. Vavilov All-Union Scientific Research Institute (VIR) at St. Petersburg, Russia and by the U.S. National Plant Germplasm System (NPGS) Ames, Iowa, U.S.A. These two institutions maintain the two largest world collections of sunflower. The VIR collection has about 2,811 accessions, including 493 accessions of wild species and 2,318 of cultivated origin, most of them collected in the former USSR (Omelchenco 2001). The NPGS sunflower collection maintained at the North Central Regional Plant Introduction Station (NCRPIS) in Ames contains 3,860 accessions from 59 countries. The collection includes 1,670 accessions of cultivated *H. annuus*, 1,006 accessions of wild *H. annuus* forms, 430 accessions from other 11 wild annual *Helianthus* species, and 754 accessions representing 37 perennial *Helianthus* species (Marek et al. 2004). It is currently the largest and most genetically diverse *ex situ* sunflower collection of the world. NCRPIS not only conserves this genetically diverse *Helianthus* collection, but conducts germplasm-related research, encourages the use of germplasm and associated information for crop improvement, and distributes the accessions all around the world.

Other large collections of wild and cultivated sunflower are maintained at the Institute of Field and Vegetable Crops, Novi Sad, Serbia, at the Dobroudja Agricultural Institute (DAI) at General Toshevo, Bulgaria, at the Research Institute for Cereals and Industrial Crops in Fundulea, Romania, and at the Station d́ Amélioration des Plantes in Montpellier, France.

6.4.1.2 Preservation of *In Situ* Resources

Preservation of in situ resources (wild species and landraces) in their natural habitats is critical, especially for wild sunflower populations, because of the lack of resources necessary to preserve all the wild species in seed banks. Moreover, a significant proportion of the wild diversity is likely to be lost while regenerating banked germplasm accessions. Unfortunately, the long-term preservation of some wild sunflower populations in their natural habitats is not always promising and some species are endangered or even extinct (Seiler and Rieseberg 1997). The U.S. Department of Interior, Fish and Wild Life Service listed several endangered and threatened species (Seiler and Rieseberg 1997). These species

included the annuals *H. paradoxus* and perennials *H. eggertii* and *H. schweinitzii*. Other candidate species for federal protection were the annuals *H. anomalus*, *H. deserticola*, *H. exilis* and *H. niveus* subsp. *tephrodes* as well as the perennials *H. laevigatus*, *H. carnosus*, *H. smithii* and *H. verticillatus*.

As for other genera, the primary obstacle for long-term preservation of wild *Helianthus* populations is human activity. This is for example the case of *H. exilis*, restricted to serpentine soils in the Inner Coastal Range of California, where mining activities destroyed several populations (Seiler and Rieseberg 1997). Another example of the impact of human activities was the case of *H. nuttallii* subsp. *parishii*, whose populations were drastically reduced by urbanizations around Los Angeles in Southern California (Rogers et al. 1982). In addition to the direct destruction of wild populations by development, their disturbance by human activity favours their hybridization with more widespread species (Rieseberg 1991). The resulting hybrid plants usually have lower fitness than locally adapted populations. For example, several rare annual sunflowers such as *H. anomalus*, *H. paradoxus* and *H. deserticola* occur sympatrically and occasionally hybridize with the common sunflower, *H. annuus*, what might imply a threat for the existence of these species. Another threat for the preservation of some rare species with small population sizes (e.g. *H. paradoxus* and *H. deserticola*) is their low level of genetic diversity (Rieseberg 1991).

6.4.1.3 Core Collections

Large germplasm collections usually contain duplications and they are difficult to manage in the evaluation of the existing variability for useful traits. Accordingly, the establishment of core subsets of the sunflower collections is imperative. Of the 1,624 cultivated accessions of the U.S. NPGS sunflower collection, a core collection of 112 accessions (7%) was established based on 20 descriptors (Brothers and Miller 1999). The accessions in this core collection represented 38 of the 57 countries of origin of the whole sunflower collection and contained 2 ornamental accessions, 7 breeding lines, 12 landraces, and 91 cultivars.

6.4.1.4 Genetic Stock Collections

Genetic stocks comprise unique mutants of different traits (morphological, chemical, physiological) as well as lines with specific characteristics (male-sterile lines, isolines, aneuploid lines) that are useful for basic research. There are about 30 sunflower genetic stocks registered by the Crop Science Society of America. Examples of these genetic stocks are characterised by nuclear male sterility (Jan 1992a), tetraploidy (Jan 1992b), dwarfness (Velasco et al. 2003a), altered seed oil fatty acid profile (Miller and Vick 2002; Vick et al. 2007), or resistance to herbicides (Miller and Al-Khatib 2002).

6.4.2 Germplasm Evaluation

A first step in any breeding program is the identification of genetic variability for different target traits. Sunflower breeders have been using a wide range of germplasm of wild and cultivated sunflower to search for variation for agronomic and seed quality traits as well as resistance to insects and diseases.

6.4.2.1 Agronomic and Physiological Traits

World collections of wild and cultivated germplasm possess a wide variability for morphological and physiological traits (plant height, flowering period, leaf and achene characteristics, etc.) of interest for sunflower breeding. A wide variety of agronomic traits was examined in wild *Helianthus* species for potential use in improving the hardiness and productivity of cultivated sunflower (Laferriere 1986). Wild *Helianthus* species have also been evaluated for resistance to several environmental stresses. Blanchet and Gelfi (1980) tested 10 species for various aspects of drought resistance and recommended *H. argophyllus* as a most likely source because of its pubescent leaves that reflect sunlight, reduced water loss, low stomatal resistance, and low transpiration rates. This species has been frequently used in breeding programs for drought resistance (Blanchet and Gelfi 1980). High variability has also been found in wild species for traits related to photosynthetic efficiency such as leaf area duration (Škorić 1988).

Several species of *Helianthus* are native to salt-impacted habitats. For example, *H. paradoxus* is found in saline marshes, where it exhibits high salt tolerance attributed to great leaf succulence and leaf sodium sequestration (Lexer et al. 2003b). Variability for salt tolerance has been also identified in germplasm of cultivated sunflower (Ashraf and Tufail 1995).

6.4.2.2 Cytoplasmic Male Sterility

Cytoplasmic male sterility (CMS) is a maternally inherited trait preventing plants from producing normal pollen. CMS is used as a tool to generate F_1 hybrid seed. Based on its origin, CMS is classified as autoplasmic or alloplasmic. Autoplasmic CMS refers to the cases where CMS has arisen within the species as a result of mutational changes in the cytoplasm. Alloplasmic male sterility arises from interspecific, intergeneric and occasionally intraspecific crosses due to incompatibility between nucleus and cytoplasm. Both types of CMS have been identified in sunflower. A type of stable alloplasmic male sterility named PET1 was reported by Leclercq (1969) in the progeny of an interspecific cross between *H. petiolaris* and cultivated sunflower. The subsequent identification of dominant fertility restoration genes, especially a source

derived from wild species (Kinman 1970) allowed for efficient and economical production of commercial hybrid seed 30 years ago. Virtually all cultivated sunflower hybrids are currently based on the CMS source derived by Leclercq (1969). The identification of additional CMS sources has been an important objective to broaden genetic diversity in cultivated sunflower. As a result of these efforts, a total of 70 CMS sources were identified (Table 6.3). Most of these sources originated from progenies of crosses between 16 different wild *Helianthus* accessions (mostly annuals) and cultivated lines (Serieys 2002), but some of them arose from mutational changes within the species *H. annuus*. Fertility restoration genes, found primarily in wild *Helianthus* species and also in related genera, have been reported for 34 CMS sources. Detailed inheritance studies have been conducted for 19 of the CMS sources (Serieys 2002). Therefore several CMS systems other than the widely used PET1 are available for practical hybrid seed production.

Table 6.3 Sources of cytoplasmic male sterility in sunflower[a]

Name	Origin (species)	FAO code[b]	Name	Origin (species)	FAO code[b]
Kouban	*H. annuus lenticularis*	ANL1	HA89	*H. annuus*	MUT 10
Indiana 1	*H. annuus lenticularis*	ANL2	HA89	*H. annuus*	MUT 11
Vir 126	*H. annuus, lenticularis*	ANL3	HA89	*H. annuus*	MUT12
397	*H. annuus* wild	ANN1	Anomalus	*H. anomalus*	ANO1
517	*H. annuus* wild	ANN2	Argophyllus	*H. argophyllus*	ARG1
519	*H. annuus* wild	ANN3	Argophyllus	*H. argophyllus*	ARG2
521	*H. annuus* wild	ANN4	Argophyllus	*H. argophyllus*	ARG3
Ns-Ann-81	*H. annuus* wild	ANN5	Arg3-M1	*H. argophyllus*	ARG3-M1
Ns-Ann-2	*H. annuus* wild	ANN6	Argophyllus	*H. argophyllus*	ARG4
	H. annuus wild	ANN7	Bolanderi	*H. bolanderi*	BOL1
	H. annuus wild	ANN8	Dv-10	*H. debilis*	DEB1
	H. annuus wild	ANN9	Exilis	*H. exilis*	EXI1
Fundulea 1	*H. annuus texanus*	AMT1	Exi2	*H. exilis*	EXI2
An-67	*H. annuus*	ANN10	Cmg2	*H. giganteus*	GIG1
An-58	*H. annuus*	ANN11	Cmg3	*H. maximiliani*	MAX1
An-2-91	*H. annuus*	ANN12		*H. maximiliani*	MAX2
An-2-92	*H. annuus*	ANN13	Mollis	*H. mollis*	MOL1
	H. annuus	ANN14	Neglectus	*H. neglectus*	NEG1
Cms-G	*H. annuus*	ANN15	Canescens	*H. niveus canescens*	NIC1
Cms-Dp	*H. annuus*	ANN16	Fallax	*H. petiolaris fallax*	PEF1
Cms-Vl	*H. annuus*	ANN17	Pet/Pet	*H. petiolaris petiolaris*	PEP1

Table 6.3 (continued)

Name	Origin (species)	FAO code[b]	Name	Origin (species)	FAO code[b]
	H. annuus	ANN18	Classical Cms	*H. petiolaris* Nutt	PET1
	H. annuus	ANN19	Cmg1	*H. petiolaris* Nutt	PET2
	H. annuus	ANN20	Petiolaris Bis	*H. petiolaris* Nutt	PET3
	H. annuus	ANN21	Pet34	*H. petiolaris*	PET4
	H. annuus	ANN22		*H. petiolaris*	PET5
Hemus	*H. annuus*	MUT1	Praecox	*H. praecox*	PRA1
Peredovick	*H. annuus*	MUT2	Phir-27	*H. praecox hirtus*	PRH1
Stadion	*H. annuus*	MUT3	Praecox	*H. praecox praecox*	PRP1
Peredovick	*H. annuus*	MUT4	Ppr-28	*H. praecox praecox*	PRP2
Peredovick	*H. annuus*	MUT5	Run-29	*H. praecox*	PRR1
Voronejskii	*H. annuus*	MUT6	Resinosus 243	*H. resinosus*	RES1
HA89	*H. annuus*	MUT7	Vulpe	*H. rigidus*	RIG1
HA89	*H. annuus*	MUT8	Rig-M-28	*H. rigidus*	RIG2
HA89	*H. annuus*	MUT9	Strumosus	*H. strumosus*	STR1

[a]Serieys (2002).
[b]The coding system for CMS sources consists of three-letter abbreviations of the cytoplasm donor species or subspecies followed by a number starting with 1, depending on the time of discovery and its reaction to restoration testers.

6.4.2.3 Disease and Insect Resistance

Diseases and insects are limiting factors of production in the majority of sunflower producing countries. The most serious diseases of sunflower are caused by fungi. They include *Sclerotinia* wilt, stalk rot, and head rot (*Sclerotinia sclerotiorum*), *Verticillium* wilt (*Verticillium dahliae*), sunflower rust (*Puccinia helianthi*), *Phoma* black stem (*Phoma macdonaldii*), downy mildew (*Plasmopara halstedii*), Phomopsis stem canker (*Diaporthe helianthi*), charcoal rot (*Macrophomina phaseolina*), alternaria diseases (*Alternaria* spp.), powdery mildew (*Erysiphe cichoracearum*, *Sphaerotheca fuliginea*, *Leveillula tarucia*), and *Rhizopus* head rot (*Rhizopus* spp.). Resistance to most of these diseases is found in wild *Helianthus* species as shown in Table 6.4 for some examples.

Genetic resistance to broomrape (*Orobanche cumana*), a parasitic plant that limited early sunflower production in the former USSR, was initially introduced into susceptible sunflower mainly from the wild species *H. tuberosus* (Pustovoit and Gubin 1974). More recently, results of evaluation of sunflower germplasm for resistance to new virulent races have shown that wild *Helianthus* species constitute the major source of resistance genes, although resistance was also found in accessions of cultivated material (Fernández-Martínez et al. 2000).

Table 6.4 Reported resistance to important diseases in wild sunflower species

Disease	Species with resistance	Reference
Downy mildew (*Plasmopara helianthi*)	*H. argophyllus* *H. annuus* *H. petiolaris* *H. praecox*	Hoes et al. (1973)
Verticilium wilt (*Vericillium dahliae*)	*H. annuus* *H. petiolaris* *H. praecox*	Hoes et al. (1973)
Rust (*Puccinia helianthi*)	*H. argophyllus* *H. annuus* *H. petiolaris* *H. praecox*	Hoes et al. (1973) Quresh et al. (1993)
Alternaria leaf spot (*Alternaria helianthi*)	*H. hirsutus* *H. pauciflorus* *H. tuberosus*	Morris et al. (1983)
Powdery mildew (*Erysiphe cochoracearum*)	*H. debilis* subsp. *debilis* *H. bolanderi* *H. praecox*	Saliman et al. (1982) Jan and Chandler (1985)
Phoma black stem (*Phoma macdonaldii*)	*H. decapetalus* *H. eggertii* *H. hirsutus* *H. resinosus* *H. tuberosus*	Škorić (1985)
Phomopsis stem canker (*Diaporthe helianthi*)	*H. maximiliani* *H. pauciflorus* *H. hirsutus* *H. resinosus* *H. mollis* *H. tuberosus*	Škorić (1985) Dozet (1990)
Rizopus head rot (*Rhizopus arrhizus*)	*H. divaricatus* *H. hirsutus* *H. resinosus* *H. x laetiflorus*	Yang et al. (1980)
Sclerotinia head rot (*Sclerotinia sclerotiorum*)	*H. decapetalus* *H. grosseerratus* *H. nuttallii* *H. pauciflorus* *H. resinosus* *H. tuberosus*	Pustovoit and Gubin (1974) Mondolot-Cosson and Andary (1994) Rönicke et al. (2004)
Sclerotinia root rot (*Sclerotinia sclerotiorum*)	*H. mollis* *H. nuttallii* *H. resinosus* *H. tuberosus*	Škorić (1987)
Sclerotinia mid-stalk (*Sclerotinia sclerotiorum*)	*H. praecox* *H. giganteus* *H. maximiliani* *H. pauciflorus* *H. resinosus* *H. tuberosus*	Škorić (1987)
Broomrape (*Orobanche cumana*)	Most of the perennial species	Fernández-Martínez et al. (2000)

Although several hundreds of insect species are associated with sunflower, only a few of them are economically important pests of cultivated sunflower (Schulz 1978). An exception was the significant yield reduction caused by the European sunflower moth (*Homoeosoma nebulellum*, Lepidoptera) in the former USSR at the end of the 19th century, which encouraged the first scientific breeding research on sunflower as early as 1890. Resistant cultivars were developed by interspecific hybridization of cultivated sunflower with *H. tuberosus*, which accumulates phytomelanin in the seed, thus reducing larval feeding (Gundaev 1971). Some wild species have been found to be resistant to insect pests attacking sunflower in North America, such as the sunflower stem weevil (*Smicronyx fulvus*, Coleoptera) (Rogers and Seiler 1985) and the sunflower beetle (*Zygogramma exclamationis*, Coleoptera) (Rogers and Thompson 1980). Evaluation of germplasm of cultivated sunflower has also revealed the existence of variability for resistance to several important insect pests (Charlet et al. 2007).

6.4.2.4 Oil and Protein Content and Quality

Oil and protein content of the sunflower achene depends on both the percentage of hull and the oil and protein concentration in the kernel. Variation for hull percentage and oil and protein content has been found in extensive evaluations of cultivated sunflower germplasm (Jiménez et al. 1985; Miller et al. 1992). Variation for oil and protein content also exists in wild species. Maximum oil content reported in wild species is lower than current standards of cultivated sunflower (around 50%). Conversely, maximum values of protein content found in wild species (35–40%) are generally higher than the typical protein content of cultivated sunflower (Seiler 1984; Ruso et al. 2000).

Variation for the seed oil quality components has been also found through the evaluation of genetic resources. The evaluation of germplasm collections led to the identification of cultivated sunflower germplasm with reduced levels of saturated fatty acids (Vick et al. 2002), high palmitic acid content (Demurin 2003) or high linoleic acid content (Miller and Vick 2001), as well as wild sunflower germplasm with reduced levels of saturated fatty acids species (Seiler 2004). Variation for increased levels of beta- and gamma-tocopherol has been found in collections of cultivated germplasm (Demurin 1993; Velasco et al. 2004a).

Sunflower germplasm is also a useful source of variation for reducing antinutritive compounds of the seeds, such as chlorogenic acid, that reduces the nutritive value of the meal. Dorrell (1976) found variation for reduced levels of chlorogenic acid in germplasm of wild and cultivated *Helianthus* species.

6.5 Major Breeding Achievements

6.5.1 Development of High Oil Germplasm in the Former USSR

After its introduction to Europe in the 16th century, sunflower was mainly grown as an ornamental. The first mention of sunflower cultivation as an oil crop was in Russia in 1779 (Gundaev 1971). The crop expanded rapidly and the first local varieties were developed by the end of the 19th century. These varieties were selected in small garden plots under different environmental conditions, which led to a wide range of variation for different traits such a maturity and seed types, including well-filled, round seeds with thin hull and oil content of about 20–30%, used for oil extraction, and large, long seeds with thick hull and oil content about 15–20%, used for direct human consumption (Gundaev 1971). Moreover, there was an important local selection for resistance to the European sunflower moth (*Homoeosoma nebulella*) and sunflower broomrape (*Orobanche cumana*), which at that time jeopardized the survival of the crop.

Scientific sunflower breeding started in 1910–1912 at Krasnodar by the academician V.S. Pustovoit, based on the local varieties developed at a local scale during the previous century (Panchenco 1966). The main efforts of breeders were initially devoted to the control of broomrape and sunflower moth, but the development of varieties with high oil content by V.S. Pustovoit constituted a crucial milestone in the development of sunflower as an oil crop not only in the USSR, but also throughout the world. The local varieties cultivated in Russia in 1913 contained only 30–33% of oil in dry seeds. This percentage increased up to 43% in 1935, 46% in 1953 and 51% in 1958, when the variety "Peredovik" was released (Panchenco 1966). This progress was obtained through the use of Pustovoit's "Method of Reserves", a method of individual selection with progeny testing and controlled pollination (Pustovoit 1967), together with the use of accurate laboratory techniques for oil content analysis. Moreover, this spectacular increase of oil content of the achenes did not caused any decline in the seed yield of the varieties released. The open pollinated Russian cultivar "Peredovik", with high oil content, introduced during the 1960s in the western countries (US, Canada, Western Europe), was the base of the first sustained commercial production of oilseed sunflower in these countries (Fick and Miller 1997).

6.5.2 Utilization of the Inbred-Hybrid Method

One of the most important breeding milestones in sunflower has been the development of hybrid cultivars that made possible the utilization of heterosis. Initial studies in the former USSR (Morozov 1947) and Canada (Unrau 1947;

Putt 1962) indicated that experimental hybrids outyielded check varieties from 160 to 189%. However, the practical production of hybrid seed was hindered by the absence of a suitable type of male sterility.

The first commercial sunflower hybrids were produced in Canada during the 1950s using a female parent with a high degree of self-incompatibility and allowing cross pollination with appropriate male parents. Although this method resulted in seed lots with hybridization rates as high as 90% under favourable environments, in general the percentage of hybrid seed was usually below 50% (Putt 1962). Thus, the seed produced by the self-incompatibility system often did not meet the legal requirements for designation as hybrid seed.

The existence of nuclear male sterility was first reported in the former USSR (Kuptok 1935) and later in France (Leclercq 1966) and Canada (Putt and Heiser 1966). In most cases the trait was controlled by a single recessive gene. Nuclear male sterility was used to produce hybrid seed in France and Romania during the early 1970s. The identification of a close linkage between genes for male sterility and anthocyanin pigmentation (Leclercq 1966) facilitated the identification and removal of the male fertile plants prior to flowering, thus allowing nearly 100% hybridization. This system allowed the development of the first commercial hybrids in Romania and in France, which yielded up to 24% more than the open pollinated varieties (Vrânceanu 1974). Even though the nuclear male sterility was an important step in the development of hybrid sunflowers, it required a considerable manual labour to remove male fertile plants.

The discovery of cytoplasmic male sterility, with its inherent advantages, provided a highly efficient method for commercial production of hybrid seed. The first stable source of cytoplasmic male sterility was discovered by Leclercq in 1968 from an interspecific cross involving *H. petiolaris* and *H. annuus* (Leclercq 1969). Subsequent identification of genes for fertility restoration in wild species (Kinman 1970) and in certain obsolete sunflower cultivars (Vrânceanu and Stoenescu 1971) allowed the efficient and economical production of hybrid seed. The development of the first sunflower hybrids based on cytoplasmic male sterility in the early 1970s intensified the interest of seed companies on the crop, which led to a considerable increase of sunflower production in many countries. When comparing sunflower yields in the countries that grew open-pollinated varieties before the introduction of hybrids, seed yields increases of about 20% were estimated (Fick and Miller 1997).

6.5.3 Development of New Types of Oil

The development of new oil types has been another important achievement for the sunflower oil industry. Sunflower breeders have been tremendously successful in developing mutants with new types of oil in the last 30 years, opening the possibility of tailoring specialty oils for specific market niches

(Fernández-Martínez et al. 2004). The most relevant was the high oleic acid mutant identified in Russia in the seventies (Soldatov 1976), which has made possible the development of commercial cultivars with mid oleic acid content (55–75%) and high oleic acid content (>85%), which are currently cultivated all over the world. In the U.S., the mid oleic acid varieties, locally known as Nusun®, are estimated to represent more than 85% of the total oilseed sunflower acreage according to the data of the National Sunflower Association.

6.6 Current Goals of Breeding

6.6.1 Seed Yield

One of the basic goals of sunflower breeding is to increase grain yield. The introduction of hybrid cultivars and the consequent exploitation of heterosis represented a breakthrough that produced an increase in yield potential around 25%. No significant improvement in grain yield potential has been observed at large scale before or after this turning point (López-Pereira et al. 1999). Even though several studies have identified yield components with a direct effect on seed yield, such as number of grains per head and grain weight (Connor and Hall 1997), major achievements in improving grain yield in sunflower have been more related to improving combining ability of hybrid parents or to selection for adaptation to limiting conditions in specific areas, e.g. shorter plant stature in areas with great lodging risk (Schneiter 1992), high degree of self-fertility in areas with limited pollinator populations (Miller et al. 1982), or pronounced head inclination in areas with high temperature and intense sunlight or with high risk of bird predation (Hanzel 1992). In many sunflower production areas, improved performance of recent hybrids was related to increased disease resistance, e.g. *Verticillium* wilt in Argentina (Sadras et al. 2000), Phomopsis stem canker in former Yugoslavia (Škorić 1985), or broomrape in several European countries (Alonso et al. 1996).

6.6.2 Morpho-Physiological Traits

6.6.2.1 Plant Height

The cultivated sunflower is a tall, erect, unbranched plant with a plant height below 75 cm in dwarf types to more than 5 m in giant varieties. Most common cultivated hybrids have a stem height of 160–180 cm, although the trait is very dependent on the environment. Several genetic sources of plant dwarfness have been identified and both semi-dwarf (100–160 cm) and dwarf (50–100 cm) hybrids have been produced and compared to standard-height hybrids. In general, no clear agronomic advantages were associated with reduced plant height in standard environments (Schneiter 1992; Velasco et al. 2003a). The

major advantage of semidwarf and dwarf cultivars is their resistance to lodging in environments with risk of heavy rains and strong winds during the growing season.

Plant height in standard sunflower types is regarded as a quantitative trait (Lay and Khan 1985). Several types of reduced plant height (<100 cm) have been developed. Reduced plant height in lines with a reduced number of leaves has been reported to be controlled by a single recessive gene (Miller and Fick 1997). Reduced plant height in genotypes with reduced internode length and a standard number of leaves has been found to be quantitatively inherited (Miller and Hammond 1991), controlled by a single dominant gene (Miller and Fick 1997), or by two recessive genes (Velasco et al. 2003b).

6.6.2.2 Head Size, Shape and Inclination

Head diameter may vary from 6 to 75 cm. The head shape presents a large gradation from concave to convex, whereas the head angle may vary from 0 (horizontal facing upwards) to 180° (horizontal facing downwards). The three traits are quantitatively inherited and subject to environmental effects (Miller and Fick 1997).

Classical Russian sunflower breeders put special emphasis on the importance of optimal head size and head shape to maximize sunflower yield. Morozov (1947) considered medium-size, thin and flat head as the ideotype for sunflower. Similarly, Pustovoit (1966) considered that a medium-size head (20–25 cm), in combination with adequate plant density, was one of the main determinants of grain yield. Larger heads would increase the percentage of hull as well as the number of empty grains in the center of the head.

Specific head shape and head inclination types have been identified as advantageous under certain environments. The incidence and severity of certain diseases such as white rot caused by *Sclerotinia sclerotiorum* and gray rot caused by *Botrytis cinerea* is directly related to the angle of the head. The lowest disease incidence is observed when the head is at an angle of 45° and remains above the foliage (Škorić 1992). A pronounced head inclination around 180°, in which the head is parallel to the soil surface, is desired to prevent sun scald in areas with high temperature and intense sunlight during seed maturation (Sailsbery and Knowles 1983), as well as to reduce bird predation in combination with a concave-shaped head (Hanzel 1992).

6.6.2.3 Flowering and Maturity Dates

Cultivation of sunflower varieties with flowering and maturity dates adapted to the particular agroecological conditions of a region is essential to ensure a high productivity of the crop. Most sunflower cultivars exhibit quantitative long-day or day-neutral responses to photoperiod although there are differences in photoperiodic sensitivity of sunflower genotypes (Connor and Hall 1997). A great variation in days from planting to maturity can be found in sunflower,

from around 75 to 150 days (Fick 1978). The genetic control of flowering date is rather complex and contradictory results have been obtained depending on the germplasm used. Vrânceanu (1974) suggested that the number of days to flowering was controlled by many genes, some of them affecting photoperiodism. However, most studies have reported a high heritability of flowering date (Miller and Fick 1997).

6.6.2.4 Pollen Self-Compatibility and Flower Characteristics

Sunflower inflorescence is a capitulum, usually referred to as head, formed by an outer whorl of sterile, ligulate flowers, known as ray flowers, and a varying number of inner whorls arranged in spiral from the head centre containing fertile flowers, known as disk flowers or florets. Flower nectaries are located at the base of the style in disk flowers and they play an important role in attracting pollinators (Tepedino and Parker 1982). Wild sunflowers have a system of sporophytic self-incompatibility that promotes insect-mediated cross-pollination. The number of loci involved in self-incompatibility has been disputed. One multiallelic locus was identified by Fernández-Martínez and Knowles (1978). Self-incompatibility systems were maintained in open-pollinated populations, but hybrid breeding has been accompanied by intense selection for high levels of self-compatibility. Nowadays, most commercial hybrids are virtually self-fertile (Fick and Miller 1997). Nevertheless, pollinator (mainly bees) attractiveness is still important in fields of hybrid seed production, where the production success depends on efficient pollen transfer from male-fertile to male-sterile parents. Selection for bee attractiveness is complex, since nearly every flower trait influences attractiveness to bees. Some of the most important traits are a short corolla length, short styles, unpigmented stigmas, and total sugar content and profile in the nectar (Montilla et al. 1988).

6.6.2.5 Male Sterility

The availability of male sterility is essential for the commercial production of hybrid seed. Both nuclear male sterility (NMS) and cytoplasmic male sterility (CMS) have been identified in sunflower. Nuclear male sterility is generally controlled by a recessive gene. Eleven different genes named *Ms1* through *Ms11* have been identified in NMS lines (Jan 1992c). A total of 72 unique CMS sources have been identified (Table 3; Serieys 2002), fertility restoration genes are available for 34 of them. Most of the CMS sources require at least two genes for fertility restoration, although some of them show single-gene restoration (Jan and Vick 2007).

6.6.2.6 Oil, Protein and Fibre Contents

The fruit of sunflower is an achene, commonly known as sunflower seed or grain. The kernel to hull ratio is one of the main parameters defining the

profitability of the crop. The percentage of hull in the achenes is very variable in sunflower germplasm, from around 10 to 60% (Miller and Fick 1997). In current oilseed cultivars, the kernel represents more than 75% of the total achene weight.

More than 80% of the economic value of oilseed sunflower cultivars is obtained from the extracted oil, whereas the rest is obtained from the protein-rich meal that remains after oil extraction. One of the major breeding achievements that facilitated the expansion of sunflower as one of the most important world oilseed crops was a drastic increase of oil content of sunflower achenes, from around 30% to more than 50% (Panchenco 1966). According to Alexander (1963), two thirds of the increase in oil content was produced by a reduction of the hull percentage in the achenes, whereas one third was produced by the increase of oil content in the kernel. The increment in the kernel to hull ratio was also associated with a concomitant increase of protein content, around 17% in current cultivars, and a reduction of fibre content. Additionally, the industry uses to hull the achenes before crushing to reduce the fibre content and improve the digestibility of the meal. Accordingly, the ease of hulling is also a selection target to take into account (Denis et al. 1994).

The hull content is a quantitative trait with high heritability mainly governed by genes with additive effects (Kovacik and Skaloud 1990). The ease of hulling is also a trait with high heritability for which variation in sunflower germplasm has been identified (Denis et al. 1994). Both oil and protein contents in sunflower achenes are traits quantitatively controlled by the genotype of the plant (sporophytic control) (Pawlowski 1964), with predominance of additive gene effects and medium to high heritabilities (Fick 1975; Alza and Fernández-Martínez 1997).

6.6.2.7 Oil Quality

Sunflower oil mainly contains molecules of triacylglycerol, composed of three fatty acids attached to a glycerol skeleton, which represent more than 95% of the total oil weight. The rest are lipid and lipid-soluble compounds, some of them of great value because of the functional and nutritional properties that confer to the oil. Breeding for oil quality in sunflower has been mainly focused in the modification of the fatty acid profile of triacylglycerols, although minor compounds with important nutritional and antioxidant value such as tocopherols and phytosterols have also attracted the attention of plant breeders in recent years.

The seed oil of conventional sunflower varieties is characterised by a high proportion of the unsaturated oleic acid (18:1) and linoleic acid (18:2), which together account for about 90% of the total fatty acids (Table 6.5). The remaining 10% correspond to the saturated palmitic acid (16:0) and stearic acid (18:0). The relative proportion of oleic acid and linoleic acid is strongly influenced by the environmental conditions, especially temperature, during seed development (Harris et al. 1978).

Table 6.5 Outstanding sunflower germplasm producing seed oil types with modified fatty acid and tocopherol profiles

	Fatty acid composition[a] (%)				
Oil type	16:0[b]	18:0	18:1	18:2	Reference
Standard[c]	7	3	30	60	
Low sat[d]	4	3	40	52	Vick et al. (2002)
High 16:0	25	4	11	55	Osorio et al. (1995)
High 18:0	5	26	14	55	Osorio et al. (1995)
Mid 18:1	4	5	55	34	Vick and Miller (1996)
High 18:1	5	3	90	2	Soldatov (1976)[e]
	Tocopherol composition (%)				
Oil type	α-T	β-T	γ-T	δ-T	Reference
Standard	95	4	1	0	
Medium β-T	50	50	0	0	Demurin (1993)
High β-T	25	75	0	0	Velasco et al. (2004b)
High γ-T	5	0	95	0	Demurin (1993)
High δ-T	5	0	30	65	Velasco et al. (2004b)

[a]Percentages for the four major fatty acids are given only.
[b]16:0 = palmitic acid, 18:0 = stearic acid, 18:1 = oleic acid, 18:2 = linoleic acid.
[c]Averaged from cold and warm environments.
[d]Low content of total saturated fatty acids.
[e]Data of hybrids developed by Fernández-Martínez et al. (1993) from a mutant originally developed by Soldatov (1976).

The tocopherols are a group of four lipid-soluble substances with molecular structure comprised of a chromanol ring and a saturated phytyl side chain. The four tocopherols, named alpha-, beta-, gamma-, and delta-tocopherol differ in the number of methyl substituents and the pattern of substitution in the chromanol ring (Packer and Obermüller-Jevic 2002). They exhibit differential in vivo and in vitro antioxidant activities. While alpha-tocopherol exerts a maximum in vivo activity, also known as vitamin E activity, but poor in vitro protection of the extracted oil, gamma-, delta- and to a lesser extent beta-tocopherol are powerful in vitro antioxidants with low vitamin E value (Pongracz et al. 1995). However, the tocopherol profile is only a part of the picture when designing a breeding programme aimed at improving the tocopherol fraction in sunflower seeds. The other part is the total tocopherol content. The seed oil of conventional sunflower varieties has an average total tocopherol content of 708 mg kg^{-1}, mainly in the alpha-tocopherol form, which accounts for more than 90% of the total tocopherols (Padley et al. 1994).

Phytosterols or plant sterols are essential components of the cell membranes. Their role as functional food components and nutraceuticals due to their ability to lower total and LDL serum cholesterol in humans is increasing in recent years. Chemically, they are steroid alcohols (triterpenes) synthesized from squalene in the isoprenoid pathway (Piironen et al. 2000). Vegetable oils are the richest natural sources of plant sterols. The seed oil of conventional sunflower varieties has an average sterol content of 3,387 mg kg^{-1}, mainly as

sitosterol (59.9% of the total sterols), Δ^7 stigmastenol (10.4%), campesterol (9.5%), and stigmasterol (9.5%) (Padley et al. 1994).

The optimal quality of sunflower oil depends on the intended use of the oil, either for food or non-food applications. The former include salad and cooking oils as well as oils for the food industry (margarines, shortenings, etc.). The latter comprises countless industrial sectors such as biofuels, lubricants, surfactants, surface coatings, cosmetics, plastics, etc. In general, those oil characteristics that are undesirable for a particular application are required for others. A clear example is the saturated fatty acid content in sunflower oil. Saturated fatty acids are regarded as detrimental on human health because of their contribution to raise cholesterol levels as compared with isocaloric amounts of carbohydrates (Mensink et al. 1994). Accordingly, the breeding objective to produce a healthy oil of direct consumption (e.g. salad oil) is to reduce total saturated fatty acid content. But on the other hand, a sunflower oil rich in saturated fatty acids is desirable for the industry of margarines and related products, because its semi-solid consistency reduces the need for transformations such as hydrogenation or transesterification that generate *trans* and positional isomers related to heart disease (Willett and Ascherio 1994). Accordingly, the development of sunflower germplasm producing oil rich in saturated fatty acids is also a breeding objective if the oil is intended to the production of semisolid fats.

Oleic acid (18:1, n-9) is nowadays considered as the preferred fatty acid for edible purposes, as it combines a hypocholesterolemic effect (Mensink and Katan 1989) with a much greater oxidative stability than polyunsaturated fatty acids. For this reason, selection for mid and high oleic acid contents has been a priority in sunflower (Table 6.5).

The tocopherols are another good example of contrasting breeding goals depending on the intended use of the oil. Selection for high alpha-tocopherol content would enhance the vitamin E value of the oil in human nutrition, but selection for low alpha tocopherol content would result in the accumulation of tocopherols with greater in vitro antioxidant effect and consequently in optimal oil properties for applications requiring high oxidative stability, e.g. deep frying or biolubricants.

A summary of outstanding sunflower germplasm producing seed oil types with modified fatty acid and tocopherol profiles is presented in Table 6.5. In general, genetic modifications altering either the fatty acid or the tocopherol profile have been found to be qualitative rather than quantitative, i.e. they are controlled by a reduced number of genes and they are less affected by environmental factors than quantitative traits such as oil content (Fernández-Martínez et al. 2004). Conversely, total tocopherol content in sunflower seeds is regarded as an oligogenic or polygenic trait, although no genetic studies on this trait have been conducted yet. Preliminary information suggests an important contribution of the genotype to the expression of the character (Alpaslan and Gündüz 2000; Velasco et al. 2002).

The high oleic acid content in sunflower was initially identified as a monogenic trait produced by dominant alleles *Ol* (Urie 1984), although more detailed studies identified several modifying genes affecting the *Ol* gene and producing reversal of the expected dominance (Miller et al. 1987; Fernández-Martínez et al. 1989b; Pérez-Vich et al. 2002b), which has complicated the practical management of the trait in breeding programmes. High palmitic acid content has been found to be controlled by alleles at three independent loci *P1*, *P2*, and *P3* in such a way that the high palmitic acid phenotype is expressed in genotypes that are homozygous recessive at the *P1* locus and either at *P2* or *P3* (Pérez-Vich et al. 1999a). Genetic characterization of high stearic acid content of the sunflower mutant CAS-3 concluded that the trait is controlled by partially-recessive alleles at two loci *Es1* and *Es2* (Pérez-Vich et al. 1999b). A third gene involved in high stearic acid content, *Es3* was identified in the mutant CAS-14. However, genetic recombination of *es3* alleles with *es1* and *es2* alleles from CAS-3 did not result in an increment of the maximum stearic acid content in the seeds compared to the maximum levels produced by the *es3* alleles alone (Pérez-Vich et al. 2006a). Reduced saturated fatty acid content was identified as a partially dominant trait controlled by more than one gene (Vick et al. 2002).

The genetic studies conducted so far have also revealed that the modified tocopherol profiles in sunflower seeds are also controlled by recessive alleles at a reduced number of loci. Demurin et al. (1996) found that recessive alleles at the *Tph1* locus produced mid beta-tocopherol levels, recessive alleles at the *Tph2* locus produced high gamma-tocopherol content, whereas their genetic recombination resulted in increased delta-tocopherol content. Hass et al. (2006) identified a third gene, named *Tph3*, which in combination with *Tph1* and *Tph2* produced a high delta-tocopherol content.

An important feature of the genetic control of the seed oil quality traits is, that in most cases they are determined by the genotype of the developing embryo with small or no maternal influence. The latter is crucial in breeding programs, as a low weight of maternal inheritance allows selection to be carried out at a single-seed level. It is also noteworthy that most of the genetic modifications are recessive, which determines that the modified alleles have to be introgressed in both parents in hybrid seed production (Fernández-Martínez et al. 2004).

6.6.2.8 Seed Meal Quality

Sunflower seed meal is extensively used as a protein supplement in animal feed because of its high protein content of around 40% if the achenes are hulled before oil extraction or around 28% if no hulling occurs (Dorrell and Vick 1997). The principal factors defining sunflower meal quality are a low fibre content, high protein content of good quality, and absence or low presence of minor components with detrimental properties from the nutritional or technological points of view.

Fibre is a heterogeneous chemical entity that includes those carbohydrates that are not truly digested by the animal and therefore do not contribute energy when consumed. High fibre content in the meal reduces its nutritive value and digestibility. It is predominantly associated with the seed hull. Because of the high percentage of hull in sunflower achenes compared to other oilseeds, hulling is a common practice necessary to render a meal with adequate quality for animal feed (Bell 1989).

Sunflower seed meal has a balanced amino acid composition except for lysine, for which it is deficient (Norton 1989). Accordingly, selection to increase lysine content in sunflower seeds is one of the major objectives to improve seed meal nutritive quality. Several studies have reported the existence of variability for lysine content in germplasm of cultivated (Ivanov 1975) and wild sunflower accessions (Christov et al. 1993). Even though selection efforts have been scarce, Ivanov (1975) reported good response to selection for this trait.

Sunflower seeds contain a series of minor compounds that remain in the meal after oil extraction and confer negative properties from the nutritional and/or technological point of view. One of these compounds is phytic acid (myoinositol 1, 2, 3, 4, 5, 6-hexakis-dihydrogen phosphate), which is present in cereals and oilseeds. Sunflower kernels contain around 2.2% phytic acid, which determines a content around 4.5% in the seed meal (Miller et al. 1986). Phytic acid is a strong chelating agent that can bind metal ions, reducing the availability of calcium, iron, magnesium, zinc and other trace elements (Oberleas et al. 1966). Additionally, phytates form complexes with amino acids, reducing the value of sunflower meal for nonruminant livestock (Erdman 1979).

Sunflower seeds contain significant amounts of phenolic compounds, mainly chlorogenic acid and caffeic acid, which reduce the nutritive value of sunflower meal for animal feed by interacting with amino acids, denaturing proteins, and inhibiting enzymes (Sozulski 1979). Additionally, chlorogenic acid produces a yellow-green coloration following oxidation in sunflower meal, which represents a serious limitation for the use of sunflower meal for human consumption in form of sunflower flour, protein concentrates and protein isolates (Dorrell and Vick 1997). Since phenolic compounds are predominantly present in the seed kernels, hulling scarcely reduces their presence in the meal (Pedrosa et al. 2000). From the evaluation of several lines with contrasting levels of chlorogenic acid in several environments and evaluation of progenies derived from crosses involving them, Dorrell (1974) concluded a significant effect of the genotype on the expression of the trait.

6.6.2.9 Disease Resistance

Most of the pathogens affecting sunflower have only economic impact at a local scale or under specific environmental conditions, but some of them have great relevance and can produce important yield losses if no adequate control measures are adopted. Breeding for resistance is considered the most effective and sustainable means of control.

Both qualitative or vertical and quantitative or horizontal genetic resistance mechanisms have been identified in wild sunflower species and successfully transferred to cultivated strains. Single dominant genes, generally associated with race-specific resistance to important diseases such as sunflower rust (Jan et al. 2004; Jan and Gulya 2006a), downy mildew (Miller and Gulya 1988; Jan and Gulya 2006a) and *Verticillium* wilt (Radi and Gulya 2007) have been identified in wild *Helianthus* germplasm and successfully transferred to cultivated sunflower. A serious problem associated with the use of major gene resistance is the regular appearance of new races of the pathogen that overcome the existing resistance genes, determining the need for identifying additional resistance genes to be introgressed into high yielding cultivars. Since resistance genes are usually identified in germplasm of wild species, the recovery of good agronomic characteristics after the introgression of the new resistance gene is a difficult task. Therefore genetic resistance based on more durable strategies such a combination of both vertical and horizontal resistance mechanisms has been proposed (Vear 2004). Pyramiding of resistance genes has been also proposed as a strategy to develop durable resistance. Tourvieille et al. (2004) compared different methods to enhance durable resistance to downy mildew, reporting that gene pyramids were less effective in reducing the appearance of new races compared to other control methods such as the use of combinations of resistance *Pl* genes, by alternation or in "multi-hybrids".

The situation has been different for diseases produced by *Sclerotinia sclerotiorum* (wilt, stalk rot, head rot), which cause the greatest losses to sunflower on a global basis. The causal agent is a polyphagous fungus that attacks many different species, including sunflower, where infection affects the root, stem, head, and seeds (Gulya et al. 1997). In this case, resistance is expected to be complex and controlled polygenically (Jan and Seiler 2007). Genetic resistance to Phomopsis stem canker (Vrânceanu et al. 1992) and *Alternaria* leaf blight (Morris et al. 1983) have been postulated to be oligogenic.

Virus diseases are not currently a major concern in global sunflower production. More than 30 different viruses have been identified on sunflower, but only a strain of tobacco streak virus has been reported as a serious disease of sunflower in all major sunflower-growing regions of India (Ravi et al. 2001). Although resistance to this virus has not been reported yet, resistance to other viruses such as the sunflower mosaic potyvirus (Jan and Gulya 2006b) and the sunflower chlorotic mottle virus (Lenardon et al. 2005) have been identified. The latter was found to be controlled by a single dominant gene.

6.6.2.10 Broomrape Resistance

Sunflower broomrape (*Orobanche cumana* Wallr.) is a weedy parasitic angiosperm that represents nowadays a serious constrain for sunflower production in Southern Europe and in the Black Sea region. Although the use

of herbicides is being considered as a promising control measure, genetic resistance offers the most effective and feasible control against *O. cumana*. However, the introduction of new resistance has been frequently followed by the appearance of new pathogenic races overcoming the resistance and there is a continuous need for new sources of resistance. Vrânceanu et al. (1980) identified five pathogenic races, named A through E, with a set of sunflower differentials carrying the dominant resistance genes *Or1* through *Or5*, respectively that provide an accumulative resistance to broomrape races. New virulent races overcoming resistance gene *Or5* have been identified in Spain (Alonso et al. 1996; Molinero-Ruiz and Melero-Vara 2005), Romania (Păcureanu et al. 2004), Bulgaria (Shindrova 2006), and Turkey (Kaya et al. 2004).

Resistance to races A through E has been found in most studies to be under the control of dominant alleles at single loci (Vrânceanu et al. 1980; Sukno et al. 1999), although two dominant genes (Domínguez 1996) and one recessive gene (Ramaiah 1987) have also been reported. Genetic resistance to Spanish race F in germplasm derived from wild *Helianthus* spp. has been found to be controlled by a single dominant gene *Or6* (Pérez-Vich et al. 2002a), which exhibited incomplete dominance in some environments due to the effect of a second gene *Or7* (Velasco et al. 2006), whereas resistance in germplasm of cultivated sunflower was controlled by two recessive genes (Akhtouch et al. 2002). A single dominant gene is involved in the resistance to race F in germplasm developed in Romania (Păcureanu et al. 2004).

6.6.2.11 Insect Resistance

Insects do not represent a serious threat to sunflower production except in North America, where insect pests of sunflower coevolved with their native hosts (Jan and Seiler 2007). Nowadays, the principal strategy in search for resistance to sunflower infesting insects is the evaluation of wild *Helianthus* spp. and cultivated sunflower germplasm.

6.6.2.12 Resistance to Bird Depredation

Bird damage causes serious losses in sunflower production, especially during the first three weeks of seed maturation (Cummings et al. 1989). Breeding for some morphological traits contributes to confer resistance to bird predation. These traits include long involucral bracts, horizontally oriented heads facing downwards, concave heads, and long head-to-stem distances (Gross and Hanzel 1991).

6.6.2.13 Resistance to Abiotic Stresses

Sunflower is grown in areas prone to several types of environmental stresses, from drought to waterlogging, from heat to frost injury, or from poor soils to

excess salinity. The development of cultivars adapted to such conditions is critical. But adaptation not only implies the ability to survive under stress, but the plants must be able to maintain adequate yields.

Several strategies have been followed to select for drought resistance in sunflower. Avoidance of high evaporative demand periods has been advocated as a strategy to increase productivity in areas prone to drought. In southern Spain, the shift in sunflower planting dates from spring to winter resulted in a substantial yield increase (Gimeno et al. 1989). Selection for early vigour has been proposed as a means for improvement of sunflower yield under water-limited environments (Agüera et al. 1998). There is also evidence that improved osmotic adjustment capacity is a trait that contributes to yield maintenance under drought conditions in sunflower (Chimenti et al. 2002). Some wild *Helianthus* spp., especially *H. argophyllus*, have been suggested as a source of useful traits to improve water use efficiency such as higher stomatal densities and leaf pubescence, which reduce transpiration rates (Blanchet and Gelfi 1980).

6.6.2.14 Herbicide Resistance

The acreage of transgenic varieties of several major crops carrying genes for resistance to herbicides have been steadily increasing in recent years in many countries. In sunflower, resistance to imidazolinone and sulfonylurea herbicides was identified in weedy populations of *H. annuus* (Al-Khatib et al. 1998). The trait has been transferred to cultivated sunflower germplasm (Miller et al. 2006a). Herbicide resistance in sunflower is mainly controlled by one gene exhibiting partial dominance and also affected by a second gene in some genetic backgrounds (Bruniard and Miller 2001). Resistance to herbicides in sunflower is also being used to control broomrape (Domínguez et al. 2004).

6.6.2.15 Nonoilseed Sunflower

Nonoilseed sunflower seeds are used as snack food, in the confectionery industry, and for feeding birds and small pets. The seeds of nonoilseed cultivars have lower seed oil content than oilseed cultivars. Breeding goals in relation to nonoilseed sunflower largely depend on the requirements of specific markets. For example large achenes are preferred for uses as snack food, but small achenes are preferred for birdseed. In some types of soils, sunflower accumulates excessive concentrations of cadmium in the seed kernels, which has became a problem for confectionery sunflower. This has encouraged breeding for reduced cadmium uptake into kernels (Miller et al. 2006b).

6.7 Breeding Methods and Techniques

6.7.1 Breeding Methods

There are comprehensive reviews of breeding methods and techniques in sunflower (Škorić 1988; Miller 1987; Fick and Miller 1997). Four important features have to be considered in breeding sunflower: (a) obtaining or developing sources of genetic variability, (b) improving source populations, (c) improving open pollinated cultivars, and (d) developing lines or parents for producing hybrid cultivars.

6.7.1.1 Obtaining or Generating Sources of Genetic Variability

The Use of Existing Genetic Variation

Many sources of genetic variability from cultivated germplasm and wild species are available in germplasm collections to be used in breeding programs. For example, open-pollinated varieties with high oil content developed in the former USSR were the base for the initial commercial production of oilseed sunflower in western countries in the 1960s (Putt 1997). Evaluation of cultivated germplasm has been extensively used to identify useful variation for many different traits such as disease resistance (Škorić 1992) or oil quality (Demurin 2003). However, wild species represent the most diverse source of genetic variability in sunflower breeding. Genes for disease and insect resistance, oil and protein content and quality, cytoplasmic male sterility, or agronomic and physiological traits have been identified in wild *Helianthus* species and transferred into cultivated lines. The main problem using wild species as sources of variability is that many of them do not cross readily with the cultivated sunflower.

Transferring genes from wild annual species into cultivated lines can be accomplished rather easily with conventional crossing and backcrossing. For example, genes conferring resistance to several diseases such as rust and downy mildew have been transferred from *H. debilis* or wild *H. annuus* to cultivated sunflower (Quresh et al. 1993; Tan et al. 1992). Conversely, crossing perennial *Helianthus* species with the cultivated sunflower is generally difficult due to early hybrid embryo abortion as well as high levels of sterility in the F_1 or BC F_1 generations (Jan 1997). These problems can be avoided with the utilization of the embryo culture technique (Chandler and Beard 1983) and subsequent chromosome doubling of the F_1 (Jan 1988). Using these techniques, amphiploids of the wild species *H. gracilentus, H. hirsutus*, *H. strumosus*, *H. maximiliani*, *H. nuttallii* and *H. grosseserratus* have been produced and used as a bridge to transfer resistance to broomrape race F (Jan and Fernández-Martínez 2002). Positive results of transfer of rust resistance genes from *H. hirsutus* and fertility restoration genes for a CMS cytoplasm derived from *H. giganteus* have also been achieved using interspecific amphiploids (Jan and Zhang 1995; Jan 2004).

Mutagenesis

Mutagenesis has been used successfully to generate genetic variability for useful traits for the improvement of sunflower. This method is especially important if variation for a given trait is not found in germplasm collections. Useful mutants with short plant height, high oil and protein content and low hull content have been obtained in sunflower using both irradiation and chemical treatments (Voskoboinik and Soldatov 1974; Leclercq 1985; Fernández-Martínez and Domínguez 1988). Other useful variability has been obtained for rust resistance (Lofgren and Rama Raje Urs 1982) and herbicide resistance (Bervillé et al. 1992). Jan and Rutger (1988) used streptomycin and mitomycin C to produce 22 cytoplasmic and 7 nuclear male sterile mutants.

Mutagenesis results in sunflower have been particularly successful in modifying seed oil quality traits. One of the most valuable mutants produced is the high oleic acid mutant (>80%), produced at the All-Union Research Institute of Oil Crops of the former USSR, after treatment with dimethyl sulfate (Soldatov 1976). High levels of either palmitic or stearic acid (>25%) were achieved using chemical and physical mutagens (Ivanov et al. 1988; Osorio et al. 1995). Mutants with increased levels of gamma tocopherol (>95%) have been isolated following chemical mutagenesis (Velasco et al. 2004b). The mutagenic treatment is usually applied to the seeds, which after treatment are named M_1 seeds. Mutants can be detected in the M_2 generation. For fatty acid and tocopherol profile, which are mainly under embryogenic control, mutants are detected analyzing M_2 half-seeds.

6.7.1.2 Methods for Improving Source Populations

The success of isolating inbred lines with good combining ability or other desired characters by the standard procedure of inbreeding and selection depends on the frequency of superior S_0 plants in the source populations. Recurrent selection is an effective method for increasing this frequency. Sunflower breeders have used several intrapopulation and interpopulation recurrent selection methods to develop improved source populations. Most relevant has been the use of the Pustovoit's Method of Reserves described below, which is a modified method of recurrent selection extensively used for developing open-pollinated cultivars.

Two types of recurrent selection have been considered in sunflower, phenotypic recurrent selection, in which the phenotype of the S_0 plant is the base of selection, and genotypic recurrent selection, in which some type of progeny test forms the base of selection. Phenotypic recurrent selection has been used by sunflower breeders to improve populations for several traits, including yield, oil percentage and disease resistance (Fick 1978; Miller 1987). For hybrid breeding, female (B) and restorer (R) populations are generally managed separately to simplify the development of inbred lines. The initial populations are often a combination of high-performing inbred lines or a composite of lines from

one or several open-pollinated cultivars (Miller 1987). The parents selected to form the initial population are crossed to produce a random-mated population. Random-mating is accomplished by emasculating, either by hand or using gibberellic acid, plants of each line and pollinating them with pollen gathered randomly and equally from other lines. The source material or initial C_0 material is grown and individual plants are selected and selfed. In the following generation, the progenies of selected plants are sown in a separate row. The progenies (rows) are crossed in all possible combinations and the resulting hybrid seeds are bulked to form the C_1 population. This completes the first cycle of selection. The C_1 plants from the first cycle are used for the second cycle of selection. Phenotypic recurrent selection has been successfully used in sunflower for different traits, for example, self-incompatibility (Kinman 1970), resistance to *Sclerotinia* stalk and head rot (Vear and Tourvieille 1984), seed yield (Gundaev 1971), and oil content (Luduena et al. 1992).

Genotypic recurrent selection methods utilizing S_1 progeny or testcross evaluation have been effectively used in sunflower hybrid breeding to improve yield and combining ability (Fick 1978) and drought resistance (Fernández-Martínez et al. 1990). The S_1 progeny recurrent selection method can also be used to develop improved open-pollinated cultivars (Miller 1987). With this method, several hundreds of individual S_0 plants of the initial source C_0 population are selected and self-pollinated. In the second generation, part of the seed is grown and evaluated for the traits of interest in replicated trials. Selected S_1 progenies are recombined to form the C_1 population, which is accomplished by random mating plants obtained from reserve S_1 seed. The recombination generation may be obtained in a greenhouse or winter nursery, which permits the completion the three generations of a cycle of selection in 2 years. By using a winter greenhouse nursery, spring evaluation, and recombination as a second crop in the fall, Fernández-Martínez et al. (1990) were able to complete one cycle per year in southern Spain.

In the test cross or half-sib progeny recurrent selection, test crosses instead of S_1 progenies are evaluated. Selected plants in the C_0 initial source population are selfed and simultaneously crossed with a tester the first year. These two operations can be done in single-headed maintainer plants by self-pollinating half of the head and emasculating and crossing the other half. In the case of multi-headed restorer plants, the main head is emasculated and crossed, whereas some secondary heads are selfed (Fick and Miller 1997). The type of tester used depends on the objectives of selection. For example, in selection for general combining ability (GCA) a broad base heterogeneous unrelated population is used as tester. There are not many reports on half-sib recurrent selection in sunflower breeding. Fick (1978) reported an increase of 3.5% in seed yield after one cycle of recurrent selection in an R-line population using testcross evaluation.

Interpopulation recurrent selection methods have also been used in sunflower. The reciprocal full-sib selection method, based in the production of hybrid (full-sib) progenies and selfed seed in the same plant, was adapted for

sunflower using a multi-headed restorer (R) and a single-headed maintainer (B) population (Miller and Fick 1978). The method is initiated by forming the R and B populations by random mating diverse collections of R and B lines to create the C_0 populations. Full-sib progenies are obtained by transferring pollen from a selected plant in the B line population to the emasculated main head of a plant in the R line population. Self-pollinated seed is obtained by self-pollinating secondary heads of the R line plant. The B line plant selected is also self-pollinated. Full-sib hybrids are tested the following year in replicated yield trials. On the basis of these evaluations, the self-pollinated S_1 seed from the best full-sib hybrids are bulked within each population and randomly mated in a greenhouse or winter nursery to form the C_1 populations to be used to start a new cycle of selection. A cycle of selection is usually accomplished in 2 years (Miller 1987), or even in 1 year (Fernández-Martínez et al. 1989a). Miller and Hammond (1985) reported a 6.3% increase in yield per cycle using this scheme of selection. Fernández-Martínez et al. (1989a) used the reciprocal full-sib selection method to maximize seed yield under environments prone to drought, with evaluations conducted under both irrigated and rainfed conditions.

6.7.1.3 Methods for Improving Open Pollinated Cultivars

Mass Selection

Mass selection implies the selection of individual plants from a population on the basis of their phenotype for the improvement of a cultivar or population for some specific traits. Seeds of the selected plants are mixed and planted the next generation in order to obtain new cultivars or to maintain the varietal purity of existing cultivars. Two methods of mass selection have been used in sunflower: phenotypic mass selection and family selection. Phenotypic mass selection was commonly used for improvement of sunflower during the early stages of cultivar development in the former USSR. Several important cultivars were developed using this procedure (Gundaev 1971).

The family selection method is a form of mass selection that involves selection of individual S_0 open-pollinated plants and classification of those plants for characteristics of interest. Each plant is harvested and its seed is bulked with other plants of phenotypically similar families. The various bulks are planted in isolation for cross pollination. Gundaev (1971) listed many cultivars developed by this method. Mass selection has also been used in Argentina (Luciano and Davreux 1967) and Mexico (Robles 1982) to produce improved sunflower cultivars. Mass selection is a simple and economical method of selection but its effectiveness depends on the heritability of the traits. In general, this method has not been effective in sunflower breeding for traits with low heritability such as seed yield, but it has been effective for improving other traits including earliness, oil content or insect and disease resistance (Morozov 1947; Vrânceanu 1974).

Head to Row Selection (Pustovoit's Method of Reserves)

The "method of reserves" was the most widely and successfully used method to develop open pollinated sunflower cultivars. It was developed in the former USSR by V.S. Pustovoit during the 1920s (Pustovoit 1967). The method is a form of recurrent half-sib selection that includes progeny testing and subsequent cross pollination among superior progenies (Alexander 1963). The "method of reserves", as outlined by Pustovoit (1967) is initiated by forming a heterogeneous population of germplasm including elite cultivars, intercross hybrids, and world collection entries. About 10,000–15,000 plants are selected from this population with the main criterion that plants have 500–2,000 seeds per head. The seeds are analyzed for hull and oil percentage and around 1,200–1,500 heads are selected for progeny evaluation. Half-sib seeds of these S_0 selections are evaluated for agronomic, disease resistance, and seed quality traits in single row plots with two replications. A check consisting of the best existing cultivars is included in every third plot as a control. On the basis of the observations from the first year of testing, 15–20% of the S_0 selections are planted in a second-year observation nursery. Remnant half-sib seed produced in season 1 is utilized also to plant this nursery and to test for disease resistance.

Based on performance in the first and second year nurseries, the original seed of the best 20–50 S_0 plants selected in season 1 are planted in a cross pollination nursery for random mating using an isolation distance of 200–300 m between nurseries. Undesirable phenotypes including disease infected, extremely tall or branched plants are removed. Seeds from random-mated plants are analyzed for oil content and the seeds of selected plants are bulked for a new cycle of selection or for testing at a larger scale. After three years of testing, superior populations are released as new open-pollinated cultivars. The "method of reserves" has been especially useful in increasing oil content and oil yield per hectare, developing early maturity, and resistance to diseases, *Orobanche*, and sunflower moth (Gundaev 1971). In the former USSR, cultivars developed by this method were grown in 4.6 million hectares in 1973 (Pustovoit and Gubin 1974), and it was successfully used in other countries such as Romania (Vrânceanu 1974) and former Yugoslavia (Škorić 1988). However, in spite of its success, the genetic gain per year in comparison with other recurrent selection methods is limited due to the number of years used for evaluation at each cycle of selection.

6.7.1.4 Methods for Improving Hybrid Cultivars

Inbreeding for improving sunflower was used by early Russian and Canadian breeders to isolate uniform strains with variation for different traits such as plant height, oil content, disease, and pest resistance (Voskoboinik and Soldatov 1974; Unrau 1947). However, the main value of inbreeding was to develop true breeding lines with desirable characteristics to be used in the production of

synthetic cultivars or hybrids. The first results involving hybridization of inbred lines showed significant heterosis for seed yield and other traits (Unrau 1947; Putt 1962).

The most common method for developing new inbred lines in sunflower is pedigree selection, but bulk selection and single seed descent have also been used. Backcrossing is frequently used for modifying existing lines. The germplasm sources are derived from open-pollinated source materials, populations improved by recurrent selection, or from planned crosses between inbred lines (Miller 1987).

Pedigree selection involves self-pollination of phenotypically desirable plants in the F_2 or S_0 generation, depending on the starting material. Selection is practiced for agronomic type, disease resistance, or other desired traits. The F_3 progeny of each F_2 plant is grown the next season. Plants are selected and self-pollinated within the best $F_{2:3}$ lines. The process of inbreeding and selection is continued for five generations. In the F_3 generation, pollen from selected plants within the $F_{3:4}$ lines may be used to cross to tester lines to produce testcross seed. The hybrid testcrosses are evaluated and $F_{4:5}$ lines with superior combining ability can be selected. In the case of B lines, pollen can be collected to cross to a CMS source to begin the conversion of the F_4-derived B line to CMS (A line) by the backcross method. This process generally requires five backcrosses using the F_4-derived line as recurrent parent.

The development of lines by the backcross method is probably used more frequently than any other method, except pedigree selection. Backcrossing is used in the context of transferring a trait from one genotype (donor parent) to a desirable genotype (recurrent parent). The trait being transferred is usually simply inherited. Backcrossing is usually a correctional breeding method that is used to enhance the performance of an elite inbred line, but it is also used to introgress a specific gene in an elite inbred line. If the backcrossed-derived line must be essentially identical to the recurrent parent, about six backcrosses must be made. The complexity of the genetic transference of a given trait to a recurrent parent depends on the number of genes involved, their dominant or recessive nature, and the presence or absence of maternal effects. Examples for dominant traits controlled by one gene involve the transfer of the high oleic trait to the sunflower lines HA 89 and RHA 271 (Fernández-Martínez et al. 1993) and the resistance of downy mildew to HA 89 (Miller and Gulya 1988). These traits can be easily transferred because plants or seeds with the desired trait can be identified in the F_1 backcross generation. However, in many cases the target traits are recessive and do not show up in the F_1 generation, which increases the duration of the backcross generation. Such a limitation can be overcome by using marker-assisted selection, which allows the recessive genes to be identified in the F_1 backcross generation.

The final value of an inbred line is determined by testing general or specific combining ability in hybrid combinations. For general combining ability, the choice of the tester (homogeneous inbred lines vs. heterogeneous sources) depends on the specific program objectives. The most common testers are

inbred lines that are being used in commercial hybrids. Miller et al. (1980) and Domínguez and Fernández-Martínez (1987) found that inbred lines are effective in identifying lines with the best combining ability. Evaluation for combining ability often begins at the S_3 or S_4 generation, but a system of early generation testing beginning after the first generation of selfing was reported to be effective in identifying lines with good combining ability (Shein 1978).

Inbred lines are used primarily in the production of single crosses or three-way sunflower hybrids using the cytoplasmic male sterility and the fertility restoration system. Single-cross hybrids are produced by crossing a male-sterile female (A line) with a male-fertile restorer (R line). A three-way hybrid is made by crossing an A line with an unrelated maintainer line (B line) to produce a male-sterile single-cross hybrid. This hybrid is crossed with an R line to produce a male-fertile, three-way hybrid. Generally, single crosses are higher yielding than three-way hybrids and have greater uniformity (Fick and Zimmer 1976; Miller 1987). Three-way hybrids are produced primarily to reduce seed cost, since seed yield of single-cross female parents is often 1.5–2.0 times greater than that of inbred lines, although inbred lines that yield up to 80% of their hybrids have been developed (Fick 1978; Škorić 1988). Three-way hybrids are considered more stable over environments than single crosses due to their greater heterogeneity (Fick and Zimmer 1976; Schuster and Friedt 1988). The use of double-cross hybrids in sunflower to further improve adaptation and yield stability has also been suggested (Vulpe 1974).

Inbred lines have also been used to produce synthetic varieties in Canada (Putt 1966) and in the former USSR (Voskoboinik and Soldatov 1974). Putt (1966) concluded that high-yielding synthetics could be developed from as few as three to five inbred lines. Synthetic cultivars have been evaluated with favourable results in countries where hybrid production is not practical, for example Nigeria (Ado et al. 1991) and Egypt (Shabana 1990).

6.7.1.5 Methods for Producing Hybrid Seed

Efficient and economical production of hybrid seed at a commercial scale was greatly facilitated by the discoveries of genetic and cytoplasmic male sterility (Leclercq 1966, 1969). Nuclear male sterility was used to produce commercial hybrid seed in the early 1970s taking advantage of a close linkage between genes for male sterility and anthocyanin pigmentation of the plants, which allows the identification and removal of male-fertile plants (Leclercq 1966; Vrânceanu 1974). Detailed methods of hybrid seed production using this system have been described by Vrânceanu (1974) and Škorić (1988). However, the use of nuclear male sterility to produce hybrid seed has been replaced by the cytoplasmic male sterility and fertility restoration system and, although new sources of cytoplasmic male sterility have been found, the source discovered by Leclercq (1969) is used almost exclusively in current hybrid seed production programs. This system requires a cytoplasmic male-sterile line (A line), which is maintained by crossing to a genetically identical male-fertile line with a fertile

cytoplasm (B line), and a restorer line (R line) which combines well with the A line and restores the fertility in the hybrid cultivar. The seed harvested from the A line is grown commercially as a hybrid cultivar.

6.7.2 Breeding Techniques

6.7.2.1 Procedures for Selfing and Artificial Hybridization

In order to carry out selfing and controlled crosses, sunflower heads must be isolated from insect pollination. Paper and cloth bags are most commonly used. Paper bags cost less and may be satisfactory in some environments if excessive rain does not occur during the latter part of the growing season, but cloth bags are more desirable for the standpoint of seed set and durability (Vrânceanu 1974; Roath and Pomeroy 1988). Artificial hybrids are produced by emasculation of the female parent followed by pollination with pollen of the desired male parent. Hand emasculation is commonly used, but chemical emasculation has also been extensively used. Hand emasculation involves removing the anthers of the disk flowers with forceps early in the morning before the anthers have dehisced and before the stigmas have elongated up through the anther tubes. A large head will flower over a five to six-day period with three to six rings of florets flowering each day. Stigmas will be fully elongated and receptive 1–2 h after emasculation and will remain receptive 4–5 days. Chemical emasculation with gibberellic acid (GA_3) is utilized by sunflower breeders to produce hybrid seed. A 50 to 100-ppm concentration of GA_3 is applied to sunflower buds of approximately 1–1.5 cm diameter with generally good results (Miller and Fick 1978). However, cultivars and inbred lines may show different responses to GA_3 requiring higher or lower concentrations and earlier or later application times (Piquemal 1970). Various negative effects such as incomplete male sterility, reduced female sterility, and stem elongation have been associated with the use of GA_3, depending on concentration and timing of application (Miller 1987).

Pollination can be accomplished by several methods. If the male plant is located adjacent to the female parent and is shedding pollen, the receptacles can be rubbed together to transfer the pollen from the anthers of the male to the stigmas of the female plant. However, the most common technique is to collect pollen from the male parent in paper bags or on a cloth or leaf from heads isolated with bags prior to flowering. Pollen in paper bags can be stored up to 4 weeks in a refrigerator at a temperature of 4–6°C and relative humidity of less than 4%, and several years at –76°C (Frank et al. 1982).

6.7.2.2 Techniques Used for Interspecific Hybridization

Direct crosses of annual wild species, except *H. agrestis* with cultivated sunflower are possible using conventional methods. However, crossing perennial

species, including diploids, tetraploids and hexaploids, is much more difficult and also produces sterile F_1 plants, requiring special techniques (Jan 1997). The two-step embryo culture procedure developed by Chandler and Beard (1983) avoids embryo abortion and seed dormancy and facilitates interspecific hybridization. With this technique, embryos are excised and cultured 3–7 days after pollination. The embryos are cultured in Petri dishes on an appropriate solid growth medium (Chandler and Beard 1983). For embryo germination and seedling growth, the cultured enlarged embryos are transferred after 1–2 weeks to a liquid germination medium in test tubes. An optimized method for culturing difficult hybrid embryos derived from perennial *Helianthus* species was proposed by Jan (1988), and additional modifications have been suggested (Kräuter et al. 1991).

Another technique to facilitate interspecific transfer is the use of induced polyploidy by chromosome doubling using colchicine applied to apical meristems. Jan (1988) described a modified colchicine chromosome doubling technique with a significant positive effect on backcross seed set. The apical meristems are submerged in a 1.5 g/kg colchicine solution with 2.0 g/kg DMSO (dimethyl sulfoxide) for 5 h in the dark. Then, chromosome doubling of each head is verified by pollen grain size and stainability.

6.7.2.3 Field Plot Techniques for Cultivar Evaluation

Newly developed hybrids or open pollinated populations must be evaluated before they can be recommended for releasing. Detailed procedures of field testing sunflower genotypes have been described by Miller (1987), Fick and Miller (1997), and Vrânceanu (1974). The testing process largely depends on the genetic control of the traits. For qualitative traits controlled by major genes and scarcely influenced by the environment, limited evaluations in the greenhouse or field may be adequate. For quantitative traits, such as yield or oil percentage, lines or cultivars must be tested over years and locations. A common plot size includes two to five rows from 6 to 10 m length spaced 0.75 m. Usually test plots are overplanted and thinned to a uniform stand soon after emergence. Preliminary yield evaluations are usually planted in plots with one or two unbordered rows with two to three replications at one or more locations using a simple lattice or augmented design. Check cultivars are sown every five to ten rows in the augmented design and randomly in the lattice design. Advanced yield trials are planted in plots with two to five rows with three to four replications at several locations. In the case of three to five rows per plot, only the center rows are harvested. Significant interplot competition effects may occur between cultivars showing wide differences in height and maturity (Domínguez et al. 1980). In these cases, the effect of interplot competition can be reduced by grouping cultivars by height and maturity. The most common experimental designs for advanced yield trials are randomized complete block design and simple lattice, the latter used when the number of entries exceeds 36.

6.7.2.4 Techniques Used for Greenhouses and Off-Season Nurseries

Greenhouse and off-season nurseries are frequently used by sunflower breeders to grow several generations per year and speed up the breeding program. Postharvest dormancy is a frequent problem in greenhouse and off-season plantings. Fick (1978) reported a technique to overcome dormancy consisting in soaking the seeds in a concentration of 0.6 mL ethrel/L of water for 16 h. Treatments with grow retardants to reduce the height of plants grown in the greenhouse and produce plants with short, thick stems that are easy to manage have been used for winter planting in the greenhouse (Fick and Miller 1997). Another technique that may have value in programs requiring short generation time is the application of growth regulators as desiccants (Rana et al. 1990).

6.7.2.5 Laboratory Techniques for Seed Quality Evaluation

Breeding programmes to improve seed oil quality traits require the availability of adequate screening techniques to measure them. In sunflower, both the fatty acid and the tocopherol profile of the seeds are mainly under gametophytic control, i.e. they are governed by the genotype of the developing embryo. Accordingly, selection for these oil quality traits can be conducted at the single-seed level. Nondestructive methods to measure these traits in single seeds have been developed. Downey and Harvey (1963) developed the half-seed technique for nondestructive analysis of the fatty acid composition of single seeds of rapeseed (*Brassica napus* L.). The technique has been adapted to sunflower (Conte et al. 1989). It consists of the removal of a small seed portion in the region extremely distal to the embryo in a way that the germination capacity of the seed is not affected. The excised half seed is used for chemical analysis whereas the other half seed containing the embryo can be sown to produce a viable plant. The half-seed technique has been used for the nondestructive analysis of fatty acid composition (Conte et al. 1989), tocopherol composition (Demurin et al. 1996) and total tocopherol content (Velasco et al. 2004b).

The use of near infrared reflectance spectroscopy (NIRS) has facilitated the screening for seed quality traits in sunflower. NIRS is a fast, nondestructive and cost-effective technique that permits the simultaneous analysis of multiple constituents in a single measurement. This requires the previous development of individual calibration equations for every constituent to transform NIRS spectral data into chemical information. NIRS was first applied to determine oil content in sunflower meal samples (Robertson and Windham 1981). The technique has been also used to analyse protein content, fiber content (Kaffka et al. 1982), tocopherol content, phytosterol content (Gotor et al. 2007), and free fatty acid content (Moschner and Biskupek-Korell 2006) in sunflower meal. However, the application of NIRS to sunflower breeding requires the use of small samples of intact achenes. Sato et al. (1995) used NIRS for the analysis of oil content in hulled sunflower seeds. Pérez-Vich et al. (1998) combined the simultaneous analysis of seed oil content and fatty acid profile in both intact and hulled sunflower kernels.

Selection for seed quality at a single seed level has been facilitated by the use of near-infrared spectroscopy (NIRS) for analyzing the fatty acid profile of intact or hulled individual kernels. Sato et al. (1995) demonstrated the feasibility of NIRS for measuring the concentration of linoleic acid in the oil of single hulled kernels of sunflower. Velasco et al. (1999) reported that NIRS permitted the discrimination of intact achenes for oleic and linoleic acid concentration in the seed oil. Velasco et al. (2004c) used NIRS for large-scale screening for high stearic acid concentration in hulled sunflower seeds.

6.7.2.6 Techniques for Disease Resistance and Broomrape Evaluation

Breeding for disease resistance requires the creation of a disease environment to differentiate between resistant and susceptible plants. Breeders and plant pathologists have developed effective procedures to evaluate breeding materials for most of the major sunflower diseases. These techniques have been described in detail by Škorić (1988), Gulya et al. (1997) and Fick and Miller (1997).

Downy mildew evaluations are sometimes conducted in lands naturally infected with the pathogen, but more often under controlled conditions in greenhouse or growth chamber. The procedure described by Zimmer (1974) is highly effective and is extensively used in breeding programs. It consists of the inoculation of germinated hulled seeds, with radicals 10–20 mm long, with a suspension of 10,000 or more zoosporangia per ml of distilled water for 18 h at 20°C. Then, the inoculated seeds are planted in sterile soil and maintained on greenhouse benches during 14 days at 20–25°C and 16 h day length photoperiod. Susceptibility is indicated by the occurrence of sporulation of the fungus on the cotyledons or the under-surface of the first true leaves after 18 h in a saturated humidity chamber. A PCR test has been developed to detect the presence of the pathogen in sunflower seeds (Ioos et al. 2007).

Researchers working with sunflower rust adopted much of the methodology developed by cereal rust pathologists. Techniques for spore collection and storage and inoculation under greenhouse and field conditions were reviewed by Gulya and Marisevic (1996). Greenhouse evaluations for sunflower rust are conducted on seedlings after inoculating with 10:1 mixture of talc and urediospores. Field evaluations are conducted by spraying 3 to 4-week-old susceptible border row plants with water suspension of urediospores collected from commercial fields the preceding season. Plants are sprayed in the evening when temperatures are lower and then covered overnight with metal or plastic containers to provide a high level of humidity. Infection of susceptible plants provides natural inoculum and subsequent spread of rust throughout the nursery allowing the selection of resistant plants (Fick 1978).

The most common procedure of evaluation for resistance to *Sclerotinia* stalk and head rot is field testing in naturally infected fields (Gulya et al. 1997), but the results depend on soil and environmental factors. Several field and greenhouse methods have been developed. They involve adding sclerotia or mycelia-infested cereal grains to the soil at planting time or directly to the basal stem to

increase the level of infection (Pirvu et al. 1985; Rashid 1992) or immersing the roots of 3-week-old plants in a *Sclerotinia* culture filtrate (Huang and Dorrell 1978). For head rot resistance, an effective procedure involving spraying an ascospore suspension onto the florets and covering the head with a paper bag was developed (Tourvieille et al. 1978).

Evaluations for Phomopsis stem canker caused by *Diaporthe helianthi* are best conducted in fields under intensive natural infection (Fick and Miller 1997). In the absence of high levels of natural infection, several artificial inoculation methods have been described consisting of placing mycelial explants on mature leaves, stem or petioles (Tourvieille et al. 1988), spraying ascospores on the leaves (Marisevic and Gulya 1992), or artificially infecting field plots by placing contaminated stalk segments in the field followed by sprinkler irrigation (Griveau et al. 1992).

Effective evaluation methods have also been developed for screening for resistance to other sunflower diseases such as *Verticillium* wilt, *Alternaria*, *Phoma* black stem and *Rhizopus* root rot. Field evaluation methods in naturally infested plots and artificial inoculation procedures have been reviewed in detail by Škorić (1988) and Gulya et al. (1997). Rani and Ravikumar (2007) suggested a combination of gametophytic and conventional sporophytic selection to improve selection efficiency for partial resistance to *Alternaria* leaf blight.

Evaluations for *Orobanche* resistance can be conducted in naturally infested fields, but more frequently breeders use artificial inoculation with seeds of the parasite collected in previous years (Škorić 1988). These evaluations are conducted in artificially infected fields or in pots in greenhouses and growth chambers. A soil mixture (sand: silt, 1:1) is homogeneously infested with broomrape seeds adding 250 mg of seeds per kg of soil (Panchenco 1975). Sunflower seedlings are planted in peat pots with the inoculated soil mixture and incubated in a growth chamber under controlled conditions of light and temperature during 15–20 days and then transplanted to pots in the greenhouse or to the field (Škorić 1988). Resistance or susceptibility is determined by the percentage of sunflower plants that are parasitized and the average number of broomrape plants per sunflower plant. Special procedures have been developed for non-destructive in situ monitoring the developmental stages of the parasite and its interaction with sunflower (Eizenberg et al. 2005).

6.8 Integration of New Biotechnologies into Breeding Programs

Despite the tremendous economic significance of sunflower, initial molecular research on this crop was considerably delayed in comparison to other crops of similar or even lower importance. In fact, the first reports on molecular markers development in sunflower emerged nearly a decade after the initial studies of restriction fragment length polymorphism (RFLP) mapping in plants. However, molecular research in sunflower has been considerably stimulated in recent years by significant contributions in the construction of saturated

genetic linkage maps, mapping and characterization of genes controlling important traits, and understanding its genetic make up. Conversely, transgenic approaches have been scarcely afforded. There is still a huge amount of innovative research to be conducted in sunflower, but many tools are being continuously developed and are available to sunflower breeders.

6.8.1 Genetic Markers and Genetic Linkage Maps in Sunflower

In sunflower, as in other plant species, genetic markers were originally used in genetic mapping to determine the order of the genes along chromosomes, and evolved from morphological markers through isozyme markers to DNA markers. The latter have evolved from hybridization-based detection to polymerase chain reaction (PCR) amplification and, most recently to sequence-based systems. Both morphological and isozyme markers are limited in number. Additionally, the morphological markers are affected by the environment, and a given marker can affect other morphological traits because of pleiotropic gene action. Consequently, genome-wide analysis is not feasible using only morphological and isozyme markers. DNA markers are typically derived from a small region of DNA that shows sequence polymorphism between individuals within a species, and may be classified into random DNA markers (also known as anonymous or neutral markers), gene-targeted markers (also known as candidate gene markers) and functional markers. Random DNA markers are derived at random from polymorphic sites along the genome, whereas gene-targeted markers are derived from polymorphisms within genes. Finally, functional markers are derived from polymorphic sites within genes causally associated with phenotypic trait variation. In this section, we will give an overview of DNA marker development and mapping in sunflower. Functional genetic linkage maps created for mapping phenotypic and quantitative trait loci will be preferentially described in a later section. Linkage group nomenclature that will be used is that of the reference public map of Tang et al. (2002).

6.8.1.1 Random DNA Markers and Maps Based on them

Restriction Fragment Length Polymorphism (RFLP) Markers

In sunflower, the first DNA-based markers developed were RFLP markers. Most of the published sunflower RFLP markers were developed with anonymous cDNA clones, which yield low copy, polymorphic restriction fragments (Berry et al. 1994; Gentzbittel et al. 1994; Jan et al. 1993). RFLP markers were initially mapped in cultivated sunflower by different research groups, and genetic maps based on them were reported. The maps by Berry et al. (1995) and Jan et al. (1998) were based on individual F_2 populations, whereas those of Gentzbittel et al. (1995, 1999) and Berry et al. (1996) were composite maps based on data of different mapping populations (Table 6.6). In general, these

Table 6.6 Characteristics of sunflower linkage genetic maps ordered according to marker system and date of publication

Reference	Mapping populations	Population type	No. of individuals	Markers	No. of mapped loci	No. of Linkage groups	Map length (cM)	Average interval size (cM)
Berry et al. (1995)	HA89 x ZENB8	One F_2	289	RFLP	234	17	1380	5.9
Gentzbittel et al. (1995)	HA89 x RHA266; CX x RHA266; PAC2 x RHA266; (HA89 x CX) x HA89; (HA89 x CX) x CX	Three F_2 and two BC_1F_1	560	RFLP	237	23	1150	7
Berry et al. (1996)	ZENB8 x HA89; ZENB8 x PAC2; ZENB8 x ZENR7; BSA52 x RHA297; HA89 x RHA271 ; ZENR7 x RHA801; HA89 x ZENR9; ZENR1 x ZENR8; ZENB4 x HA300	Nine F_2	1287	RFLP	635	17	1472	2.3
Jan et al. (1998)	RHA271xHA234	One F_2	93	RFLP	271	20	1164	4.6
Gentzbittel et al. (1999)	HA89 x RHA266; CX x RHA266; PAC2 x RHA266; SD x PAC1 ; SD x CP73; CP73 x PAC1 ; GH x PAC2	Seven F_2	1115	RFLP	238	17	1534	6.7
Ungerer et al. (1998)	(*H. anomalus* ANO-1497 x *H. anomalus* ANO-1506) x CMS89		56	RAPD, AFLP, isozyme	701	17	1983	6.2

Table 6.6 (continued)

Reference	Mapping populations	Population type	No. of individuals	Markers	No. of mapped loci	No. of Linkage groups	Map length (cM)	Average interval size (cM)
Peerbolte and Peleman (1996)	CX x RHA266; PAC2 x RHA266 from Gentzbittel et al. (1995)	Two F_2	184	RFLP, AFLP	437	19[a]	1144	
Gedil et al. (1999)	HA370 x HA372	One F_2	108	RFLP, AFLP	400	17	1326	3.3
Flores-Berrios et al. (2000)	PAC-2 x RHA-266	One RIL	99	AFLP	264	18	2558	9.9
Langar et al. (2003)	HA89 x LR4	One R_9 RIL	171	DALP, AFLP	305	18	2169	6.1
Tang et al. (2002)	RHA280 x RHA801	One R_7 RIL	94	SSR	459	17	1368	3.1
Yu et al. (2003)	HA370 x HA372 from Gedil et al. (2001a)	One F_2	94	RFLP, SSR	202	17	1275	6.3
Yu et al. (2003)	RHA280 x RHA801 from Tang et al. (2002)	One R_7 RIL	94	SSR, INDEL	577	17	1423	2.5
Yu et al. (2003)	PHA x PHB	One RIL	94	SSR	264	20	1199	4.5
Rachid Al-Chaarani et al. (2002)	PAC-2 x RHA-266 from Flores-Berrios et al. (2000)	One RIL	123	AFLP, SSR	367	21	2916	7.9
Lai et al. (2005)	RHA280 x RHA801 from Tang et al. (2002)	One RIL	94	SNP, SSR	439	17	1349	
Hu et al. (2004)	83HR4 x RHA345	One RIL	129	TRAP	160	17[b]	1140	9.0
Hu (2006)	RHA280 x RHA801 from Tang et al. (2002)	One F_7 RIL	92	TRAP, SSR	760	17	1747	

[a] The authors describe 19 stable linkage groups (3 markers or more) in a total number of 23 linkage groups.
[b] The authors describe 17 linkage groups and 4 pairs of markers not assigned.

maps comprised 17 or more linkage groups that presumably correspond to the 17 haploid chromosomes of sunflower, and covered distances close to the estimated length of the sunflower genome (1,650 cM; Gentzbittel et al. 1995) (Table 6.6). Distorted segregation and duplicated RFLP loci were detected by Berry et al. (1995, 1996) and Gentzbittel et al. (1995).

Random Amplified Polymorphic DNA (RAPD) Markers

Despite the dominant and low reproducible nature of random amplified polymorphic DNA (RAPD) markers, they were used in early genetic studies in sunflower. High levels of RAPD variation were reported in sunflower species (Lawson et al. 1994; Teulat et al. 1994) with the proportion of polymorphic loci averaging more than 50% for most domesticated lines. Methods such as bulked segregant analysis (BSA) allowed the rapid identification of RAPD markers associated with agronomically important traits in sunflower, such as rust resistance (Lawson et al. 1996) or broomrape resistance (Lu et al. 2000). To overcome RAPD limitations, RAPD bands can be converted into sequence-characterized amplified region (SCAR) markers. In sunflower, Lawson et al. (1998) developed SCAR markers from RAPD bands linked to two rust resistance genes, demonstrating their robustness for the detection of these resistance genes in different genetic backgrounds, and Lu et al. (2000) reported SCAR markers linked to the Or5 gene conferring resistance to race E of broomrape.

RAPDs have been used for mapping in sunflower, particularly in wild species. Rieseberg et al. (1993) constructed a *Helianthus anomalus* map based on 161 RAPD markers and one isozyme locus. Later on, this map was expanded and now includes 549 RAPD, 151 AFLP, and one isozyme locus (Rieseberg et al. 1995; Ungerer et al. 1998), covering 17 linkage groups and 1,983 cM (Table 6.6). RAPD maps were also developed for wild *H. annuus* and *H. petiolaris* (Rieseberg et al. 1995), based on 212 and 400 RAPD loci, respectively. These authors reported 17 linkage groups for both species covering 1,084 cM for *H. annuus* and 1,761 cM for *H. petiolaris*.

Amplified Fragment Length Polymorphism (AFLP) Markers

AFLP are powerful markers for genome mapping and genetic variability studies, since they are highly reproducible, require no prior sequence information, and have a high multiplex ratio. However, AFLP markers are typically dominant and therefore their utility is greatest for projects where dominance is not disadvantageous. AFLP markers have been used to fingerprint elite sunflower inbred lines (Hongtrakul et al. 1997), to construct new genetic maps, and to increase the density and to fill gaps of already developed genetic maps. Peerbolte and Peleman (1996) added 291 AFLP loci to two of the F_2 populations used by Gentzbittel et al. (1995) (Table 6.6). These markers pulled two linkage groups together and permitted several previously unlinked RFLP marker loci to be mapped (Knapp et al. 2001). Gedil et al. (2001a) added 296 AFLP loci to a

104 RFLP loci map based on markers from Berry et al. (1996) and Jan et al. (1998), and constructed an AFLP-RFLP map that comprised 17 linkage groups, had a mean density of 3.3 cM, and was 1,326 cM long. Other AFLP maps have been developed. Flores-Berrios et al. (2000) constructed an AFLP map of 2,833.7 cM using 99 recombinant inbred lines (RILs) (Table 6.6), which was later improved with additional AFLP markers (Rachid Al-Chaarani et al. 2002). Langar et al. (2003) constructed a genetic map 2,169 cM long combining direct amplification of length polymorphism (DALP) markers and AFLP markers (Table 6.6).

Simple Sequence Repeats (SSRs) or Microsatellites

Microsatellite markers are ideal DNA markers for genetic mapping and population studies because of their abundance, high levels of polymorphism, multiallelic nature, codominant inheritance, wide dispersion in genomes, ease of assay using PCR, and ease dissemination across laboratories (Powell et al. 1996). Early studies demonstrated the presence of SSRs in the sunflower genome with (A)n, (GA)n, and (CA)n being the most abundant motifs (Dehmer and Friedt 1998a). Later on, different research groups have described the development and characterization of SSR markers (Gedil 1999; Paniego et al. 2002; Yu et al. 2002; Tang et al. 2002), summing up a total of 2,040 markers (Paniego et al. 2007).

Tang et al. (2002) constructed the public-reference SSR map of sunflower using 94 F_7 recombinant inbred lines (RILs) and 408 polymorphic SSR markers (Table 6.6). The map was 1,368 long and had a mean density of 3.1 cM. Yu et al. (2003) provided the first sunflower cross-referenced maps by mapping 701 new SSR and 89 RFLP or INDEL marker loci into three populations, two of them previously used by Gedil et al. (2001a) and Tang et al. (2002) (Table 6.6). From these maps, Tang et al. (2003a) developed a composite SSR linkage map of sunflower that integrated 657 loci in a 1,423 cM map with a mean density of 2.2 cM per locus. This map allowed the selection of 95 single-locus SSRs at an average spacing of 12.9 cM representing a near-genomewide collection for a first-pass scan of the sunflower genome, from which 13 six-locus PCR multiplex sets including 78 SSRs were developed. A different set of 78 SSR markers was selected by Zhang et al. (2005) for sunflower variety identification and diversity assessment.

The AFLP map developed by Rachid Al-Chaarani et al. (2002) was improved by increasing the number of AFLP markers and integrating 38 SSR markers (Rachid Al-Chaarani et al. 2004). In the new map, 367 AFLP and SSR marker loci were placed in 21 linkage groups covering 2,916 cM (Table 6.6). An additional improvement of this SSR-AFLP map has recently been reported by Paniego et al. (2007), who integrated 161 new SSR markers from different sources, and cross referenced this map to the public SSR map of Tang et al. (2002).

6.8.1.2 Gene-Targeted Markers and Maps Based on Them

Markers Based on Sequenced RFLP-cDNA Probes

INDEL (Insertion-Deletion) markers were developed from 81 RFLP markers by sequencing the cDNA clones, aligning sunflower cDNA and *Arabidopsis* genomic DNA sequences, predicting from such an alignment intron sites in sunflower genes, and designing flanking primers to amplify the introns and flanking coding regions spanned by the primer pairs (Yu et al. 2003). The genetic linkage map position of these markers (ZVG1 through ZVG81) integrated in the public SSR map of Tang et al. (2002) is described in Yu et al. (2003). Recently, Kolkman et al. (2007) resequenced in different inbred lines and wild sunflower populations essentially these 81 genes previously mapped as RFLP markers and identified 1078 single nucleotide polymorphisms (SNPs) and 178 INDELs.

Markers Based on ESTs

Expressed sequence tags (ESTs) are typically unedited, automatically processed, single-read sequences produced from cDNAs, and are currently the most widely sequenced nucleotide element from the plant genomes. Different EST sequencing programs have been carried out in sunflower, including the Compositae Genome Project, and other programs reported by Fernández et al. (2003), Tamborindeguy et al. (2004), and Ben et al. (2005). These programs have produced 94,111 EST entries for Helianthus annuus in GenBank (last accessed October, 2007). A comprehensive annotated sunflower EST database can be found at the database of the Compositae Genome Project (http://compgenomics.ucdavis.edu/).

EST constitutes a novel source of DNA-based markers that are physically associated with coding regions of the genome. In sunflower, EST resources have been used to develop SNP and SSR markers. Pashley et al. (2006) developed a novel suite of 48 polymorphic SSR markers surveying sunflower EST sequences to identify those containing SSRs. These authors found that SSRs based on ESTs exhibited higher transferability across species as compared to anonymous SSRs. SNP/INDEL markers from sunflower ESTs were developed by Lai et al. (2005). These authors identified 605 ESTs that displayed SNP or small insertion-deletion variation *in silico*, had apparent tissue-specific expression patterns, and/or were ESTs with candidate gene function for development, cell transport, metabolism, plant defence, and tolerance to abiotic stress. Primer pairs for 535 of these loci were designed from the ESTs. Two hundred and forty-three of these markers were mapped within a 196 SSR loci framework map from the RIL population reported by Tang et al. (2002) and Yu et al. (2003). The SNP/INDEL-SSR map was 1,349 long (Table 6.6), and constitutes the first functional map based on sunflower ESTs.

Target region amplification polymorphisms (TRAPs) are derived from a rapid and efficient PCR-based technique, which uses bioinformatics tools and EST database information to generate polymorphic markers around targeted candidate gene sequences (Hu and Vick 2003). The TRAP technique has been employed in sunflower to construct a linkage map based on 160 TRAP markers (Hu et al. 2004) (Table 6.6), and to define the sunflower linkage group ends through the use of TRAP markers based on *Arabidopsis*-type telomere repeat sequences (Hu 2006).

Sunflower ESTs have also been used as a source for developing universal markers useful for comparative mapping and phylogenetic analysis within the sunflower family, Asteraceae. Chapman et al. (2007) used alignments of a conserved orthologous set of ESTs from sunflower and lettuce and genomic sequences of *Arabidopsis* to design a suite of primer pairs that were conserved across species, but which were predicted to flank introns (variable regions) and therefore to detect polymorphism within species. One hundred and ninety-two of these primer pairs were tested for amplification across eight diverse members of the Asteraceae. From these, 85% amplified in at least one taxon, and 20% amplified in all the eight taxa tested, the majority of these loci being polymorphic within species.

6.8.1.3 Functional Markers

Functional markers are derived from polymorphic sites within the genes known to be causally involved in phenotypic trait variation. The development of functional markers requires allele-specific sequences of functionally characterized genes from which polymorphic, functional motifs affecting plant phenotype can be identified. Functional markers have been developed in sunflower for traits determining oil quality (Tang et al. 2006b), or herbicide resistance (Kolkman et al. 2004). A detailed description of these markers will be given in the section on molecular breeding.

6.8.2 Molecular Breeding

6.8.2.1 Germplasm Characterization

The characterization of genetic structures in cultivated sunflower was one of the first aims of genetic fingerprinting using molecular markers. Initial studies using RFLPs consistently separated sunflower inbred lines into sterility maintainer (B-line) and fertility restorer (R-line) groups (Gentzbittel et al. 1994; Zhang et al. 1995), reflecting breeding strategies that maximize heterosis. AFLP and SSR analyses confirmed these results (Hongtrakul et al. 1997; Paniego et al. 2002; Yu et al. 2002). Tang and Knapp (2003) performed

phylogenetic analyses on 47 domesticated and wild germplasm accessions using 122 SSR markers distributed throughout the sunflower genome. These authors found extraordinary allelic diversity in the Native American land races and wild populations, and progressively less allelic diversity in germplasm produced by successive cycles of domestication and breeding, suggesting that the contemporary oilseed sunflower pool could profit from an infusion of novel alleles from the reservoir of latent genetic diversity present in wild populations and Native American land races. Finally, Zhang et al. (1995) used RFLPs to screen inbred lines for intra-line polymorphisms. Although they found polymorphism within the four lines screened, these lines presented good uniformity of morphological characters in the field. It was concluded that the polymorphisms stemmed from residual heterozygosity or outcrossing, and proposed using RFLPs for distinctness, uniformity, and stability testing in sunflower.

Heterotic group modelling in sunflower using molecular tools has been reported (Hongtrakul et al. 1997; Cheres et al. 2000), although it failed to reveal clear heterotic groups. Cheres et al. (2000) estimated the correlation between genetic distance, heterosis, and hybrid performance using AFLPs and coancestries, and found that genetic distance alone was a weak predictor of hybrid performance in sunflower.

6.8.2.2 Molecular Mapping of Simply Inherited and Complex Traits

Oil Content

Oil content in sunflower is considered to be quantitatively inherited, and depends on both the percentage of hull weight in relation to whole seed weight and the concentration of oil in the kernel. Leon et al. (1995b) identified six QTL associated with oil content with predominant additive gene action, which accounted for 56% of the genetic variation. Two of these QTL were associated to kernel oil percentage, two of them with kernel percentage, and two of them with both components. Later studies reported the identification of three (Mestries et al. 1998), six (Mokrani et al. 2002), and eight (Leon et al. 2003) QTL associated to achene oil content, some of them consistently identified across environments. Recently, Tang et al. (2006a) identified six QTL for achene oil concentration on LG 1, 4, 9, 10, 16, and 17 in a RIL population developed from the cross RHA280 (confectionery line) x RHA801 (oilseed line). The QTL individually explained 3.1–22.5% and collectively explained 55.7% of the phenotypic variability for achene oil concentration. QTL on LG 10, 16, and 17 were centered on the phenotypic loci *B* (apical branching), *hyp* (hypodermal pigment), and *P* (phytomelanin pigment), respectively. Hajduch et al. (2007) using a proteomic approach reported 77 protein spots differentially expressed in the high oil line RHA 801 versus the low oil line RHA 280. Identification of 44 of these proteins indicated that the two main processes affecting low or high oil concentration in these lines were glycolysis and amino acid metabolism.

Oil Quality

Fatty Acids

The molecular basis of modified fatty acid profile in the seed oil of sunflower has been studied through a QTL and a candidate gene approach. A number of sunflower genes, coding for enzymes involved in the fatty acid biosynthetic pathway in seeds, have been cloned and their polymorphism studied in cultivated sunflower. Hongtrakul et al. (1998a) reported the isolation of two stearoyl-acyl carrier protein (ACP) desaturase genes (*SAD17* and *SAD6*) in sunflower that were highly expressed in seeds. The SAD enzyme desaturates stearoyl-ACP to oleoyl-ACP. Candidate gene and QTL analysis revealed the co-location of a major QTL associated to stearic acid content in the high stearic acid mutant CAS-3 (genotype *es1es1es2es2*) with a *SAD17* gene. The *SAD17A* locus was found to co-segregate with *Es1* (Pérez-Vich et al. 2002b). Using RFLP-AFLP linkage maps constructed from two different mapping populations derived from CAS-3, the *SAD17A* locus was mapped to LG 1, and it was found to underlie the major QTL affecting the concentration of stearic acid. This QTL explained about 80% of the phenotypic variance of this fatty acid (Pérez-Vich et al. 2002b) and it was named *st1-SAD17A*. Other minor QTL affecting stearic acid content, which mapped to LG 3 (*st2.1*), LG 7, and LG 13 (*st2.3*), were detected in that study, although none of them was consistent enough to be considered as a strong candidate for the *Es2* locus (Pérez-Vich et al. 2002b).

Since the highly significant effect of the macromutation *Es1* reduced the power of the QTL analyses to identify QTL with smaller effects on stearic acid levels, another mapping population in which stearic acid segregated independently of *Es1* was developed from the CAS-20 line (genotype *Es1Es1es2es2*) (Pérez-Vich et al. 2004a). An RFLP-SSR genetic linkage map from this population allowed the identification of three QTL affecting stearic acid, located on LG 3 (*st2.1*), LG 11 (*st2.2*), and LG 13 (*st2.3*). The three QTL collectively explained 43.6% of the phenotypic variation. On the basis of positional information, QTL on LG 11 was suggested to be a *SAD6* locus (Pérez-Vich et al. 2004a).

Very high stearic acid content in the sunflower mutant line CAS-14 is determined by the *Es3* gene (Pérez-Vich et al. 2006a). Using bulked segregant analysis, Pérez-Vich et al. (2006b) mapped *Es3* to LG 8 of the sunflower genetic map, and identified SSR markers closely linked to this gene (Fig. 6.1). *Ms11*, one of the genes determining nuclear male sterility in sunflower, was also mapped to LG 8 at a genetic distance of 7.4 cM from *Es3*.

Marker studies related to high oleic acid content in sunflower began with the identification of two RAPD makers linked to the *Ol1* gene (Dehmer and Friedt 1998b). Subsequent studies demonstrated that the *Ol1* gene cosegregates with a seed-specific oleoyl phosphatidyl-choline desaturase gene (*FAD2-1*) that is strongly expressed in normal-type (low oleic) and weakly expressed in mutant (high oleic) lines (Hongtrakul et al. 1998b; Lacombe and Bervillé 2001; Martínez-Rivas et al. 2001). The *Ol1-FAD2-1* locus mapped to

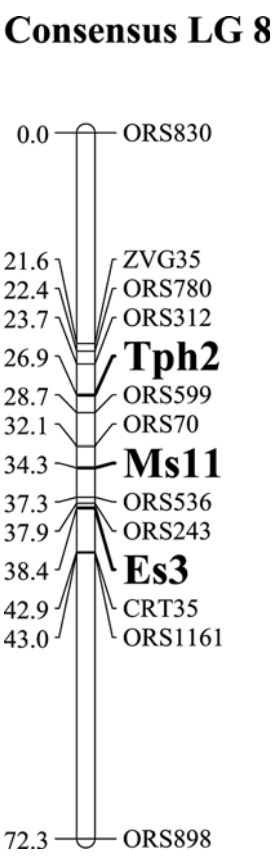

Fig. 6.1 Composite map of LG 8 containing the Tph2, Es3, and Ms11 genes determining high gamma-tocopherol content, very high stearic acid content, and nuclear male sterility, respectively. The map was constructed from the F2 mapping populations P21 × CAS-14 (Pérez-Vich et al. 2006b) and CAS-12 × IAST-540 (García-Moreno et al. 2006). The ORS and CRT prefixes denote SSR marker loci, and the ZVG prefix denotes INDEL marker loci. The cumulative distances in centimorgans are shown at the left of the map

LG 14 (Pérez-Vich et al. 2002b) of the public sunflower genetic map, and was found to underlie a major oleic acid QTL explaining 56% of the phenotypic variance for this character (Pérez-Vich et al. 2002b). Schuppert et al. (2006a) determined the physical structure of the *FAD2-1* locus and developed polymorphic sequence-tagged-site (STS) DNA markers diagnostic for the *Ol* mutation. Schuppert et al. (2005) indicated that the mechanism underlying the *Ol* mutation was a *FAD2-1* silencing by RNA interference.

Several studies have also been conducted to characterize modifying genes affecting oleic acid content. Pérez-Vich et al. (2002b) described the existence of a minor QTL on LG 8 which showed an epistatic interaction with the major QTL for oleic acid at the *FAD2-1* locus on LG 14. Lacombe et al. (2001, 2002) identified a locus that suppressed the effect of the *FAD2-1* locus, probably through a mechanism of gene silencing. Schuppert et al. (2003, 2006b) described the effect of at least seven genes from the fatty acid biosynthesis pathway, including another oleate desaturase gene (*FAD2-2*) on LG 1, acting epistatically with the *Ol1-FAD2-1* locus on LG 14.

Tocopherols

Hass et al. (2006) and García-Moreno et al. (2006) mapped the *Tph2* gene determining high gamma-tocopherol content in sunflower seeds to LG 8 of the sunflower linkage map (Fig. 6.1). In addition, Hass et al. (2006) isolated and characterized two paralogs of the gamma-tocopherol methyltransferase gene (*gamma-TMT-1* and *gamma-TMT-2*), that mapped to LG 8 and cosegregated with the *Thp2* locus. These authors also developed STS markers diagnostic for *Tph2*. However, none of these DNA polymorphisms found between wild type and mutant *gamma-TMT-1* and *gamma-TMT-2* alleles were associated with the mutant phenotype, suggesting that the mutation may disrupt regulatory sequences very tightly linked to the *gamma-TMT* locus (Hass et al. 2006).

Tang et al. (2006b) and Vera-Ruiz et al. (2006) mapped the *Tph1* gene controlling high beta-tocopherol content in sunflower seeds to LG 1. Tang et al. (2006b) determined that the *Tph1* mutation associated to the modified tocopherol phenotype was a non-lethal knockout mutation in a 2-methyl-6-phytyl-1,4-benzoquinone/2-methyl-6-solanyl-1,4-benzoquinone methyltransferase (*MPBQ/MSBQ-MT*) locus of LG 1 (*MT-1*) caused by the insertion of a 5.2 kb *Ty3/gypsy*-like retrotransposon, and developed STS markers diagnostic for wildtype and mutant *MT-1* alleles. A second *MPBQ/MSBQ-MT* locus mapping to LG 4 (*MT-2*) was also described to be associated to tocopherol composition (Hass et al. 2006; Tang et al. 2006b). This locus was epistatic to the *MT-1* locus on LG 1 and had no effect in *Tph1Tph1* or *Tph1tph1* individuals, but significantly increased beta-tocopherol content in *thp1thp1* individuals.

Disease Resistance

Resistance to downy mildew is probably one of the best examples of the use of major gene resistance. There are up to 10 resistance genes described, denoted Pl, carrying resistance to various downy mildew races and mapped to genetic maps (Vear 2004). Research on these genes has shown that there are at least three clusters of genes plus several others that segregate independently from these clusters. A cluster on LG 8 includes the Pl1, Pl2, Pl6 (from wild *H. annuus*), and Pl7 (from *H. praecox*) genes covering a large area of about 0.5 cM with two genetically distinct regions conferring resistance to different Plasmopara races (Vear et al. 1997; Bouzidi et al. 2002). A second cluster on LG 13 includes the Pl5 (from *H. tuberosus*) and Pl8 (from *H. argophyllus*) genes (Bert et al. 2001). The Pl_{arg} (from *H. argophyllus*) gene was solely mapped to a telomeric region of LG 1 (Dussle et al. 2004). Numerous resistance gene analogues (RGAs) have been found clustered and linked to the Pl clusters on LG 8 (Gentzbittel et al. 1998; Gedil et al. 2001b; Bouzidi et al. 2002; Slabaugh et al. 2003) and LG 13 (Radwan et al. 2003), and to Pl_{arg} (Radwan et al. 2007).

Resistance to the parasitic weed *Orobanche cumana* appears to follow a similar pattern to that of downy mildew. Dominant resistance genes *Or1* through *Or5*, conferring resistance to races A through E, respectively, have

been described (Vrânceanu et al. 1980). The *Or5* gene has been mapped to a telomeric region of LG 3 of the sunflower genetic map (Lu et al. 2000; Tang et al. 2003b; Pérez-Vich et al. 2004b). However, recent genetic and molecular studies have revealed a more complex genetic control of broomrape resistance. Pérez-Vich et al. (2004b) reported that phenotypic variance for race E resistance was mainly explained by a major QTL on LG 3 (*Or5* gene) associated to the resistance or susceptibility character, while race F resistance was explained by QTL with small to moderate effects, mainly associated with the number of broomrapes per plant.

Lawson et al. (1998) developed SCAR markers linked to the R_1 and R_{adv} genes conferring resistance to rust. Subsequent studies demonstrated that one of the SCAR markers linked to the R_1 gene mapped to LG 8 and was closely linked to the downy mildew cluster on this LG (Slabaugh et al. 2003). Lenardon et al. (2005) mapped the *Rcmo-1* gene for resistance to sunflower chlorotic mottle virus to LG 14.

Resistance to other diseases, such as *Sclerotinia*, Phomopsis stem canker, and black stem is more complex, involving several loci with different effects and highly dependent on environmental conditions. For this quantitative resistance, there are no specific genes and races described, although lists of QTL are becoming available. QTLs for resistance to *Sclerotinia* concerning the capitulum reaction to the ascospore test have been identified on 14 of the 17 sunflower linkage groups in different crosses, explaining individually less than 20% of the phenotypic variance (Bert et al. 2002, 2004; Yue et al. 2007). One particular strong QTL was found on LG 8, linked to a protein-kinase gene (Gentzbittel et al. 1998), but while it explained 50% of the variability in one cross, in other crosses it explained only 15% or was not present. QTLs for reaction to mycelium tests on leaves and capitula and for natural attack on terminal buds have also been reported (Mestries et al. 1998; Bert et al. 2002, 2004), which often appear to co-localise with the QTLs for resistance to the ascospore test (Vear 2004).

QTL studies on *Sclerotinia* midstalk rot resistance reported six to nine QTL for each of the three resistance traits evaluated (leaf lesion, stem lesion, and speed of fungal growth), each with a small effect. In total, between 24.4 and 33.7% of the genotypic variance for resistance against *Sclerotinia* could be accounted for by these QTL (Micic et al. 2004). QTL for stem lesion detected by these authors on LG 8 and 16 were demonstrated to be consistent across generations (Micic et al. 2005a). Micic et al. (2005b) determined the effect of three to four QTL associated to *Sclerotinia* resistance by selective genotyping in a mapping population derived from crosses with a different resistant line. In addition, these authors cross-referenced previous studies of Mestries et al. (1998), Bert et al. (2002), and Rönicke et al. (2005) and found that the same six linkage groups carried QTL for *Sclerotinia* resistance in more than one population, and that LG 1, LG 9, and LG 10 had a significant effect in the majority of the populations considered. Despite the complex genetic architecture of *Sclerotinia* resistance, QTLs consistent across

environments (Bert et al. 2002), generations (Micic et al. 2005a), and mapping populations (Rönicke et al. 2005; Micic et al. 2005b) have been identified, which constitute valuable tools for the establishment of marker assisted selection programs aimed at improving *Sclerotinia* resistance.

Bert et al. (2002) found three QTLs explaining up to 20% of the variability for resistance reaction to natural attacks of Phomopsis stem canker, and other three QTL for resistance reaction to artificial infections, one of them for both types of infection. These authors also reported the co-localisation of a QTL affecting resistance to *Sclerotinia* mycelium in leaves and another QTL for resistance to Phomopsis in leaves, suggesting that these QTL could result from the same components in the mechanism of resistance to these two facultative parasites.

In two independent studies, a significant number of QTL (four to seven) with moderate effects on resistance to black stem were identified, explaining each QTL from 5 to 17.5% of the phenotypic variance (Rachid Al-Chaarani et al. 2002; Bert et al. 2004). Subsequent studies on the population from Rachid Al-Chaarani et al. (2002) testing different *Phoma macdonaldii* isolates allowed the identification of a total of 27 resistance QTL for 4 isolates (Abou Alfadil et al. 2007) and 10 resistance QTL for 2 isolates (Darvishzadeh et al. 2007), with moderate individual effects ranging from 6 to 29%. Alignan et al. (2006), using a cDNA microarray approach, identified several genes regulated in response to *Phoma macdonaldii*. These authors proposed a model in which negative regulation of a dual-specificity mitogen-activated protein kinase (MAPK) phosphatase could be implicated in the defence mechanisms against this pathogen via activation of a MAP kinase cascade that could trigger defence responses such as thaumatin biosynthesis and phenylalanine ammonia lyase (PAL) activation.

Developmental and Agronomic Traits

Male Sterility

Three out of 11 recessive genes controlling nuclear male sterility in sunflower have been mapped. The *Ms9* gene was mapped to LG 10 using TRAP and SSR markers (Chen et al. 2006), whereas the *Ms10* and *Ms11* genes were mapped to LG 11 and LG8, respectively using RFLP, SSR and INDEL markers (Pérez-Vich et al. 2005).

Molecular studies have examined the nature of different CMS sources available in sunflower. Köhler et al. (1991) suggested that a new open reading frame, orfH522, in the 3′-flanking region of the *atpA* gene was associated with the CMS phenotype. Further studies using mtDNA genes and three probes for the open reading frame clearly distinguished CMS sources by their mtDNA organization and CMS mechanism (Horn 2002). Kusterer et al. (2005) developed PCR-based markers closely linked to the fertility restoration gene *Rf1*.

Self-incompatibility and Seed Dormancy

Wild populations of *H. annuus* are self-incompatible and have deep seed dormancy, whereas modern sunflower cultivars are self-compatible and have short-lived seed dormancy. Gandhi et al. (2005) mapped QTL for self-incompatibility and seed dormancy in a backcross population from parents showing contrasting characteristics for both traits. A single locus S for self-incompatibility was identified and mapped to LG 17. The locus was tightly linked to a cluster of QTL for several domestication and postdomestication traits. Three QTL for seed dormancy with small individual effects in the predicted direction (wild alleles decreased seed germination) were identified.

Embryogenesis

Plant regeneration by in vitro organogenesis offers the possibility of obtaining a high number of regenerated shoots. Flores-Berrios et al. (2000) developed an AFLP genetic linkage map from a RIL population exhibiting variability for organogenesis traits. Six putative QTL for number of shoots per explant plated and seven putative QTL for number of shoots per regenerating explant were identified. For each trait, QTL explained 52 and 67%, respectively of the total phenotypic variance.

Days to Flowering

Days to flowering is an important trait primarily controlled by the genotype, photoperiod, and temperature. Few genetic factors for days to flowering have been reported. Mestries et al. (1998) identified two QTL that accounted for 30% of the phenotypic variation in a single environment. Leon et al. (2000) identified five QTL that accounted for 73 and 89% of the phenotypic and genotypic variations, respectively, across four locations with limited range of photoperiod. When evaluating the same population in locations with more different photoperiods, Leon et al. (2001) found that the two QTL with the strongest association with days to flowering were responsive to photoperiod.

Resistance to Abiotic Stresses

Poormohammad Kiani et al. (2007) conducted a pot experiment for mapping water status traits and osmotic adjustment associated with drought tolerance. The plants were induced to water deficit to compare QTL detection under well-watered and water-stressed environments. In general, most of the QTL detected under well-watered conditions were not detected in water-stressed conditions. Eight QTL were detected for osmotic adjustment. The largest one, located at LG 5 and accounting for 29% of the phenotypic variation, overlapped with QTL for other water status traits such as leaf water potential, relative water content, osmotic potential, and osmotic potential at full turgor.

Resistance to Herbicides

Sunflower biotypes resistant to two classes of acetohydroxyacid synthase (AHAS)-inhibiting herbicides such as imidazolinones (IMIs) or sulfonylureas (SUs) have been discovered. Kolkman et al. (2004) identified, cloned and sequenced three AHAS sunflower genes: AHAS1, AHAS2, and AHAS3, which were mapped to LG 9, 6, and 2, respectively. In addition, these authors identified mutations in codons 197 and 205 in AHAS1 that conferred resistance to IMI and SU herbicides, respectively, and developed a SNP genotyping assay diagnostic for the codon 205 mutation.

6.8.2.3 Marker Assisted Selection

In contrast to the high number of reports on mapping of important traits controlled by major genes and QTL, literature on practical application of those markers in sunflower breeding programs remains very limited. There are probably several scientific and logistical issues that must be still resolved before practical marker assisted selection (MAS) strategies can flow from mapping studies. Therefore in this section we will deal with factors determining enhanced power of MAS and how they are faced in sunflower. Moreover, the few examples of practical use of molecular markers in breeding programs available so far will be highlighted.

MAS Optimization

Marker Validation and Refinement

Marker validation and refinement is one of the main factors enhancing selection power of MAS. For markers associated to simply inherited traits, marker validation and reduction of the distance between the marker and the gene of interest is fairly straightforward. Examples of marker validation in different genetic backgrounds have been reported for the Pl_2 gene determining resistance to different downy mildew races (Brahm et al. 2000), to the R_1 and the R_{adv} genes conferring rust resistance (Lawson et al. 1998), and to the *Or5* gene conferring resistance to race E of broomrape (Tang et al. 2003b; Pérez-Vich et al. 2004b). Improvement of marker accuracy for the *Rf1* gene restoring pollen fertility in PET1 based material was improved by using enlarged mapping populations (Kusterer et al. 2005).

In many cases, the marker identified in the process of fine-mapping may not be polymorphic in all the populations tested, thus requiring the identification of alternative markers for those populations. Ideal markers for selection are those based on gene mutations underlying the trait of interest. This kind of markers has been developed in sunflower for oil quality traits and other simple traits. For example, Tang et al. (2006b) determined that a non-lethal knockout mutation in a *MPBQ/MSBQ-MT* locus on LG 1 (*MT-1*) was underlying beta-tocopherol accumulation in sunflower seeds, and robust STS markers

diagnostic for wildtype and mutant *MT-1* alleles were developed. Similarly, Kolkman et al. (2004) identified a mutation in codon 205 in the acetohydroxyacid synthase gene *AHAS-1* that confers resistance to imidazolinone (IMI) herbicides, and developed a SNP genotyping assay diagnostic for it.

The situation becomes more complicated for QTL markers for complex traits. Factors such as population structure and size, parental selection and genetic background effects, epistasis, inaccurate phenotyping, or QTL x environment interactions contribute to bias the estimation of QTL effects, thus reducing the likelihood of successful use of these QTL in MAS programs. QTL validation in independent samples and in different genetic backgrounds and environments is therefore necessary before using them in MAS breeding programs. There are some good examples of QTL validation in sunflower, making the validated QTL ideal targets for MAS. For *Sclerotinia* resistance, QTL have been validated across environments (Bert et al. 2002), generations (Micic et al. 2005a), and genetic backgrounds (Rönicke et al. 2005; Micic et al. 2005b). For oil content, QTL have been also validated across generations, environments, and mapping populations (Leon et al. 2003; Tang et al. 2006a).

In addition to QTL validation, fine-mapping of QTL is very useful for identifying tightly linked markers that will not suffer from loss of linkage due to recombination between marker and QTL, and that will allow to minimize the size to the introgressed fragment. The development of a high density sunflower genetic map (one marker per 0.8 cM) through the mapping of 2,495 high-throughput DNA marker loci (Knapp et al. 2007) will contribute to map QTL with a high level of resolution. The development of specific genetic resources such as near-isogenic lines (NILs), differing in a genomic segment containing a target QTL, and RILs will also contribute to fine-mapping of QTL. In sunflower, Micic et al. (2005a) re-estimated position and effects of a number of QTL for *Sclerotinia* resistance in a RIL population developed from F_3 families where the QTL were originally identified. However, they only obtained partial recovery of QTL detected in the earlier F_3 generation in the RILs for traits such as stem lesion. Pizarro et al. (2006) have developed QTL-NILs varying in a target QTL for seed oil concentration, which allowed the authors to determine its effect with higher resolution.

Association mapping has great potential for higher-throughput QTL detection. The method relies on linkage disequilibrium (LD) to study the relationship between phenotypic variation and genetic polymorphism. The LD extent and the application of association mapping in sunflower have not been studied in depth. Recent reports by Liu and Burke (2006) and Kolkman et al. (2007), who studied patterns of nucleotide diversity in genic loci from both wild and cultivated sunflower, demonstrated that SNP frequencies and LD decay were of sufficient magnitude in wild populations (1 SNP/19.9 bp and LD decay within ~200 bp), exotic germplasm accessions (1 SNP/38.8 bp and LD decay within ~1,100 bp), and modern sunflower cultivars (1 SNP/45.7 bp and LD decay within ~5,500 bp) for high-resolution association mapping.

Assays Optimization and Cost Reduction

After the development of molecular markers and validation of their power for selection for the trait, it is often necessary to optimize the assays, with driving criteria being to reduce unit costs and turn around times while increasing throughput and minimizing errors. Technologies that speed up the implementation process, reduce laboratory requirements or errors, and lower the cost associated with scaling-up, are crucial to the success of MAS. One of the main priorities included in the "White paper: Priorities for research, education and extension in genomics, genetics and breeding of the Compositae" (The Compositae Genome project, http://compgenomics.ucdavis.edu/; 2007) for translating sunflower genomics into practical breeding programs was the reduction of total marker costs. Advances in sunflower marker technologies have been carried out in recent years. For SSR markers, PCR multiplexes for a genome-wide framework of SSR marker loci developed by Tang et al. (2003a) increased genotyping throughput and reduced reagent costs, which is essential for repetitive genotyping applications. In addition, multicolour assays, SSR primer design to facilitate "pooled amplicon multiplexing" by length in SSR development, and SSR analyses in semi-automated, high-throughput genotyping systems (Yu et al. 2002; Tang et al. 2002) resulted in time-saving and reduced costs for SSR assays. Currently, different techniques for SNP detection are being used in sunflower to type SNPs in a high-throughput, time-saving and cost-effective fashion, including denaturing high-performance liquid chromatography (DHPLC) (Lai et al. 2005), and single-base extension or allele specific primer extension (Knapp et al. 2007).

Improved QTL detection methods that reduce costs have also been proposed. Micic et al. (2005b) used selective genotyping for detecting QTL for *Sclerotinia* resistance in sunflower. This method exploits the concentration of most of the information for QTL effects in the "tails" of the quantitative trait distributions. Accordingly, population sizes can be reduced to those individuals found in these "tails". The authors concluded that selective genotyping can be efficiently used for QTL detection and analysis of congruency for resistance genes across populations, despite the limited sample size and the non-random sampling used.

MAS in Sunflower Breeding Programs

The most common application of MAS is marker assisted/accelerated backcross breeding for gene introgression. Optimally, this is based on positive foreground selection for donor trait, positive background selection for the recurrent parent genome, and negative background selection against undesirable donor parent alleles (Frisch et al. 1999). In general, marker assistance is expected to provide higher efficiency, reduced cost, and/or shorter duration of the backcross breeding scheme, compared with conventional methods.

In sunflower, marker assisted backcross breeding is currently being carried out in private breeding companies to accelerate the introgression of target genes into elite germplasm. Traits such as downy mildew resistance, high oleic acid content, and herbicide resistance are currently the main targets, although complex traits such as resistance to *Sclerotinia*, *Phoma* and Phomopsis stem canker are also taken into account. Despite there are no reports on such programs, it seems that markers routinely used in plant cultivar development in private programs are used mainly for selecting alleles with large effects on traits with relatively simple inheritance. However, dissection of complex traits such as oil content using molecular markers in sunflower is contributing to implement MAS for such traits. For example, QTL associated to different components that determine oil content (kernel percentage in the achene and kernel oil percentage) have been identified and validated. Some of these QTL are associated to the phenotypic loci *B* (apical branching), *hyp* (hypodermal pigment), and *P* (phytomelanin pigment) (Leon et al. 1996, 2003; Tang et al. 2006a). This fact was explained by Tang et al. (2006a) as a pleiotropic effect of such phenotypic loci on oil content, and allowed Leon et al. (1995a) to establish combined marker and phenotypic (based on the *hyp* locus) assisted selection for high oil content during the backcross process.

Codominant markers are most useful for marker-assisted backcrossing because selection amonguy backcross progeny involves selection for heterozygous progeny. If a dominant marker is used for selection, it will remain informative in subsequent backcross generations if the dominant allele (conferring band presence) is linked to the donor parent allele. If the recessive allele is linked to the donor parent allele, then progeny testing of each individual in each backcross generation would be required, thereby doubling the number of generations required for backcrossing. Panković et al. (2007) proposed increasing MAS efficiency in backcross programs to introgress the *Pl6* gene conferring resistance to downy mildew race 730 by using a combination of closely linked codominant cleaved amplified polymorphic sequence (CAPS) markers with dominant markers developed from resistance candidate genes.

Marker-assisted backcross breeding is also very effective in transferring genes or QTLs determining valuable traits from wild donor genotypes into elite breeding lines, reducing both the time required and the risk of undesirable linkage drag with unfavourable donor attributes. To facilitate and accelerate the introgression process of genes related to disease resistance, Slabaugh et al. (2003) proposed the identification of allelic variation in wild species for specific candidate genes such as RGAs, to identify potentially useful resistance genes through disease screening, and to use markers developed from these RGAs to track the gene in the introgression process. QTL and candidate gene analyses in wild sunflower species is contributing to identify genes and QTL for adaptation to salt or drought tolerance (Lexer et al. 2003a, b; Kane and Rieseberg 2007) that could be exploited as a source of new genes to be introgressed into cultivated sunflower. Despite the use of molecular markers to assist backcross introgression of specific genes from wild species is still scarce, they have been

useful for the identification and characterization of interspecific hybrids (Natali et al. 1998; Binsfeld et al. 2001).

Gene pyramiding is a useful approach to enhance the durability and degree of pest and disease resistance, or to increase the level of abiotic stress tolerance. Genes controlling resistance to different races or biotypes of a pest or pathogen and genes contributing to agronomic or seed quality traits can be pyramided together to maximize the benefit of MAS through simultaneous improvement of several traits in an improved genetic background. Vear (2004) suggested that major genes need to be backed up by quantitative, non-race specific resistance QTL for increasing resistance durability. For this purpose, the use of molecular markers is essential, since partial resistance conferred by these QTL can not be determined phenotypically if combined with major resistance genes. Molecular markers will be very useful to pyramid resistance genes tightly linked in clusters, a virtual impossibility in practice using phenotypic analysis alone (Slabaugh et al. 2003). For partial resistances such as *Sclerotinia* and Phomopsis stem canker, a very important step towards the improvement of the level of resistance is the use of MAS to combine different resistance QTL.

Different strategies are currently being carried out to enhance the efficiency and scope of molecular breeding. The development of BAC (bacterial artificial chromosome) and BIBAC (binary-bacterial artificial chromosome) libraries (Gentzbittel et al. 2002; Özdemir et al. 2004; Feng et al. 2006; Tang et al. 2007) and linkage group-specific clones (Jan and Seiler 2007) is providing resources and tools essential for comprehensive research of the sunflower genome. These libraries are being used for isolating and physical mapping of loci such as the *FAD2-1* locus (Tang et al. 2007), or the fertility restorer *Rf1* locus (Hamrit et al. 2006). In addition, combination of QTL mapping and gene expression analysis and function elucidation is becoming an excellent tool for dissecting QTL into Mendelian factors and improving the efficiency of molecular breeding of complex traits in sunflower, such as drought tolerance (Poormohammad Kiani et al. 2007).

6.8.3 Transgenic Breeding

Despite the increasing success of transgenic varieties of some major oilseed crops such as soybean and canola, transgenic breeding research in sunflower has been rather limited so far in comparison with the mentioned crops. A major constraint for the advance of transgenic breeding have been the limitations of the initial regeneration systems as well as problems in combining regeneration and transformation within the same cells. Nevertheless, efficient transformation protocols with high reproducibility and high transformation frequency have been developed (Mohamed et al. 2006).

Most of the development of transgenic varieties has been conducted by seed companies, which have incorporated agronomically important traits.

The most important ones for which information is available are tolerance to the herbicide glyphosate, by expression of 5-enolpyruvishikimate-3-phosphate synthase (*EPSPS*) genes from *Agrobacterium tumefaciens*, tolerance to the herbicide glufosinate ammonium by expression of phosphinothricin acetyltransferase (*PAT*) genes from *Streptomyces* spp., resistance to Lepidoptera by expression of *Cry* toxins genes from *Bacillus thuringiensis* (*Bt* genes), resistance to Coleoptera by expression of trypsin inhibitor genes from cowpea (*Vigna unguiculata*) plus lectin genes from snowdrop (*Galanthus* spp.), resistance to *Sclerotinia* by expression of oxalate oxidase genes from wheat or barley, and enhanced rubber production by expression of genes from guayule (*Parthenium argentatum*) (Cantamuto and Poverene 2007).

Transgenic lines have also been produced at the public sector. Rousselin et al. (2002) developed transgenic sunflower lines with reduced stearic acid content by expression of a delta-9 stearoyl desaturase gene from castor bean (*Ricinus communis*). Sawahel and Hagran (2006) produced transgenic plants resistant to *Sclerotinia* by expression of the human lysozyme gene.

The risks associated with the gene flow from transgenic cultivars to the wild flora are a matter of general controversy. In the case of sunflower, the risk is particular high in North America, centre of origin for the genus, but also in many other parts of the world where feral and naturalized populations of wild *Helianthus* species are present (Bervillé et al. 2005). Examples of adaptative advantages associated with the flow of transgenes to wild *Helianthus* populations have been reported by Snow et al. (2003) and Burke and Rieseberg (2003).

6.9 Seed Production

The sunflower breeder identifies inbred lines or open pollinated varieties that have better performance than the currently used ones. Once the preliminary trials suggest that an inbred line or open pollinated variety has potential, the breeder increases the seed supplies and produces larger quantities of seed for expanding testing. Seed at this stage is referred as "Breeder seed" because the breeder is responsible for maintaining purity and increasing seed supplies of the line or variety. When applied to hybrid varieties, it refers to the seed of male-sterile, maintainer, and restorer lines. The initial increase of breeder seed is known as "Foundation seed". It is handled to maintain specific genetic purity and identity. "Registered seed" is the progeny of breeder and foundation seed handled under procedures acceptable to the certifying agency to maintain satisfactory genetic purity and identity. "Certified seed" is the progeny of breeder and foundation seed handled to maintain satisfactory genetic purity and identity which is approved by the certifying agency.

Hybrid seed is the first generation of seed of a cross produced by controlling the pollination and by combining two or more lines. Single hybrid sunflower seed is produced through the controlled crossing of male (restorer or R) and

female (male-sterile or A) lines. The A-line is maintained by crossing to a genetically identical male-fertile line with a fertile cytoplasm, referred to as maintainer or B-line. The commercial seed is usually grown from hybrid seed and it is planted for any use except for seed production.

Increasing breeder seed and foundation seed of parental lines and certified seed of hybrids is a time-consuming and critical operation in breeding programs requiring full-time personnel to be in charge of operations. It requires systematic planning and management on the part of seed producers. The production, processing and marketing of the certified seed is exclusively the responsibility of the seed producers. The seed-certifying agencies set up the procedures by which each class of seed may be produced, the standards of purity and identity, and also assume the responsibility for inspecting, sampling testing, and certifying that the seed meets certification standards. Exact certification procedures vary from country to country.

6.9.1 Maintenance and Increase of Parental Lines

Increases of A, B, and R lines are initially accomplished under bags in nurseries to check for purity and stability of the cytoplasmic male sterility and to eliminate off-types. These increases are often carried out under isolation in screened cages to eliminate bagging. A hive of bees is placed inside each cage to pollinate lines to be crossed and to eliminate hand crossing. An increase of the R-line may not require pollination by bees. Usually lines are planted on different dates so that these cages can be utilized for several increases each season. For small increases, hand crossing of the A-line with the B-line may be done. Hand pollination should be carried out in the morning on all days throughout the flowering period.

Field increases of breeder/foundation seed of the female (A) line involves planting the A and B lines using a ratio 1:1 or 2:1 (A:B) at low plant populations in rows spaced 75–90 cm apart. The production field is isolated at least 6–8 km from commercial fields or wild populations in countries such as USA where those are frequent. One hive of bees per hectare is placed in the field for pollination of the female parent.

An occasional problem in converting certain lines to cytoplasmic male sterility is the occurrence of fertile plants in the progeny of crosses between maintainer and male-sterile lines. The frequency of fertile plants was estimated 5.7% in a study involving 500 inbred lines (Vrânceanu and Stoenescu 1980). Because of potential problems with fertile plants in increasing foundation seed and in hybrid seed production, many breeders use the system of paired crosses (Vrânceanu 1974) in converting and maintaining A- and B-lines. In this system, the identity of individual A- and B-line plants is maintained and only those B-line plants that produce completely sterile progeny are used for further multiplication.

6.9.2 Commercial Hybrid Seed Production

6.9.2.1 Isolation

Similarly to the multiplication of parental lines, maintaining the recommended isolation from other sunflower crops or wild species is a crucial requirement in hybrid seed production to maintain genetically pure hybrid seed. Pollen from external sources will contaminate the crop, causing tall plants, reduced disease resistance, reduced yield potential, and in the case of wild sunflowers, multi-headed plants. Seed companies try to avoid this carrying out seed production in regions with no major commercial production to eliminate the problem of isolation from cultivated sunflower. In the USA, where wild sunflower is commonly present, wild plants must be removed from the area of seed production before flowering to avoid undesirable cross-pollination (Miller 1987). Considering the role of honeybees in pollination and their flight range, seed certifying agencies have established minimum isolation requirements of 1.6–4.8 km between seed production fields and those of commercial sunflower in order to maintain the genetic purity of parental lines or hybrids. Space isolation is the most important factor to be considered for the production of quality seed. When space isolation is not possible, time isolation of about 30 days is satisfactory. This means that the flowering stage of the parental lines in the seed production field should be at least 30 days earlier or later than that of other varieties grown within the area to avoid contamination by pollen.

6.9.2.2 Plant Population and Planting Methods

Optimum plant population for hybrid seed production depends on the characteristics of the female and male parents, environmental conditions, and desired seed size. For most of the oilseed type hybrids, plant population varies from 40,000 to 60,000 plants under irrigation. Optimum populations for confectionary type hybrids are higher when smaller seed is desired for the grower. However, when the market demands larger seeds, plant populations of 40,000 plants and wider spacing between rows are used. The ratio of female to male rows usually ranges from 2:1 to 6:1, depending on the pollination ability of the male parent, similarity in the flowering dates of the male and female parents, and number of row units used (Vrânceanu 1974). For example, if the restorer line incorporates the recessive branching trait, which allows the production of pollen during longer periods, the number of female rows can be increased. One important consideration in planting the seed production field is the staggered sowing of the parental lines in order to achieve flowering synchronization between the female and R-line to avoid problems in hybrid seed set.

6.9.2.3 Pollination

In the maintainer as well as in the hybrid seed production plots, pollination is a crucial aspect to be considered. Hives of honeybees are placed in the hybrid seed production field at the beginning of anthesis. The number of hives depends on the plant population and stage of flowering. Seed producers generally use one to four hives per hectare during the heaviest pollinating period. An adequate number of hives is important since placing too many may force bees to forage other sources of pollen and increase the percentage of outcrosses.

6.9.2.4 Roguing

Roguing is an essential practice in sunflower hybrid seed production for obtaining physical and genetic purity. The objective of roguing is to remove before anthesis the plants that do not meet the expected characteristics of the line (off-types). It has to be strictly carried out in all the stages of crop growth. A north–south orientation of the rows to improve its efficiency is recommended (Miller 1987). In breeder/foundation seed as well as in certified seed production, male-fertile plants within the male-sterile line are easily identified by dark anthers and pollen production. In addition, plants with other morphological deviations have to be removed in A- and B-lines before flowering. In breeder and foundation seed production of restorer lines as well as in certified seed when R-lines are involved, off-types have to be removed before anthesis. A row spacing of 76–90 cm facilitates walking between rows for observing plants. Twin rows, consisting of two rows of the female parent planted 60 cm apart and 90 cm from the next twin-row set, have also been used.

6.9.2.5 Harvesting and Processing

Processing hybrid seed from the field to the bag requires many operations. Harvesting seed production fields is a critical operation. It should not start until moisture has reached 11–13%, and much care must be taken to prevent excessive damage to the achenes. Rows of the male parent generally are removed before the female rows are harvested to avoid contamination. In some cases, seed producers remove rows of the male parent after pollination. After harvesting, the seed is transported to the seed processing plant where it is cleaned, graded, treated, and bagged. Quality control is required in order to maintain the seed quality certification standards. Seed is stored under adequate conditions of temperature and relative humidity to avoid deterioration of quality. Commercial seed lots are generally treated with a combination of insecticides and fungicides conferring protection against damage and stand loss by early season soil and foliar insects as well as early season diseases, particularly downy mildew.

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Chapter 7
Flax

Scott D. Duguid

7.1 Production and Utilization

Over the centuries the production of flax (linseed, *Linum usitatissimum* L.) has spread across Europe, Africa and finally North America where it was the first oilseed to be widely grown in western Canada. Today, world flaxseed production has ranged between 2.0 and 3.0 million tonnes over the last 10 years. In 2006–2007, the production of flaxseed was 2.7 million tonnes, which represents about 0.7% of the world production of oilseeds. Canada is the world's largest producer representing 40% of world production and most of the export trade. China, the United States of America and India together account for 40% of the world production. Within the European Union, the main producers of flaxseed are Germany, United Kingdom, and France. Production has remained relatively stable in China, India, and the European Union over the last decade (Table 7.1).

Primarily, flax is grown for seed with fibre production as a by-product. Today, the unique properties of flaxseed with high levels of alpha linolenic acid (ALA) in the oil differentiate it from other oilseeds in the industrial, human food and animal feed market. Industrially, the oil is a major ingredient in linoleum flooring and is also used in paints, stains and surface coatings as well as in the oleo-chemical industry due to its rapid oxidation properties. Technological developments since the 1950s, such as increased usage of latex paints and petroleum based floor covering has reduced industrial demand. Since the turn of the century, the trends to green and health oriented products have resulted in new opportunities for flax, for instance the non-allergenic and biodegradable characteristics of linoleum, along with quality improvements have led to a resurgence in demand for linoleum flooring.

The use of flaxseed as a functional food has been traditional in many parts of the world such as Northern Europe, however, the functional properties of flax have only recently received considerable attention in North America due to the

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Table 7.1 World flaxseed supply and disposition

	2005–2006	2006–2007 (estimated)	2007–2008 (forecast)
	Million tonnes		
Carry-in stocks	**0.09**	**0.53**	**0.70**
Production			
Canada	1.08	1.04	0.60
China	0.48	0.48	0.45
United States	0.48	0.35	0.23
India	0.23	0.21	0.21
EU	0.18	0.18	0.17
C.I.S.	0.10	0.10	0.10
Bangladesh	0.05	0.05	0.05
Argentina	0.05	0.05	0.04
Other	0.20	0.21	0.28
Total production	**2.85**	**2.67**	**2.13**
Total supply	**2.94**	**3.20**	**2.83**
Crush	2.09	2.19	2.15
Other	0.32	0.31	0.35
Total use	**2.41**	**2.50**	**2.50**
Carry-out stocks	**0.53**	**0.70**	**0.33**
Trade	0.80	0.86	0.86

Source: Bi-weekly Bulletin – Flaxseed Situation and Outlook, Agriculture and Agri-Food Canada.

numerous health benefits attributed to this oilseed. Flax is classified as a functional food or nutraceutical product primarily because of the presence of significant quantities of three of the most physiologically-active components: (1) the omega-3 essential fatty acid, alpha linolenic acid, (2) lignans, a major class of phytoestrogens and (3) soluble fibre primarily in the form of mucilage. Benefits from flaxseed can be introduced to the diet through flaxseed oil, whole or milled flaxseed, or through many products such as omega-3 eggs produced by hens on flaxseed-fortified rations or in products that are readily available in supermarkets and that contain flaxseed include breads, cereals, crackers, energy bars, baking mixes, snacks, soups and waffles.

Flaxseed oil and meal have for many years been recognized as valuable components in animal nutrition. The animal feed market offers significant opportunities for flax to: (1) improve animal productivity as an integral component of the feed and (2) to develop healthier food applications for humans from flax fed animals. The benefits of flaxseed as animal feed include improving growth and lessening disease and stress related conditions. For poultry, flaxseed in the laying hens' rations results in eggs that are higher in omega-3 fatty acid, which health conscious consumers want in their diet. For swine, the inclusion of flaxseed in the diet not only changes the nutritional quality of the pork by making more omega-3 fatty acid available to the consumer, but studies also

suggest that flaxseed in the diet of breeding sows produce larger and healthier piglets. Flaxseed is routinely included in premium pet foods to improve the overall health and appearance of cats and dogs. Research continues on how flaxseed could be incorporated into rations for dairy cattle to create products such as omega-3 enriched milk and cheese. In general, flaxseed is milled before it is included in animal rations, to ensure maximum absorption of the nutrients in the flaxseed.

Flax straw contains long stem fibres with unique properties that make it useful in the production of fibre products for textiles but it is also used in the paper and pulp industry as a pulp sweetener to strengthen recycled paper, geotextiles, absorbent products, insulation. New developments are focusing on using flax straw as an alternative fuel, which has a per tonne heating value similar to soft coal and appears to be a carbon neutral method of producing heat.

7.2 Origin and Taxonomy

The flax family Linaceae is geographically widespread with about 300 species worldwide. *Linum* is the largest genus within the family with three geographic centers of diversification: (1) the Mediterranean area; (2) southern North America and all of Mexico; and (3) South America as well as in other areas of Europe, Asia and the Americas. Several proposals for dividing the genus into sections exist (Diederichsen and Richards 2003) and the status of many species remains to be clarified. The diversity of species in the genus *Linum* differs in various parts of the world. Chennaveeraiah and Joshi (1983) conducted cytological studies on 19 species and proposed phylogenetic relationships based on chromosome numbers and similarities. Within the genus *Linum*, the chromosome number varies between 2n = 16 and 2n = 60, with most of the species having either 2n = 18 or 2n = 30 chromosomes. Gill (1987) presented chromosome numbers of 41 species of the genus ranging from 2n = 16 to 2n = 80. Further clarification to the phylogenetic relationships within the genus is required as the systems proposed are artifical, in nature. Cross hybridization between cultivated and wild species has found that interspecific hybridization between cultivated flax and *Linum angustifolium* have been successfully conducted. In addition Gill and Yermanos (1967) reported successful hybridization with 3 different *Linum* species. However, it should be pointed out that none of these interspecific crosses has had any practical use in flax breeding. Plant systematics attempts to establish a system reflecting the evolutionary relationships among plant species. As Diederichsen and Richards (2003) suggested, natural systems in plant taxonomy are supportive for plant breeding because they facilitate the targeted search for desirable traits of economic importance in closely related species. The challenge for plant breeders is to introduce the desired traits from the wild species (Table 7.2).

Table 7.2 Distribution of species in the genus *Linum* for different areas of the world

Area	Number of species
Northern America and Mexico	63
Former Soviet Union	45
Turkey	38
Europe	36
France	24
Italy	20
Bulgaria	19
Morocco	19
Lebanon and Syria	17
Algeria	15
Iran	13
Germany	10
Portugal	10
USA, North-East	10
Central Asia	9
Canada	8
Switzerland	8
Afghanistan	7
Alava/Spain	7
Mallorca/Spain	6
Australia	6
Libya	6
Pakistan, West	6
Cyprus	5
India	5
Egypt	4
Tropical East Africa	4
Belgium	3
Iraq	3
Arabia	2
Japan	2
Scandinavia	2

Source: Diederichsen and Richards (2003).

7.3 Variety Development

Cultivar development for Canada and the USA has been largely a task of public breeding programs. Four major breeding programs develop flax varieties for Canada and the USA: Agriculture and Agri-Food Canada program located at the Morden Research Station in Morden, Manitoba; Crop Development Centre program located at the University of Saskatchewan in Saskatoon, Saskatchewan and the Viterra program located at the Alberta Research Council site at Vegreville, Alberta; and finally the North Dakota State University program at Fargo, North Dakota, USA.

Since the early 1900s, Agriculture and Agri-Food Canada and its predecessors have been active in the development of new flax varieties for Canada, and, in particular, for the Canadian prairies. The initial program at the Central Experimental Farm in Ottawa produced varieties such as Diadem Ottawa 770B, Ottawa 829C, and Novelty. During the 1950s, this program was particularly active, releasing varieties such as Linott, Raja and Rocket. The 1950s and 1960s also marked the beginning of an evolution and transition in flax breeding in Canada. A new program was initiated at the Indian Head Experimental Farm and the Winnipeg Cereal Breeding Laboratory which led to the development of the variety Cree. In the 1960s, a breeding program was also conducted in Alberta at the Fort Vermillion Experimental Farm and Beaverlodge Research Station, producing the variety Noralta, the predominant variety grown in northern Alberta and Saskatchewan. The breeding programs of Agriculture and Agri-Food Canada were moved and consolidated to Winnipeg in 1960; then moved to Morden, Manitoba, where they still exist. The varieties Dufferin, McGregor NorLin, NorMan, AC Linora, AC McDuff, AC Emerson, AC Carnduff, Lightning, Hanley, Macbeth, Prairie Blue, Prairie Thunder, and Prairie Grande have been released by Agriculture and Agri-Food Canada (Table 7.3).

A modest breeding program was carried out at the University of Saskatchewan from the 1920s to the 1960s, which produced the varieties Royal and Redwood 65. The program was enlarged in 1974 when the

Table 7.3 North American flax cultivars released since 1990

Cultivar	Pedigree	Year of release	Breeding program	Reference
AC Linora	Linott/NorMan	1991	Agriculture and Agri-Food Canada	Kenaschuk and Rashid (1993)
Neche	CI2847/Culbert 79	1991	North Dakota State University	Hammond et al. (1991)
AC McDuff	FP766/FP775	1992	Agriculture and Agri-Food Canada	Kenaschuk and Rashid (1994)
Omega	CI3036/Flor	1992	North Dakota State University	Miller et al. (1992)
Verne	Culbert/Hioil 5017	1992	Minnesota Agriculture Experiment Station	Comstock and Putnam (1992)
Linola™ 947	McGregor/Zero// 84495/3/McGregor^2	1993	Viterra	Dribnenki and Green (1995)

Table 7.3 (continued)

Cultivar	Pedigree	Year of release	Breeding program	Reference
AC Emerson	Noralta/Vimy	1994	Agriculture and Agri-Food Canada	Kenaschuk and Rashid (1996)
Prompt	BFP/Culbert	1994	South Dakota State University	Grady and Lay (1994)
Day	N707//CI2777/N419	1994	South Dakota State University	Grady and Lay (1994)
Linola™ 989	McGregor/Zero// CPI8495/3/ McGregor[3]	1995	Viterra	Dribnenki et al. (1996)
CDC Normandy	Tissue Culture Derived Somaclonal Variant of McGregor	1995	Crop Development Centre	Rowland et al. (2002)
CDC Triffid	Sulfonylurea Herbicide Resistant – Transgenic Never produced commercially	1996	Crop Development Centre	McHughen et al. (1997)
CDC Valour	NorLin/Vimy	1996	Crop Development Centre	Rowland et al. (2002)
AC Watson	NorLin//FP775/ CI2941	1997	Agriculture and Agri-Food Canada	Kenaschuk and Rashid (1998)
AC Carnduff	Dufferin//NorLin/ Culbert 79	1998	Agriculture and Agri-Food Canada	Kenaschuk and Rashid (1999)
CDC Arras	Vimy/FP833	1998	Crop Development Centre	Rowland et al. (2002)
CDC Bethune	NorMan/FP857	1998	Crop Development Centre	Rowland et al. (2002)
Taurus	Cebeco 3100	1999	Cebeco Zaden B. V., Netherlands	
Cathay	M23/CI2932	1998	North Dakota State University	
Pembina	FP805/SD8308	1998	North Dakota State University	

Table 7.3 (continued)

Cultivar	Pedigree	Year of release	Breeding program	Reference
Linola™ 1084	Flanders/FP946	1999	Viterra	Dribnenki et al. (1999)
Linola™ 2047	989//Windemere/ M2702	2000	Viterra	Dribnenki et al. (2003)
Hanley	Flanders/AC Emerson	2001	Agriculture and Agri-Food Canada	Duguid, S. D. et al. (2003)
Lightning	AC McDuff/AC Watson	2001	Agriculture and Agri-Food Canada	Duguid, S. D. et al. (2003)
CDC Mons	Flanders/FP926	2002	Crop Development Centre	Rowland et al. (2003)
Macbeth	M2701/AC Linora	2002	Agriculture and Agri-Food Canada	Duguid, S. D. et al. (2003)
Prairie Blue	Flanders/FP956	2003	Agriculture and Agri-Food Canada	Duguid et al. (2004)
CDC Gold	ED-48/YSED-19	2003	Crop Development Centre	
Linola™ 2090	92-7335/92-7337	2003	Viterra	Dribnenki et al. (2004)
Linola™ 2126	SP992/94-7889	2004	Viterra	Dribnenki et al. (2005)
Nekoma	U605/Bison	2004	North Dakota State University	Hammond et al. (2004)
York	U23/CI2929// McGregor	2004	North Dakota State University	Hammond et al. (2004)
CDC Sorrel	FP956/Vimy	2005	Crop Development Centre	
Linola™ 2149	SP1084/96-32-F_3	2005	Viterra	Dribnenki et al. (2007)
Prairie Thunder	FP974/FP1043	2006	Agriculture and Agri-Food Canada	
Prairie Grande	AC Watson/CI3395	2007	Agriculture and Agri-Food Canada	

Crop Development Centre (CDC) initiated a flax breeding program. It has since produced the cultivars Vimy, Somme, Flanders, CDC Normandy, CDC Valour, CDC Arras, CDC Bethune, CDC Mons, and CDC Sorrel as well as the solin variety CDC Gold. Other varieties produced at the Crop Development Centre include Andro (tissue culture derived) and CDC Triffid (first transgenic flax cultivar – never commercially produced). Both of these varieties have now been deregistered and are not commercially available (Table 7.3).

In 1987, a solin (low alpha linolenic acid) breeding program was initiated by Biotechnica Canada in cooperation with Australia's Commonwealth Scientific & Industrial Research Organization to develop the low alpha linolenic acid flax, subsequently known as solin utilizing a double mutation. In 1990, UGG Ltd. (now known as Viterra) purchased Biotechnica's interest in the program and moved the program from Calgary to the AAFC research station located at Morden, Manitoba and the research and evaluation farm at Rosebank, Manitoba. In 2005, the program moved to the Alberta Research Council research facility at Vegerville, Alberta. This breeding program has produced the solin cultivars Linola^{TM} 947, 989, 1084, 2047, and 2090 (Table 7.3).

Over the last number years the flax breeding has decreased significantly in the United States of America with the only remaining active program being located at North Dakota State University. In 1984, with the retirement of breeder at the Minnesota Agriculture Experiment Station, the program was terminated and 1997 was the last year that material was evaluated in South Dakota State University program. The USDA/ARS flax improvement research at North Dakota State University was reduced to maintenance of the Flax World Collection and coordination of regional testing several years ago. Several years ago the collection was moved to Ames, Iowa (Table 7.3).

Developing improved flax cultivars is a continuing process using the germplasm base, created by the cumulative efforts of flax workers over many years. Each improvement in germplasm represents an increment added to the flax gene pool. Many lines from the Canadian and USDA germplasm collections have provided valuable sources of variation that were successfully used to develop improved cultivars. Valuable germplasm lines that researchers developed are freely exchanged among flax researchers, which increases the diversity of the genetic base of gene pools of each program. Continued progress in the improvement of yield, quality and disease resistance demonstrated by a succession of cultivars in all flax growing areas suggest further progress can be anticipated. In addition, identification of genetic variation for useful, unique characteristics of flax may provide a germplasm base for developing cultivars suitable for new uses for the flax crop.

7.4 Variety Development Objectives

Breeding objectives vary among flax breeding programs depending upon the particular problems that exist in the specific production area. Objectives that are common to most flax breeding programs are high yield, suitable maturity, lodging resistance, good grain quality and disease resistance. Some problems may be unique to a specific area and of little concern in other areas. For example, breeding for chlorosis resistance is an important objective in Manitoba but of little consequence in Saskatchewan and Alberta where the soil is not as calcareous as in Manitoba.

7.4.1 Yield

Breeding for high grain yield is clearly the most important objective for flax improvement. Over the past century, flax breeders have been extremely successful in exploiting the germplasm base to produce the currently available cultivars but if flax is going to compete with other oilseeds for acreage then substantial improvements in yield will be necessary. The primary yield components of flax are: (1) the number of bolls per unit area, (2) the number of seeds per boll, and (3) seed weight. Fortunately, flax has the capacity of compensation among the yield components. Although it is possible to breed for high yield per se, the flax breeder must select for other qualities such as disease resistance, lodging resistance, and maturity adaptation to develop cultivars that consistently produce high yields.

7.4.2 Maturity

Flax is a cool season crop, so maturity suitable for the intended production area is important. The development of an early maturing variety with specific adaptation to northern conditions and that matures in about 90 days instead of 100 days should result in yield increases. There has been a tendency over the years to breed flax for early maturity. The reasoning behind is that early maturing versus late maturing varieties have a better opportunity to escape damage or stress from abiotic stresses such as heat and drought, cold, frost and diseases. Flax maturity is highly heritable and available germplasm is adequate for developing cultivars with maturity suitable for the intended area of production.

7.4.3 Lodging Resistance

Lodging caused by severe storms, high soil fertility or delayed harvest frequently causes serious flax losses and grain quality reductions particularly

if it occurs early during grain filling. Severely lodged flax is also more susceptible to diseases such as pasmo and sclerotinia. Lodging resistance helps to ensure good grain filling and minimal harvest losses. In 1993, 80% of the flax acreage in Manitoba was grown to medium-early maturing varieties, predominantly NorLin, NorMan and Somme. These varieties are more susceptible to lodging than the late maturing varieties such as McGregor and AC McDuff. Yield losses in flax resulting from lodging have not been well documented, and yields of susceptible variety Vimy in Manitoba are often more than 50% of the yields of other varieties of similar maturity in cooperative tests grown under severe lodging conditioning.

Grain yield losses caused by lodging in small plot breeding trials generally are not indicative of losses that occur under practical conditions on the farm, and consequently, some breeders may underestimate the importance of lodging resistance especially if wet conditions occur for several days after lodging did. Flax breeders certainly have been aware of the need for better lodging resistance in flax cultivars, but there have been few programs where improvement of that trait was the top priority for the following reasons: (1) developing cultivars with resistance to major diseases has required a major portion of the limited resources available for flax improvement, (2) heritability of lodging resistance is relatively low, (3) the trait is difficult to measure because of undependable, highly variable occurrence of stresses causing lodging, and (4) combining lodging resistance with high yield and good seed quality is difficult.

7.4.4 Grain Quality

Grain quality factors such as high oil content and desirable fatty acid composition and high protein content have received increased attention from flax breeders in recent years. The important grain quality factors are under genetic control. There is sufficient genetic diversity in the cultivated and germplasm collections to ensure continued improvements of most quality traits.

7.4.4.1 Oil and Fatty Acid Composition

The most important quality parameter for flax is high oil content. Over the past 10 years, Canadian flaxseed contained, on average 44% oil (Table 7.4). Oil content on individual farm samples can vary by as much as 15% with a range of 35–50% being reported for farm grown samples tested over the past 5 years. As the seed from individual farm samples moves through the handling system, it is combined and the range of oil content decreases so that the range of oil content in Canadian exports of flaxseed has only been 3.5%, from 42 to 46%. Our

Table 7.4 Flaxseed, No 1. Canada Western Seed Quality for 1997–2006 harvest survey

Year	Oil content %	Protein content %	Iodine value Wijs	Fatty acid, % in Oil					Free fatty acid, %
				Palmitic	Stearic	Oleic	Linoleic	Linolenic	
1997	43.9	23.5	193					58.0	0.20
1998	43.6	22.9	190	5.5	3.6	19.4	14.3	56.8	0.19
1999	43.9	21.8	196	5.4	3.1	17.1	14.7	59.6	0.17
2000	44.1	22.4	194	5.4	3.2	17.9	14.2	58.9	0.26
2001	44.1	24.1	190	5.2	3.7	19.5	15.1	56.3	0.40
2002	45.5	23.7	195	4.9	3.1	17.3	15.1	58.9	0.29
2003	44.2	25.6	184	5.2	3.7	22.4	15.0	52.9	0.15
2004	44.8	22.1	201	4.9	3.0	14.5	15.8	61.6	0.26
2005	46.2	22.0	194	5.0	3.3	16.8	16.3	57.7	0.18
2006	45.9	23.6	190	5.0	3.6	19.5	15.6	55.8	0.16
2007	44.7	24.3	184	5.0	3.6	20.6	16.2	52.6	0.16
1997–2006	44.7	23.2	193	5.2	3.4	18.2	15.1	57.7	0.22

Source: Canadian Grain Commission.

highest oil producing variety at present is AC McDuff with an oil content of 48%. Recently, Agriculture and Agri-Food Canada received a recommendation to register FP2188 which is the first flax variety with a oil content of 50% of which 59% is alpha linolenic acid.

The nutritional quality of a vegetable oil for human and animal consumption or as industrial oil are determined by its fatty acid composition. Flax oil is characterized by high alpha-linolenic acid contents (>50%) and moderate concentrations of oleic (approximately, 18%) and linoleic acid (approximately, 14%). Palmitic (about, 5%) and stearic (about, 3%) fatty acids are also present (Tables 7.4 and 7.5). Flax oil is one of the most unsaturated common plant oils, and its iodine value is usually greater than 185 Wijs units. The level of unsaturation varies with both variety and environment. The level of linolenic acid over the last 10 years (1997–2006) from Canadian flax according to the Canadian

Table 7.5 Flaxseed, No 1. Canada Western Solin Seed Quality for 1998–2005 Harvest survey

Year	Oil content %	Protein content %	Iodine value Wijs	Fatty acid, % in oil				
				Palmitic	Stearic	Oleic	Linoleic	Linolenic
1998	42.8	23.3	140	6.4	4.1	16.2	70.0	1.9
1999	43.5	21.7	143	6.1	3.5	14.6	72.2	2.2
2000	44.6	22.5	143	5.7	3.6	15.4	72.0	2.1
2001	44.8	22.3	141	5.5	4.2	17.5	69.7	2.0
2002	46.2	22.7	144	5.3	3.5	15.7.	72.6	2.1
2003	46.4	26.0	139	5.9	4.0	18.3	68.3	1.8
2004	48.0	23.4	148	5.5	2.8	12.8	75.2	2.4
2005	49.1	22.0	145	5.7	3.2	14.4	73.3	2.2
1995–2004	45.1	22.9	143	5.9	3.7	15.8	71.2	2.0

Source: Canadian Grain Commission.

Grain Commission was 57.7% with an iodine value of 192.7 Wijs. Environmental studies have demonstrated that flax grown under cool conditions has higher levels of linolenic acid and overall iodine value.

The need for various fatty acid modifications in flax is well established and research efforts are underway to develop germplasm with the various fatty acid profiles for use by the world flax industry whether as a functional food, pharmaceutical or industrial uses because of their improved biodegradability. Various sources of higher levels of linolenic acid are available in flax and these are being introgressed into elite germplasm. Lines with approximately 70% ALA have also been developed but require agronomic improvement. Flax breeders, using mutation selection processes have developed lines of flax with low levels of alpha linolenic acid (< 3%) which have been given the common name solin to differentiate them from traditional flax varieties. Examples of solin varieties include Linola 947, 989, 1084, 2047, 2090, 2126 and CDC Gold (Tables 7.3 and 7.5).

7.4.4.2 Protein

Historically, the flaxseed crush in Europe was driven by the demand for linseed oil to be used in the production of linoleum, paints and other industrial products. Recently, however, the demand for non-genetically modified high protein meal (45–50%) is driving the crush and the production of linseed oil. The linseed meal is fed to livestock, primarily in Western Europe, while surplus linseed oil is sold to distant markets such as China and North Africa. In general, linseed meal (1.2 Mt in 2001–2002) is consumed in the country in which it is produced, but only 82,000 t were exported from the producing country.

As with other oilseeds, there is an inverse relationship between oil and protein in flaxseed. The relationship for flaxseed however, is not as strong as for other oilseeds such as canola and, unlike in canola, the meal protein is not related to the oil content. Seed proteins in flax consist of about 20% albumins and 80% legumin-like proteins and are more liophilic than soybean proteins. The legumin-like fraction is rich in sulphur amino acids. Over the past 5 years, Canadian flaxseed has been found to contain about 23% crude protein that translates into a meal protein content of 43–46% (Tables 7.4 and 7.5). On individual farm samples, protein ranged from 17 to 29% while on exported flaxseed the range was 20.9–25.1%. Various sources of higher protein content are available in flax and these are being introgressed into elite germplasm. FP2188, the recently recommended line for registration for Agriculture and Agri-Food Canada, is an example of one of these sources of higher protein content (47%) in the meal but also breaking the inverse relationship between oil and protein content.

7.4.4.3 Mucilage

In flax, sugars and starch (digestable carbohydrates) are minimal to none whereas the majority of carbohydrates are resistant to the action of digestive enzymes. Flaxseed mucilage is an excellent source of dietary fiber and due to its highly viscous nature also shows potential for being used as a food gum. Flaxseed mucilage is a complex mixture of two polysaccharides, which differ in physiochemical properties such as composition, molecular size, structural conformation and rheological properties. Genetic variability exists for the content (4–7%) and physiochemical properties of flaxseed mucilage (Diederichsen et al. 2006). This variability would allow the development of cultivars that contain mucilage with rheological and functional characteristics for specific end uses.

7.4.4.4 Lignan

There has been considerable interest in the inclusion of flaxseed in western diets or as an isolated and purified compound for improvement of human health. Part of this interest comes from studies indicating that there may be a significant beneficial effect resulting from the biological activity of the lignan secoisolariciresinol diglucoside (SDG). Although the level of SDG found in Canadian cultivars is significant (13 mg/g for Flanders – 22 mg/g Linola 947 of the seed), an increase in this level would be beneficial. Of the six major groupings of flax, the Indian, the Mediterranean and Spring collections contain accessions with the highest SDG levels, while Winter, Fiber and Forage types contain lower levels of SDG on average. If a variety was developed with a meal SDG content of approximately 40 mg/g, it would represent more than double the SDG content of commercial flax meal which is typically in the 1.2–1.7% range.

7.4.4.5 Anti-nutritionals

Characteristics that limit utilization of flaxseed meal include the presence of anti-nutritional factors such as cyanogenic glucosides and cadmium. As in the case of canola, a leading factor in international competitiveness would be the development of improved varieties low in anti-nutritional factors such as cyanogenic glucosides and cadmium. Hydrolysis of cyanogenic glucosides can produce hydrogen cyanide, a potent respiratory inhibitor, and thereby limiting the quantity (8–10%) of flaxseed that can be used in food products or feed rations. Lowering of cyanogenic glucosides may also increase the value of other value added products in the food and pharmaceutical markets such as proteins, gums and lignans. Typically, varieties such as AC McDuff have a total level of 467 mg/100 mg of seed (390 mg/100 mg linustatin and 70 mg/100 mg of neolinustatin). Accessions have been identified with lower levels of cyanogenic glucosides and crossed with adaptable material. These crosses are beginning to reach the yield trial level and have a 60% reduction in total cyanogenic glucosides.

Cadmium content in flaxseed is influenced by geography, soil type, fertilizer usage, etc. All current varieties are high cadmium accumulators (range 1.3–2.0 ppm) (Oomah et al. 2007; Grant et al. 2008). Flax is currently receiving increasing attention from health professionals as a possible food supplement in human diets, especially because of the evidence of its substantial health benefits. This will open the way for acceptance of flax as a high quality source of improved protein meal and value added products. The current limitations can be removed by improving the quality of flaxseed meal through applied plant breeding programs. Accessions have been identified with lower levels of cadmium uptake and crossed with adaptable material. However, the lack of an inexpensive technique to evaluate the level of cadmium has slowed the effort. With the development of an inexpensive testing along with traditional breeding and double haploid techniques it should be possible to develop low accumulating cadmium varieties of flax.

7.4.4.6 Seed Color

There is a growing market for yellow flaxseed in the baking industry and health food trade. The market is expected to increase significantly because of the interest shown in flaxseed as a dietary source of linolenic acid in human nutrition. Most of the present market is in Western Europe with an increasing market in Canada, Japan and USA. Both whole seed and flour are used in the baking industry and yellow seed is preferred because of its aesthetic appeal in bakery products. At the present time, there are varieties available in North America that are grown under contract which are poorly adapted, disease susceptible and low yielding (65–85% of brown seeded registered varieties). Because of low yields a higher price is paid. Higher yields from improved varieties will reduce the price and make Canadian flaxseed more competitive in the world market and should result in higher production.

7.4.4.7 Disease Resistance

Flax diseases are a potential constraint to flax production in nearly all areas where flax production occurs throughout the world. A significant portion of flax improvement programs has been to increase resistance to pathogens and thus to stabilize production and crop quality. The development of genetic resistance is the most efficient and cost effective method to prevent losses in yield and quality to diseases. Diseases that have received most attention are flax rust, fusarium wilt, powdery mildew and pasmo and are caused by fungi. Resistance to these diseases is under genetic control, and satisfactory sources of resistant germplasm are available for use in flax breeding programs for flax rust, fusarium wilt and powdery mildew. At this point in time a comparable level of resistance to pasmo is not available. Breeding for resistance to some of the diseases, especially the rusts in the past, has been hampered by continual development and spread of new physiological biotypes of the causal disease organism. Various sources of resistance to these diseases are available in flax

and are being introgressed into elite germplasm. Resistant germplasm, if properly used in conjunction with innovative breeding procedures, should provide excellent disease protection in the years ahead. The development of molecular markers for known resistance to flax pathogens will be extremely useful to breeders and pathologists attempting to incorporate resistance to multiple pathogens into high yielding varieties. The severity of crop loss to disease varies significantly among regions and years because development and spread of pathogens depends on various environmental conditions, the proportion of the flax acreage planted to resistant cultivars, and the effectiveness of the resistance.

7.5 Germplasm Sources

A wealth of diverse flax germplasm is available to flax breeders. The total number of flax germplasm accessions existing in the world gene banks is about 53,000 as shown in (Table 7.6). There are at least 81 genebanks, institutions and breeding stations in the world, which maintain collections of flax germplasm. The total number of flax germplasm accessions is large, but caution should be used as there is a high degree of duplication between collections such as those at the All Russian Flax Research Institute and the N.I. Vavilov Institute for Plant Industry. In some cases germplasm accessions have been integrated into breeding programs such as that of the German breeding company Deutsche Saatveredelung. Ethiopia has a large collection but access has been restricted. The collections for flax germplasm of the United States National Plant Germplasm System and Plant Gene Resources of Canada and that of the Research Institute for Technical Cultures are to a great extent

Table 7.6 Genebank collections of *Linum* germplasm

Name of institution	Country	Number of accessions
All Russian Flax Research Institute, VNIIL, Torzhok	Russia	6,100
N. I. Vavilov Institute of Plant Industry, VIR, St. Petersburg	Russia	5,700
Research Institute for Technical Cultures, RITC	China	4,000
Breeding Company DSV, Lippstadt	Germany	3,500
Ethiopian Genebank, Addis-Ababa	Ethiopia	3,100
North Central Plant Introduction Center, Ames, Iowa	USA	2,800
Plant Gene Resources of Canada, PGRC, Saskatoon	Canada	2,800
Research Institute for Cereals and Industrial Crops, RICIC, Funduela	Romania	2,700
Other collections (81)		22,300
Total		53,000

Source: Diederichsen and Richards (2003).

duplicates of one another. According to Diedrichsen and Richards (2003) about 30% of the world flax germplasm can be considered unique. They suggest this duplication helps to ensure preservation of diversity as continued preservation in genebanks can be severely affected by political change, genebank priorities and problems with rejunvenation. The wild species in the genus *Linum* are rarely found in collections because they are perennials and are difficult to regenerate due to cross pollination. The German genebank at Gatersleben has the largest collection of these species.

Released cultivars are also maintained by the originating breeding program or by the Plant Gene Resource Centre of Agriculture and Agri-Food Canada in Saskatoon, Saskatchewan or by the United States Department of Agriculture. Germplasm developed in the individual breeding programs is often exchanged informally with other breeders or is formally released and registered to make it available to all breeders.

7.6 Breeding Procedures

Flax breeding procedures usually have been grouped into three general categories: introduction, selection and hybridization followed by selection. Selection from introduced accessions and pedigree selection following hybridization have been the predominant methods of flax breeding. Prior to 1936, most of the cultivars recommended for production in North America were derived by mass or single plant selection from introduced accessions. Although many different breeding procedures and combinations of procedures are used by flax breeders today, a universal feature of all current programs is hybridization followed by selection. However, there are notable exceptions: (1) Norland which was selected from Victory, a variety that was heterogenous for both plant height and maturity; (2) Redwood 65 which was selected from an x-irradiated population of Redwood and represented an improvement in oil content; (3) Andro; (4) CDC Normandy somaclonal variation from tissue culture and (5) CDC Triffid, a genetically transformed cultivar with sulfonylurea herbicide resistance.

7.6.1 Selection of Parents

The initial step of any flax breeding program is to clearly define the objectives which are both economically and biologically reasonable. Then the breeder must choose the parents for creating segregating populations from which a potential cultivar will be selected and to a great extent this will be based on the specific breeding objectives of the program and availability of germplasm to meet those objectives. A flax breeder can only use a small part of the parental material available; the success of the program

depends upon the ability of the breeder to select parents that will produce superior progenies. The parents are then crossed. Flax breeders commonly choose and hybridize the parents with complementary traits with the objective of selecting individual progeny lines that combine those traits.

7.6.2 Methods of Combining Parents

The methods used to make a cross and proper cultural practices have been summarized by Beard and Comstock (1980). An unopened bud (prior to anthesis) that has petals protruding 30–60 mm past the sepals is chosen as the female parent and emasculated. Pollen from the male parent is transferred as soon as the anthers dehisce. Typically, 70% of the pollinations result in viable seed which are usually grown in the greenhouse or in the field.

An understanding of the genetics of desirable new traits is of practical value to the flax breeder since such information greatly assists in determining breeding objectives, developing breeding strategies, and utilizing resources efficiently. Knowledge of the genetic relationships among different traits can also be useful since desirable new traits may have undesirable pleiotropic effects on other important traits or be linked to undesirable traits. New sources of resistance or other desirable traits often occur in unadapted, agronomically inferior germplasm with poor quality, and must be incorporated into a more useful genetic background before they can be used in breeding programs. Rapid advances are currently being made using molecular markers and successful transformations have been reported in flax. These powerful tools have great potential to improve understanding of flax genetics and accelerate the development of improved germplasm and cultivars with novel genes. It is important to participate in these new developments if the flax industry is to be competitive at home and abroad. However, much work still needs to be done in identifying desirable new traits and determining their inheritance so that biotechnological techniques can be effective.

The incorporation of desirable new traits into acceptable flax cultivars requires considerable effort over a long period of time. The backcross method is effective when the new trait is simply inherited, easy to detect and occurs in an unadapted genotype. When unadapted germplasm is introgressed into adapted germplasm for population improvement, one backcross to the adapted parent for population improvement or a three-way cross are more effective than a single cross. When both parents contribute to a number of desirable traits, a single cross should be used with standard breeding methods, such as the pedigree or bulk methods. Double crosses can be used to accumulate both major and minor genes. Several generations of selfing and selection may be required particularly if a number of minor genes are involved. A number of breeding cycles may also be needed to introgress desirable traits such as linolenic acid into an acceptable flax cultivar. A double haploid recurrent selection procedure that takes only 2 years for each complete cycle has been devised.

7.6.3 Methods of Breeding

The choice of breeding method to handle the segregating populations depends upon factors such as the breeding objectives, nature of the crop, parental lines available, number of traits, relationships among the traits, mode of inheritance and ease of selection for a particular trait, diversity and number of parents and resources available to the breeder. Methods commonly used in flax breeding include the pedigree method with various modifications, bulk selection, backcrossing, and single seed descent. While conventional breeding practices are primarily used, anther culture or microspore techniques are also now being used to accelerate the development of new flax cultivars and improved germplasm, and to facilitate genetic studies.

7.6.3.1 Pedigree

The pedigree method is the most widely used flax breeding procedure. The pedigree method is effective when the traits of interest in the breeding program are easy to identify, highly heritable, and thus can be effectively selected in early generations. For example, the pedigree system has been especially effective in flax for rust resistance, oil content, and fatty acid composition. The pedigree method of breeding, like any other method, has certain advantages and disadvantages. The method is labour intensive and more expensive than other methods, but it can systematized with the assistance of computer programs to simplify the record keeping and data collection so that large numbers of crosses can be handled efficiently. A major advantage of the method is that undesirable progenies are eliminated early, and only the superior progenies are advanced to the next generation. The intensive selection in early generation selection may result in undesirable limitations on recombination, especially in cases where traits of interest are linked with undesirable genes for other important traits.

When using the pedigree method, the breeder emphasizes intensive selection in segregating generations and the establishment of homozygosity and homogeneity within lines before entering them into advanced yield trials. This method consists of making a cross between two genotypes possessing the characters to be combined in a new variety and increasing the F_1 seed in the greenhouse, growth room or field. The main purpose of the F_1 generation is to provide sufficient seed for the F_2 population, therefore, any method which does this is quite satisfactory. The size of the F_2 population will depend on many factors including breeding objectives, availability of seed, genetic differences between the parents, and resources available for selecting and handling individual plants. Selection begins in the F_2 generation and progenies from the selected F_2 plants are grown and selected in each subsequent generation until near homozygosity or at least until individual lines appear uniform for visual characters. Selection may be practiced both between and within progeny rows.

Visual selection is conducted for desirable agronomic characteristics such as early maturity, plant height, disease resistance, high number of bolls which has been shown to be a good indicator of yield potential. A common practice is to select several plants from the progeny rows or hills that appear most promising for the traits of interest and increase the seed in a winter nursery or greenhouse. During the winter increase, the reserve seed is evaluated for resistance to rust, oil content and fatty acid composition. Those plants which are rust resistant, having high oil content and oil quality are grown to maturity in the winter nursery/greenhouse. This cycle is repeated for each generation. The bolls from the individual plant are threshed and maintained separately, examined visually, and selected ones saved for the next generation. The same procedure is followed until the desired level of homozygosity is reached. At this point, each selected progeny row is bulk harvested and used for further seed increase and performance testing. Most breeders do not increase individual lines for advanced yield testing before the F_5 to F_7 generation.

The remaining lines are then evaluated in preliminary yield trials. Data collected for days to maturity, plant height, 1,000 seed weight, lodging resistance, oil content, oil quality and resistance to rust, powdery mildew and pasmo. The lines are also grown in the wilt nursery to confirm resistance to wilt. The preliminary yield trials serve to reduce the number of lines to a quantity which can be handled in regional trials conducted by the individual breeding institutions. These trials serve to determine the performance of experimental lines in flax growing areas in Canada. The very best candidate lines from each breeding institution are then entered into the Cooperative Test which is grown at 20 locations across Canada with 15 locations in three Prairie Provinces. Each participating breeding institution, university or private industry grows a uniform set of entries at each location and the data is compiled and analyzed. These results are ultimately used in support of the licensing of varieties in Canada.

7.6.3.2 Single Seed Descent

Single seed descent is a form of the pedigree selection method that is being used by breeding programs to rapidly advance germplasm through segregating generations to produce varieties or generate genetic material for research purposes. A single seed from each F_2 plant and their progenies are advanced through succeeding generations by harvesting one seed from each plant to provide the population for the next generation. The procedure is repeated for several generations to obtain a population of near-homozygous plants for selection. Individual plants are then harvested and planted in individual rows and the selected lines are harvested for further seed increase and testing. The single seed descent method is especially well adapted for generation advance in the greenhouse. No selection is generally practiced and since only one seed is harvested, optimum plant growth is not required.

7.6.3.3 Backcross

Generally, the backcross breeding method is used to transfer one or at most a few genes controlling a simply inherited trait into an otherwise outstanding cultivar or elite germplasm. The line possessing the desirable trait is the recurrent parent and the parent contributing the simply inherited trait is the non-recurrent or donor parent. In a typical backcrossing program, the F_1 of a single cross is crossed back to the recurrent parent, and in subsequent backcrosses only selected plants having the desired traits from the donor parent are crossed back to the recurrent parent. With each successive backcross, the genetic contribution from the donor parent is reduced by one half resulting in rapid recovery of the recurrent parent. Most breeders believe that four to six backcrosses are sufficient to recover the prototype of the recurrent parent. The backcross method has been used to transfer single genes for disease resistance from unadapted to adapted varieties of flax. This was typical of the approach taken by flax breeders incorporating new rust resistance genes into varieties or for the generation of genetic material such as the set of rust differentials developed by Dr. H.H. Flor by backcrossing individual genes into the cultivar "Bison".

7.6.3.4 Bulk

In the bulk method, seed harvested from the F_1 is bulk seeded in the field, and the F_2 is bulk harvested to provide seed for the next generation. The entire plot from each cross is harvested in each generation, and a random sample of the bulked seed is used to plant the next generation. This usually is repeated until F_5 to F_7 after which individual plants are selected from the bulk population. These selections are grown in individual row or hill plots and lines with desired traits are harvested and advanced to yield tests.

7.6.3.5 Doubled Haploids

Doubled haploids in flax can be produced by anther culture (Chen et al. 2003). All methods require the use of tissue culture techniques, precise growing conditions, specialized technical skills, and considerable time and effort. The efficiency of doubled haploid production will ultimately determine its success. There are large genotypic effects on response to anther culture and the populations derived from anther culture may not represent an unbiased sample of parental gametes which is a disadvantage in conducting genetic studies. The use of doubled haploids would save the breeding program 2–3 years in developing new cultivars and increase cultivar uniformity. The complete homozygosity of doubled haploid lines results in greater genetic uniformity of new cultivars and makes screening for genes of interest more reliable. The capability to produce doubled haploids would allow new cultivars to be developed more rapidly in

response to changing market demands, new *niche* markets and disease problems. It provides ideal material to study the genetics of important traits such as disease resistance and quality, and to develop molecular markers to facilitate marker assisted selection when it becomes feasible. While the use of doubled haploids has the potential to speed up and increase the efficiency of plant breeding program, it is more costly to implement, uses more growth facility space, and requires more highly trained technical support than conventional plant breeding methods. As a result, the breeder may not be able to select among as many lines per cross in as many crosses, which may reduce the probability of success. It may also be more difficult to break unfavorable linkages using doubled haploids since there is less chance for recombination.

7.7 Summary

Canada leads the world in flax production and exports. Today, the unique properties of flax differentiate it from other oilseeds and a new spectrum of food, animal feed and industrial uses is expanding markets and adding value to the crop. As demands of the industrial and functional food arenas change, both nationally and internationally, further improvements in flax production and bioproduct quality will need to occur in order to capture the market. The flax breeding programs contribute to the increasing and sustaining flax production by developing varieties and germplasm with improvements in yield but also by reducing the risk of abiotic and biotic stresses through the incorporation of genetic resistance. Further enhancing the competitiveness of the cultivars and germplasm is the genetic improvement of oil and meal quality along with the reduction in anti-nutritional substances. This will create new opportunities for cultivars with specific end uses which are environmentally friendly and safe.

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Chapter 8
Cotton

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8.1 Introduction

In the 19th century, uses emerged for cottonseed left over from ginning harvested cotton (Altschul et al. 1958). Oil from cottonseed was determined to be useful in a range of food products, and cotton is now considered second only to soybean in the value of its oil products. Seed of the cotton plant (*Gossypium* spp.) generally has an oil content of around 21% and protein content near 23%. The fatty acid profile of cottonseed includes about 55% polyunsaturated fatty acids, 18% monounsaturated fatty acids, and 27% saturated fatty acids (Lukonge et al. 2007; National Cottonseed Products Association 2007). Use of cottonseed has been hampered because it contains gossypol, which is a toxic compound. Nevertheless, ruminant animals can digest gossypol in limited quantities, and it can be removed during oil crushing processes. Cottonseed oil has good stability as cooking oil and can withstand high temperatures without deterioration. Because of the stability of cottonseed oil, partial hydrogenation is not necessary, which enables manufacturing of many non-transfat products. Moreover, cottonseed is a very popular feedstock for ruminant animals such as cattle. Cottonseed value as a protein source for dairy cattle milk production is equivalent to that of other high quality oil seeds such as canola (*Brassica napus*) and soybean (*Glycine max*) (Sanchez and Claypool 1983; Brito and Broderick 2007), and can be an economically lucrative alternative feedstock in areas where it is available (Blackwelder et al. 1998). While the crude protein level of cottonseed is relatively high for a livestock feed, value of the protein is limited by low levels of the amino acids methionine, threonine, valine, and most critically lysine (Nagalakshmi et al. 2007).

Despite the extra profit from the sale of cottonseed and the emergence of new markets for cottonseed, historical production practices have been focused almost exclusively on fiber yield and quality because cottonseed is typically

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only worth about 12% of the crop's value (Elam 1995). There is an economic disconnect between producers and incentives for higher quality seed. Producers are paid for their cottonseed based on volume with little regard for quality parameters. When prices for seed go up or down due to market value and/or seed quality, economic consequences are spread across all producers who use a common gin and not to a specific producer or even less so to a particular field of cotton.

New market opportunities for cottonseed appear to be rapidly developing. Cottonseed is a renewable fuel alternative to fossil fuels, and there is surging interest in cottonseed oil for use as a biodiesel (Karaosmanoglu et al. 1999; He and Bao 2005; Putun et al. 2006; Royon et al. 2006; Smith 2007). In addition, new opportunities for food use are now possible because of recent biotechnological advances in producing gossypol-free seed (Sunilkumar et al. 2006; Townsend and Llewellyn 2007). The process currently used to remove gossypol from cottonseed damages protein value (Freidman 1996). If seed can be produced without gossypol, then protein value of cottonseed should inevitably improve.

8.2 Origin and Domestication

Cotton is historically referred to four species of the genus *Gossypium*: *G. hirsutum, G. barbadense, G. arboreum* and *G. herbaceum*, all of which produce long fibers on seeds, suitable for spinning and weaving into fabrics. These four species are cultivated between 37°N and 32°S latitude worldwide. The genus *Gossypium* includes 44 recognized diploid ($2n = 26$) and five tetraploid ($2n = 52$) (Fryxell 1992; Percival et al. 1999) species in drier tropical and subtropical regions worldwide. Plants of this genus all produce perfect, often highly visible flowers that are normally self-compatible (Fig. 8.1). Cross-pollination is limited to insect vectors. Most species are perennial shrubs or sub-shrubs adapted to flower and set seed according to native photoperiod and rain seasons. This genus is distinguished from other genera in the *Malvaceae* family by distribution of gossypol-producing glands throughout plant tissues.

The genus is thought to have originated 10–20 million years ago when continents were closer in proximity. Breakup of the continents divided *Gossypium* into easily recognized groups of species and genomes that subsequently evolved independently. In Australia, 18 species exist and are recognized by the C, G and K genomes. In Asia and Africa, 13 species are found of the A, B, E and F genomes. In Central and South America, 13 species of the D genome and five species of the tetraploid AD genome are found. These genome types were assigned by cytological observations of chromosome size, structure, and behavior in meiosis with intra- and interspecific hybrids. Cytogenetic and molecular evidence suggests that long ago a progenitor of each of the A and D genome

Fig. 8.1 The cotton flower is a perfect flower having all male and female parts. Cross-pollination generally is facilitated by bees, otherwise, the flowers will self-pollinate

species united to form a tetraploid AD genome (Wendel and Cronn 2003) that subsequently evolved into the five tetraploid species known today.

Two A genome diploid species (*G. arboreum* and *G. herbaceum*) and 2 tetraploid species (*G. hirsutum* and *G. barbadense*) have been domesticated for thousands of years in the Old and New World, respectively. Considerable introgression and overlapping either within or between the new and old species has occurred because of cultivation and manipulation by man. These four species have become adapted to both feral and disturbed habitats and have changed from perennial, often photoperiodic plants, to day-neutral annuals.

Popularity of these four species is attributed to seed fibers that are highly elongated and to convoluted single cells that are mostly cellulose crystals at maturity. Other species produce extremely poor fiber and, in some cases, no fiber at all. The wealth of habitats and morphological diversity of undomesticated species still provides ample genetic diversity for cotton taxonomic studies and breeding.

8.2.1 Taxonomy

Early classification of cotton by Todaro (1877) and Watt (1907) applied many taxonomic divisions to the morphological diversity in what is now known by

just four species. Zaitzev (1928) was among the first to recognize separate diploid and tetraploid species in the genus. Many subspecies were still applied to these species and now are recognized as discrete lineages within each species having distinct morphological and agronomic characters.

G. herbaceum has been described into five forms: *acerifolium, africanum, kuljianum, persicum*, and *wightianum* (Hutchinson 1963; Brubaker et al. 1999). Only var. *africanum* is recognized by taxonomists (Fryxell 1992) and considered to truly exist in the wild. It is the most variable form and is found in southern Africa. *Acerifolium* is the most primitive cultivated form that still grows as a perennial in areas of Northern Africa and adjacent Arabia. The three other forms are annuals in their environment: *kuljianum* in western China, *persicum* in Iraq and the Levant, and *wightianum* in western penninsular India. *G. arboreum* has been described into four mostly perennial forms (*burmanicum, cernuum, indicum* and *soudanense*) and two annual forms (*bengalense* and *sinense*). *Burmanicum* and *cernuum* originated in Northeastern India. *Indicum* is the most primitive form and is found in western penninsular India and eastern Africa. *Soudanense* grows to tree size in northwestern Africa and Sudan. *Bengalense* originates in Northern India and *sinense* originates in China, and both are areas of extremely early maturity. China, India, and Pakistan still maintain germplasm of these diploids that are valuable sources of trait improvement for Upland cotton cultivated in arid environments.

G. barbadense originated in northwestern South America, but it has been cultivated throughout South America and Central America. Originally, *G. barbadense* was classified into three forms: *barbadense, brasilense* and *darwinii*. The 'moco' or kidney cottons of Brazil were commonly associated with *brasilense* but later were recognized as just a local cultivar of *G. barbadense* not worthy of subspecies status. *Darwinii* was later classified separately as another species of tetraploid cotton, *G. darwinii*. 'Tanguis' cotton varieties of Peru and 'Sea Island' types from the Caribbean and southeastern US are common early types of *G. barbadense*. Muhammed Ali Pasha developed the foundation of modern Egyptian cotton varieties by combining Sea Island varieties with other strains of *G. barbadense*. The boll weevil (*Anthomonous grandis*, Boheman) ended the *G. barbadense* industry in the southeast US because these cottons were extremely susceptible to this pest. Despite a brief revival in that area, *G. barbadense* cottons were soon relegated to Arizona and California. The USDA started a breeding program in Arizona with *G. barbadense* and Egyptian cottons to develop 'Pima' cottons. These became known as extra long staple (ELS) cottons and now occupy a small, high quality cotton niche in the global cotton market.

The first efforts to classify *G. hirsutum* led to a large array of taxonomic subdivisions. As late as 1963, Hutchinson still recognized seven forms of *G. hirsutum*: *latifolium, marie-galante, morrilli, palmeri, punctatum, richmondii*, and *yucatanense*. Later taxonomists would disregard these subspecies names, but their morphology and geographical distribution are still distinguishable by these names and may hold clues to ancient cultivation and distribution by

peoples in Central and South America. *Marie-galante* is strongly arborescent and originated on the west coasts of southern Central America and northern South America. It was likely distributed by natives and/or explorers to the West Indies and northwestern South America. *Punctatum* describes smaller, bushier *G. hirsutum* cotton found along the coasts of eastern Mexico, Florida, Yucatan, and the Bahamas. It was discovered early by explorers and brought to West African colonies where it naturalized and contributed to cotton breeding for bacterial blight disease resistance. *Morrilli* describes *G. hirsutum* with bushy growth, large and intensely hairy leaves that grows in the present day Mexican States of Oaxaca, Puebla, and Morelos. The state of Guerrero contains *G. hirsutum* form *palmeri*, with deeply dissected and glabrous (i.e., okra) leaves, pyramidal growth, and strong anthocyanin production. In a restricted area of northern Yucatan, *G. hirsutum* of the form *yucatanense* grows wild, whereas most other forms usually grow in association with humans or in disturbed areas. In the Mexican state of Chiapas and regions of Guatemala, *G. hirsutum* form *latifolium* is found. These forms bear the most fruit in their first season, and thus they are easily treated as annuals in cultivation. Most of the early improvements in US cotton varieties depended on introductions of *G. hirsutum* form *latifolium*.

8.2.2 Domestication

Archaeological evidence dates domestication at least as early as 2,000–3,000 BC for *G. arboreum* in regions of India (Gulati and Turner 1928) and for *G. barbadense* in Peru (Stephens 1958). Each of the four cotton producing species are thought to have been cultivated independently in their original habitats and altered by human selection during cultivation and distribution to new habitats (Hutchinson et al. 1947; Hutchinson 1951). The toxicity of gossypol in seeds suggests that cotton was primarily grown for fiber rather than food.

It is difficult to truly determine wild habitats or progenitors of these four species due to extensive cultivation by man. For example, *G. barbadense* is considered to have originated in northwest South America, but forms have been found throughout South and Central America. In Brazil, local cotton with the distinct characteristics of large leaves and kidney shaped seeds appears to be a subspecies of *G. barbadense*, but it was selected for this growth habit to facilitate easier harvest and removal of fibers. Conquests by Alexander the Great helped reveal the existence of cotton fabrics in India to the rest of the Old World. Continued development of trade routes to India prompted cotton to be cultivated throughout the Old World and also increased demand for products produced with cotton fibers. Great Britain strongly influenced distribution and demand for cotton. The British established the Empire Cotton Growing Company which sponsored cotton

cultivation and improvement in areas they colonized (e.g. Sudan, Uganda, and the West Indies). In Trinidad and Sudan, a large collection was established of cotton cultivars and species collected by the Empire Cotton Growing Company. Continued domestication of cotton adapted these four species to regions outside their natural tropical habitats and even to more temperate northern or southern latitudes.

European settlement of the Americas led to discovery and dispersal of tetraploid species of cotton. Likely all four species were grown in the US, but soon it became apparent the tetraploid species were superior in yield and quality to the two Old World diploids (Watt 1907). In colonial North America, early cotton culture was focused on limited cultivation for local uses. In mild coastal climates of Georgia and the Carolinas, *G. barbadense* cultivars from the Caribbean were cultivated and called Sea Island cotton. These cultivars were likely derived from prior cultivation in the West Indies and produced very high quality fiber but with limited yields. They were poorly adapted to other mainland areas. Britain was among the few countries with a significant industry manufacturing cotton products and needed to import cotton. British merchants valued the high quality lint of *G. barbadense*. *G. hirsutum* was cultivated further inland, throughout the Southeast and as far west as Texas, thus earning the name 'Upland' cotton. 'Georgia Green Seed' and 'Creole Black Seed' were two early varieties of Upland cotton planted in the South and Southeast. Creole Black Seed was introduced from Siam into Louisiana by the French and was easy to gin. Georgia Green Seed was introduced from the West Indies and had better yields (Moore 1956). After the Revolutionary War, cotton was valued as an export crop, but it was difficult to produce and process the quantities needed for export. Invention of the Whitney Gin in 1793 was among many improvements to make cotton production more profitable in the US. Development of cotton in the US would have a large impact on the growth of the cotton industry and cultivation worldwide.

In 1806, Walter Burling, a planter from Natchez, Mississippi, successfully smuggled seed of valuable cotton germplasm out of Mexico City. This germplasm and progeny resulting from its hybridization with Creole and Green Seed was the first step to improving Upland cotton in the US. Better quality fiber, earlier maturity, and resistance to diseases were noticed in the newly created cultivars (Wailes 1854). Burling's Mexican cotton was grown in the Mexican Highlands and exhibited earliness and an annualized growth habit, which distinguished it from most *G. hirsutum*. It likely represented significant progress by Mexicans or their fore bearers to adapt *G. hirsutum* to cultivation. Local reselection within this novel germplasm enabled adaptation and improvement of Upland cotton in the US. Early cotton breeders practiced visual selection for lint quality and quantity and eventually learned to select the best individual plants (Moore 1956). Large, easily picked bolls with long fibers were emphasized in selection of cottonseed for local cultivation. In time, four general types of cotton came into use in the United States, and they were named

according to their general geographical areas of production: 'Eastern Big Boll,' 'Delta,' 'High Plains' and 'Western Acala' (Ware 1951; Smith et al. 1999).

Emergence of pests and diseases would shape further domestication of cotton in the United States. Infestation by the boll weevil in the late 1800s prompted a large scale change in cotton germplasm and practices. Left unchecked, the boll weevil could easily render most US cotton fields completely barren. Vital cooperation among federal, state, and private institutions helped to combat the boll weevil and preserve the cotton industry. Early maturing cotton crops were the best strategy for coping with the boll weevil at this time. Lint yield and fiber quality were sacrificed in order to produce a harvestable crop before boll weevil pestilence became too great to produce a profit. Due to increased boll weevil pressure later in the growing season, public and private cotton breeding programs shifted to developing early-maturing germplasm. The federal government sponsored plant explorations into Mexico, the natural habitat of *G. hirsutum*, in the hopes of finding host plant resistance to boll weevil and potential for cotton breeding improvement. Substantial genetic resistance to the boll weevil was never identified, but Burling's original Mexican Stock and other Mexican cottons were imported to the US to improve earliness, yield, and harvestability of cotton varieties.

Reduction in cotton production from the Civil War and boll weevil outbreaks gave impetus to other nations to increase cotton cultivation to meet worldwide demand for cotton (May and Lege 1999). These countries without a doubt benefited from Upland cotton varieties produced in the US and from varieties and species distributed worldwide. Upland cotton production became established in Africa, Southeast Asia, Egypt, South America, portions of the Mediterranean and the Middle East. The former USSR had already explored Central America for cottons to augment their cotton breeding programs (Mauer 1930). India, China, Pakistan, and other countries were replacing Old World and obsolete tetraploid varieties with Upland varieties developed in the US and elsewhere. Many of these countries benefited from increased earliness in Upland cottons in order to expand cotton production to temperate areas. With an emerging global cotton market and improved ginning and spinning technologies, the US cotton production industry is now faced with the need to increase the quality and productivity of its cotton.

8.3 Varietal Groups

G. hirsutum cultivars comprise over 90% of the cotton grown worldwide, followed by *G. barbadense*, and to a very limited extent, *G. arboreum* and *G. herbaceum* in remote localities of Asia and Africa. As stated earlier, Upland cotton (*G. hirsutum*) grown in the US can be broadly divided into four groups: Eastern Big Boll, Delta, High Plains, and Western Acala (Ware 1951; Smith et al. 1999). Eastern Big Boll reflects an early emphasis on large bolls in the

east-southeast part of the Cotton Belt. Delta cottons often were grown in the fertile alluvial soils in the Mississippi River floodplain and sported high-yielding, high-quality crops. Due to the long growing season and therefore large plant sizes often obtained in this region, more emphasis could be placed on yield. High Plains cottons are characterized by more compact growth habits with stormproof bolls that retain cotton fibers to prevent losses due to wind or rain and by adaptation to partial or no irrigation. Stripper harvest machinery was engineered to remove cotton from the stormproof bolls. Western Acala cottons were developed from high-quality cottons collected from the Acala region of Chiapas, Mexico. Acala cottons are primarily grown in Arizona, California, New Mexico, and western Texas. They are well-adapted to long, dry summers and irrigation practices. Fiber quality of these cottons command a premium compared to most other Upland cottons. California became noted for mandating a one-variety law for Acala cottons in order to distinguish themselves from other cotton producing states. This action secured a premium for all California growers. Over time, historical distinction among cotton variety groups became blurred with the advance of the boll weevil, which made it necessary to grow early-maturing varieties in both the eastern and the southern regions of the Cotton Belt. Pedigrees of these four groups became more intertwined with additional needs for disease resistance and uniform fiber quality. Bacterial blight, Fusarium and Verticillium wilts, nematodes, etc. would affect large areas of the Cotton Belt and require simultaneous improvement of much of the regional germplasm base. Also, formation of national and global seed companies restricted some local genetic diversity but also integrated novel gene sources (i.e. recombinant DNA technology) into their cultivars (Stewart 1995).

Interspecific hybrids have demonstrated potential for combining the high yield of *G. hirstum* and the quality of *G. barbadense*. Additional labor for manual pollinations and/or development of a male sterility trait necessary to facilitate production of hybrid seed has prevented widespread adoption of commercial hybrid seed production in the US. However, in India and China, cheaper labor and integration with value-added recombinant DNA traits (transgenic technology) has allowed the prospects of hybrid cotton to remain positive (Barwale et al. 2004; Dong et al. 2004).

8.4 Genetic Resources

Cotton germplasm of the four cultivated species were enhanced in their natural ranges long ago by human cultivation (Hutchinson 1963). A mix of economically acceptable and wild forms still exists for three of these species in their natural tropical ranges (Brubaker et al. 1999). Only cultivated forms of *G. arboreum* have been found. The earliest known collection of cotton was established by J.P.B. von Rohr in the late 18th century under a commission

from the Danish King. He maintained a garden of cottons from the Caribbean and South America at St. Croix in the Danish West Indies (now the Virgin Islands) (Rohr 1791–1793). In the mid 19th century, the Italian botanists, Parlatore and Todaro, assembled a collection of cotton (Todaro 1877). The first major contributor to genetic resource accumulation and disbursement was the former Empire Cotton Growing Company, chartered by Britain to grow cotton in areas settled outside the US. This company introduced cotton to Africa, the Middle East, and Australia, and established germplasm collections in Trinidad and Sudan. Upon the closing of this company, the Trinidad collection was dispersed and the Sudan collection was maintained by the Sudanese Department of Agriculture. A number of major cotton collections exist worldwide that each contains thousands of accessions of cultivated and wild species of cotton. Some of these major collections are located in the following countries: China, the former USSR (Vavilov Institute of Research, Tashkent and Uzbekistan), India (Central Institute for Cotton Research in Nagpur), Pakistan (Cotton Research Institutes of Multan and Sakrand District), France (Institute de Recherches du Coton et des Textiles Exotiques), and the US. The US Cotton Germplasm Collection is the largest single collection of cultivated and wild cotton species worldwide (Percival et al. 1999). This collection contains over 9,300 accessions of 46 species of *Gossypium* from 101 countries or political jurisdictions and from a range of latitudes from 40°N to 40°S. Even remote tropical areas, such as the Galapagos Islands, harbor valuable germplasm and continued exploration into these regions is justified. The collection historically has been grouped into seven sub-collections:

1. *Variety Collection*: over 3,100 accessions of obsolete or publicly donated *G. hirsutum* cultivars; originally established by the Delta Branch Experiment Station in Stoneville, Mississippi; accessions are catalogued with the prefix 'SA';
2. *Primitive Race Collection*: over 2,100 accessions of wild or primitive races of *G. hirsutum;* created at the Texas A&M University Experiment Station in College Station, Texas, from plant explorations and germplasm exchanges with other collections; accessions are prefixed by 'TX';
3. *G. barbadense Collection*: over 1,500 accessions of primitive germplasm, obsolete and current cultivars; originally established at the Cotton Research Center in Phoenix, Arizona; accessions are prefixed by 'GB';
4. *Asiatic Collection*: includes accessions of *G. herbaceum* (194 accessions, 'A1' prefix) and *G. arboreum* (1,729 accessions, 'A2' prefix); established at College Station from germplasm exchanges;
5. *Wild Species Collection*: over 500 accessions representing 42 species of wild diploid and tetraploid *Gossypium* species; established at College Station from explorations, exchanges and donations; accessions are prefixed by the genome designation of the species;
6. *Genetic Marker Collection*: includes *G. hirsutum* and *G. barbadense* lines of special interest; and

7. *Base Collection*: required backup collection that includes permanent storage of all materials listed above and any new Plant Introductions; all materials are maintained at the National Center for Genetic Resources Preservation (NCGRP) in Fort Collins, CO.

Access to the US collection is freely available worldwide to persons with a *bona fide* interest in cotton. In 2006 alone, seed of 4,160 accessions were distributed to 105 researchers and institutions worldwide. Seeds are stored at 4°C and 23% relative humidity to prolong seed viability. Seed propagation routinely is performed on a subset of the collection to increase the seed viability and quantity available for distribution. A longstanding arrangement between the USDA, the National Cotton Council, and INIFAP (the Mexican agricultural ministry) established a cotton winter nursery in Mexico for mass seed propagation of the germplasm collection. This location is ideal for seed and fiber production, especially of photoperiodic or slow maturing accessions. Other species are intensively cared for in greenhouses to generate the necessary seed. Descriptor, fiber quality, and agronomic evaluation data on accessions in the collection is shared with researchers to assist in reviewing and requesting seed of cotton germplasm.

In 1946, 'A Memorandum of Understanding' was formalized by 11 State Agricultural Experiment Stations (SAESs) and the USDA (Cotton Division). This established yearly meetings to arrange research priorities in cotton. One major outcome was the development of germplasm resources for cotton. The station at Stoneville, MS, was assigned the task of accumulating obsolete cultivars of *G. hirsutum*, while the station in College Station, TX, was assigned to maintain *G. hirsutum* and other species collected from the wild. The station at Phoenix, AZ, focused on *G. barbadense* accessions. Beginning in 1960, collections along with their characterization data were deposited at NCGRP (formerly the National Seed Storage Laboratory). The International Board for Plant Genetic Resources established a set of cotton descriptors in 1980. All these collections were combined in 1985 at the USDA-ARS facility in College Station, TX. This collection focused worldwide with emphasis on germplasm exchange and conservation, while still fulfilling the mission of maintaining the germplasm for use in cotton improvement.

This germplasm resource is utilized extensively with worldwide requests for germplasm and numerous trait evaluations and introgressions into improved cotton germplasm. Tri-species hybrid crosses were made to introduce increased fiber strength into Upland cotton (Fryxell 1976). Resistance to bacterial blight (Brinkerhoff 1970), nematodes (Robinson and Percival 1997), pyramiding of multiadversity resistance (El-Zik and Thaxton 1989), development of male sterility for hybrid production (Meyer 1975) and evolutionary studies (Wendel and Cronn 2003) are just a few of the accomplishments made possible with this germplasm resource.

8.5 Major Breeding Achievements

Whether improving yield, fiber quality, or reducing the cost of production, increasing profit per acre has been the ultimate objective for cotton breeders. In 1900, average cotton fiber yield in the US was 195 lbs. per acre (Ware 1951); by 2005, it was 831 lbs. per acre (Meyer et al. 2006). While not all the gain can be attributed to genetic improvement, certainly cotton breeding programs deserve much credit. Meredith and Wells (1989) reported that in comparing modern cultivars to antiquated cultivars, newer germplasm is capable of higher yields because of greater partitioning of dry matter to reproductive structures and away from vegetation. Certainly, specific improvements in cotton cultivar yield beyond a more favorable ratio of fruiting to vegetative tissue have led to this dramatic improvement. Areas of improvement include host plant resistance, abiotic stress tolerance, agronomic adaptation, improved fiber quality, and enhanced seed traits.

8.5.1 Host Plant Resistance

Several insect, nematode, and disease maladies afflict cotton. Perhaps none is as damaging as the boll weevil. Its invasion of the US Cotton Belt at the end of the 19th century financially ruined much of the cotton industry. The boll weevil created a societal upheaval and prompted scientists to begin searching for host plant resistance to this pest (Jones 2006). While no cotton germplasm provided substantial genetic resistance, earlier maturing varieties were developed that provided some temporal resistance to the boll weevils (Walker 1979). By developing cotton varieties that produced a greater portion of their fruit before the 2nd and 3rd generations of post-diapause boll weevils could reproduce, an economically acceptable cotton cultivar could be grown in many areas of the US Cotton Belt.

In the 1960s, efforts to identify mechanisms of resistance to other insect pests were sought. Several biochemical and morphological traits associated with cotton host plant resistance were identified with varying degrees of usefulness (El-Zik and Thaxton 1989). Butler and Henneberry (1984) determined that fewer leaf trichomes resulted in greater cotton host plant resistance to the whitefly (*Bemisia tabaci*). Increased tannins (Lege et al. 1992) and gossypol (Bottger and Patana 1966), as well as glabrousness (Lukefahr et al. 1971) and the nectariless trait (Butler et al. 1972) all reduced *Heliothis* spp. damage. Schuster et al. (1976) reported that plants without floral nectaries also were found to be less attractive to plant bugs (*Lygus hesperus)*. The okra-leaf trait confers resistance to pink bollworms (*Pectinophora gossypiella*) (Wilson et al. 1986), and it is a valuable means of improving pesticide penetration into the lower cotton canopy (Fig. 8.2).

Fig. 8.2 Upland cotton leaf shapes include the normal shape on the *left*, the okra-leaf shape is on the *right*, and in the *middle* is the heterozygous intermediate-shape

Considerable breeding efforts have been directed towards improving nematode and disease resistance of cotton germplasm. R.L. Shepherd (1979) outlined methodology for screening germplasm for resistance to root-knot nematodes (*Meloidogyne incognita*) as well as developing several resistant cotton lines. Improving resistance to Verticillium wilt has been a major goal of several breeding programs throughout the world. In areas where *Verticillium* is prevalent, breeders often establish highly inoculated nurseries to screen early-generation lines. Occasionally, breeders are even capable of developing host plant resistance to poorly defined diseases. In 1995, a new and devastating disease, Bronze wilt, afflicted hundreds of thousands of US acres (Bell et al. 2002). Although Koch's postulates were never satisfied, most breeders realized a strong relationship between cotton varieties and disease incidence. Consequently, breeders soon purged much of their breeding material that was susceptible to Bronze wilt. No major outbreak of the disease has been reported since 2000. Breeders have successfully used host plant resistance against other cotton diseases including bacterial blight (*Xanthomonas campestris*), Fusarium wilt, reniform nematodes (*Rotylenchulus reniformis*), blue disease, and boll rot.

8.5.2 Abiotic Stress Tolerance

Soil moisture deficits generally are the most yield-limiting stress factor for cotton. In certain growing areas, other abiotic stresses are of major

concern,including excessive salt and extremely high or low temperatures. Abiotic stress screening usually entails subjecting early-generation lines to extreme drought, heat, or cold conditions; and then selecting the most productive plants to advance in breeding programs. Often, all advanced lines in a program are tested under drought conditions to evaluate performance. Heat tolerance generally is identified in plants by rating pollen production. Breeding programs approach screening for heat tolerance at different stages of cultivar development. Some emphasize screening early-generation lines that are still segregating, while others wait to identify tolerance or sensitivity until later generations. Seed increases are grown in extreme high-temperature areas of Arizona to help identify tolerance or sensitivity. Cool temperature stress typically receives little attention during early stages of development. High levels of cold tolerance are often associated with larger seed size and tolerance to seedling disease complexes. For salt tolerance, usually minor efforts are made for selection and improvement. Gossett et al. (1994) indicated varietal differences between New Mexico State University's Acala varieties and those varieties developed in the Mid-South, which suggests serendipitous enhancements for salt tolerance due to the New Mexico breeding program environment.

8.5.3 Agronomic Adaptation

Early maturity can enhance yield and reduce the risk of late-season pest damage and aberrant weather in areas with a short growing season. Conversely, late-maturing varieties can improve drought tolerance (Dumka et al. 2004). In latitudes closer to the equator, later-maturing varieties also can exploit more heat units from the growing season, recover from fruit shedding, and translate that opportunity into greater yield potential than more determinant fruiting varieties.

The ideal boll type depends on location. On the High Plains of Texas and Oklahoma where strong windstorms frequently occur, a tight storm-proof boll is preferred. Necessity for such a boll, however, has decreased with the adoption of chemical harvest aids, which shorten exposure time between defoliation and harvest. In the Mid-South and Southeast, growers prefer 'looser' bolls, which are less prone to hard-locking and will 'fluff-out.' This type of boll can be easily pulled out of the burr by mechanical pickers, which results in greater harvesting efficiency and higher quality fiber with less trash. In addition, bolls need to open easily in the presence of low sunlight and high humidity, which are common in these regions during the harvest season (Fig. 8.3).

Fig. 8.3 A fully mature cotton plant has open bolls that are 'fluffed-out' and ready to harvest

8.5.4 Fiber Quality

Because cotton's ultimate value is as a fiber crop, much attention has been given to improving fiber traits. Fiber quality is more heritable than yield potential and can affect crop quality in the most environmentally diverse and challenging conditions (Krieg 2002). Fiber quality has dramatically improved in almost all US production regions. By the early 1960s, high-volume instrument (HVI) testing was introduced and changed the way cotton fiber quality was measured. With this advent, breeders could affordably evaluate fiber from individual plants and breeding lines, and then assign absolute and objective numbers to fiber properties. This technological innovation helped make rapid improvements in fiber traits.

Concomitant with HVI development, new strategies were developed to break negative linkages among fiber properties and yield (Miller and Rawlings 1967; Meredith and Bridge 1971; Culp and Harrell 1973). Fiber length and strength typically were characteristics most often targeted for improvement because of their importance to spinning quality, quantification by HVI, and the existence of alleles for fiber quality improvement. Today, many breeders employ sophisticated crossing schemes designed to break linkages between yield and fiber qualities. Other fiber properties such as

micronaire, elongation, length uniformity, fineness, and maturity also garner attention from breeders. Because of the low cost and accuracy of fiber analysis, even on a single plant basis, most lines are evaluated during every generation of development.

8.5.5 Seed Traits

Minor breeding attention has been given to improving seed quality because seed represents only a small fraction of a cotton crop's total value. Seed size typically has been a trait receiving the most attention by breeders because small seed size can result in poor seedling vigor (Quisenberry and Gipson 1974). More importantly, small seeds can cause problems at the gin by increasing seed coat fragments and neps (Bargeron and Gardner 1991). Cotton breeders monitor seed size through a seed index, which is the weight of 100-fuzzy seeds. Smaller seed size is often associated with a greater lint-to-seed ratio, which is an important yield component. Therefore, a tendency exists among breeders to opt for smaller seed size as a means to improve yield.

A major devaluating characteristic of cottonseed is gossypol, a toxic secondary metabolite. Gossypol makes oil extraction and utilization more expensive and less competitive with other oil crops such as soybeans and canola. The first cotton variety with glandless seed was described by McMichael (1959). Less than two decades later, several commercial glandless varieties were available (Halloin et al. 1978), but none of these lines was very successful because of pest damage and lack of value returned to growers.

8.6 Current Goals of Breeding

Breeding goals vary depending on organizations and their missions. Cotton breeding programs typically fall into three categories: private, state or provincial, and federal. In the United States, private seed companies focus on short-term, high-value objectives. State university programs address regional problems with development of germplasm and varieties. Federal US Department of Agriculture (USDA) breeding programs conduct long-term improvement programs and more basic research projects that have a broad, national scope.

8.6.1 USDA

Early on, the national interests of cotton were promoted by the USDA with creation of the Division of Cotton and other Fiber Crops and Diseases. USDA began sponsoring germplasm exploration trips in the early 1900s to the centers of cotton diversity. These efforts resulted in nearly 1,000 accessions added to the

national collection (Percival and Kohel 1990). Later, USDA breeding stations were established to address the need for cotton varieties with greater yield and better fiber qualities in Shafter, CA; Las Cruces, NM; Florence, SC; and Sacaton, AZ. Once fiber quality was improved; however, maintaining the high quality properties of these cottons after ginning became a problem. In response, USDA created uniform testing procedures to class cotton fiber as well as classing offices to assist plant breeders in predicting spinning performances of breeding lines.

In 1943, the USDA-Agricultural Research Service (USDA-ARS) became the dominant agency to conduct crop research within the USDA organization. USDA-ARS plays a major role in cotton improvement along with plant introduction, exploration, and germplasm maintenance activities. Through multistate research activities, the USDA-ARS, universities, and other stakeholders coordinate activities to evaluate and develop cotton germplasm and genetic resources the industry can use to develop cultivars. Some of the accomplishments and ongoing efforts include:

- Release of over 100 breeding lines, often in concert with universities, with enhanced fiber properties (CCGC 2005).
- Development of cytogenetic stocks useful as molecular breeding tools and sources of diverse and valuable alleles.
- Creation of a saturated genetic map whereby genes associated with traits can be identified in the genome and tagged for possible selection (Frelichowski et al. 2006; Ulloa et al. 2007).
- Release of lines converted from photoperiodic landraces into day neutral forms.
- Introgression of Upland cotton and *G. barbadense* to produce substitution lines (Saha et al. 2006), aneuploid stocks for genetic mapping, and improving yield and quality of both species (Percy et al. 2006).
- Evaluation and breeding for resistance to nematodes and *Fusarium* wilt.
- Continued expeditions into Mexico to explore for additional *Gossypium* species.
- Negotiations with Chinese and Uzbekistan authorities for access to at least 1,000 accessions from their respective cotton collections.
- Evaluation of cultivated and exotic breeding lines for resistances to insects and heat stresses.
- Investigations of variability of cottonseed properties and sources of improvement (Stipanovic et al. 2005).

8.6.2 State Universities

The establishment of land grant universities subsequent to the Morrill Acts of 1862 and 1890 began the involvement of universities in cotton research. State universities disseminate information about agricultural advances to producers,

apply the scientific method and innovation to solve problems related to cotton, and educate and train the future labor force to support the cotton industry. State Agricultural Experiment Stations (SAES) are branches of US university systems. Phenotypic cotton breeding programs of SAES exist in New Mexico, Texas, Louisiana, Arkansas, Mississippi, Georgia, and North Carolina. Private cotton breeders conduct research into traits they deem to be the most profitable for the companies they represent (i.e. yield, fiber quality, transgenic herbicide and insect resistance). Public breeders generally cannot compete in this arena because of the resources available to private breeders; therefore, public breeders embrace research into characteristics with less industry-wide, but more regional importance, while developing breeding lines and cultivars with acceptable values for yield and fiber quality measures. Reviews of cotton breeding research activities and objectives were documented by Bowman (1999) and Calhoun and Bowman (1999).

Some breeding programs concentrate on identifying useful traits in obsolete and wild cottons accessed from germplasm collections. Traits being evaluated in breeding programs include insect and disease resistance and tolerance to environmental stresses including drought, salt, and cold temperatures (Basal et al. 2005). Such traits could be novel sources of variation for breeders to use in developing new cultivars. Other programs focus on enhancing germplasm to improve yield and fiber characteristics (i.e. improved fiber length and strength, reduced micronaire and short fiber content, etc.). Some breeding programs release cultivars while others release unique germplasm useful to other public and private breeders. One of the most understated roles of the SAES breeding programs is training students to become plant breeders.

State and federal institutions play a vital role in developing technologies and germplasm resources that have been adopted by private companies. These companies have assumed an increasingly larger role in cotton breeding world-wide through the further enhancement of these resources to generate savings, profits, or greater utility for the cotton industry (Heisey et al. 2001).

8.6.3 Private Companies

Since most cotton acreage in the lucrative markets of the United States, Australia, Brazil, and Argentina are planted to transgenic cotton cultivars, the primary focus of commercial seed companies is to provide a mechanism of delivering technology to cotton growers and capturing value from seed technology as revenue from chemical technology decreases. Most transgenic cotton in the United States has a herbicide-resistant transgene and a transgene for producing the proteins of *Bacillus thuringiensis* (Bt) (Table 8.1; USDA 2006). Current commercial cotton planting seed markets have injected tremendous profits for seed companies capable of delivering transgenic technology. More than ever, speed of delivering new and improved cotton cultivars with transgenic

Table 8.1 Upland cotton acreage planted to genetically engineered (GE) varieties planted by state and across the United States, 2000–2007 (USDA-NASS estimates)

	Insect-resistant (Bt) only								Herbicide-tolerant only							
State	2000	2001	2002	2003	2004	2005	2006	2007	2000	2001	2002	2003	2004	2005	2006	2007
	Percent of all upland cotton planted															
Alabama[†]						10	10	10						28	25	25
Arkansas	33	21	27	24	34	42	28	32	23	29	37	25	15	12	21	16
California	3	11	6	9	6	8	9	4	17	27	26	27	39	40	40	51
Georgia	18	13	8	14	13	29	19	17	32	43	55	32	23	11	13	10
Louisiana	37	30	27	30	26	21	13	17	13	14	9	15	7	10	13	11
Mississippi	29	10	19	15	16	14	7	16	13	15	22	16	23	23	22	19
Missouri[†]						20	32	13						59	40	63
North Carolina	11	9	14	16	18	17	19	13	29	37	27	29	27	24	19	16
Tennessee[†]						13	16	10						8	10	17
Texas	7	8	7	8	10	14	18	16	33	35	40	39	40	35	34	36
Other States[‡]	17	18	19	18	22	18	21	27	21	33	35	32	24	26	24	20
US	15	13	13	14	16	18	18	17	26	32	36	32	30	27	26	28
	Stacked gene varieties								All GE varieties							
Alabama[†]						54	60	60						92	95	95
Arkansas	14	28	26	46	45	42	45	47	70	78	90	95	94	96	94	95
California	4	2	1	3	7	5	8	6	24	40	33	39	52	53	57	61
Georgia	32	29	30	47	58	55	64	68	82	85	93	93	94	95	96	95
Louisiana	30	47	49	46	60	64	68	68	80	91	85	91	93	95	94	96
Mississippi	36	61	47	61	58	59	69	62	78	86	88	92	97	96	98	97
Missouri[†]						16	25	23						95	97	99
North Carolina	36	38	45	48	46	54	60	64	76	84	86	93	91	95	98	93
Tennessee[†]						75	67	71						96	93	98
Texas	6	6	4	6	8	14	18	28	46	49	51	53	58	63	70	80
Other States[‡]	36	33	32	38	45	46	45	42	74	84	86	88	91	88	90	89
US	20	24	22	27	30	34	39	42	61	69	71	73	76	79	83	87

[†] Estimates published individually beginning in 2005.

[‡] Includes all other states growing Upland cotton.

technology is imperative to the profitability of private seed companies. Consequently, great resources are made available to commercial breeding programs to hasten development of transgenic cultivars.

The next generation of commercial transgenes will likely be for additional insect pest control, abiotic stress tolerance, and unique fiber properties. Aside from transgenic traits, commercial breeding objectives are improving lint yield, fiber properties, and agronomic adaptability. Little regard is given to seed qualities. Private companies strive to develop cultivars that are adapted to the broadest possible range in an effort to limit their product portfolio and simplify seed production operations.

8.7 Breeding Methods and Techniques

Breeding methodology is standard among programs whether they are public or private. The greatest differences between public and private programs generally are the greater size and scope of private programs and their use of transgenic technologies. Nevertheless, basic techniques of creating genetic variability, selection practices, and evaluation procedures are similar. Cotton is an autogamous crop, and breeding methods used in cotton are variations on or combinations of methods normally utilized with self-pollinating species (Fehr 1991).

Early generations of breeders employed mass selection within a heterogeneous population to improve cotton. With this technique, the breeder selected individual plants of the desired phenotype, and sowed seeds of these plants for the next generation. Then the breeder again selected individual plants with the desired phenotype. This selection cycle was repeated to increase the percentage of desirable genotypes while forming a homozygous population.

Most cotton breeders today use a pedigree method of breeding, first described by Newman (1912), or slight derivations thereof. After initial hybridization, counter-season nurseries, especially for first filial increases, are frequently used to hasten development. Individual plants are selected in second filial or later generations after additive genetic effects are expressed. Initial testing of selected lines is done in well-controlled, high-yield potential environments where the greatest expression of alleles contributing to yield and fiber traits is possible. As testing continues on advanced generation lines, trials tend to include more diverse growing environments to determine performance stability. The entire process generally takes at least ten years from an initial cross-pollination to a finished cotton cultivar.

A yield plateau was thought to have been reached in the late 1990s due to several factors. Chief among these factors was a lack of genetic diversity caused in large part to, as Meredith (2002) suggested, breeding efforts directed toward added value traits. Genetic diversity is an important component for advancing breeding populations of cotton germplasm. Commercial plant breeders are

pressed to quickly release high-performing varieties. Consequently, private breeders tend to use more in-house elite germplasm with a narrow genetic base in comparison to public cotton breeders (Bowman 2000). Commercial cotton breeding programs are becoming more global in nature. Major cottonseed companies such as those owned by Monsanto, Bayer, and Dow recently established cotton breeding stations around the world. These operations are now exchanging breeding lines, which will enhance diversity and increase opportunities for quantitative genetic improvement. In addition, their extensive testing networks allow great insight into performance capabilities of elite germplasm.

The cost of commercial cotton breeding has risen dramatically in recent years due to transgenic technology. Concomitantly, return on investment has risen as well. In some cases, the amount a US cotton grower pays for transgenic cottonseed is 2000% more than they were paying for conventional cottonseed in the early 1990s. This has had profound effects on the cottonseed industry. Most seed companies are now owned by large chemical companies willing to invest in biotechnology to offset losses from chemical sales. Because almost 90% of cottonseed sold in the United States contains transgenic technology (USDA 2006) and much of the rest of the world is rapidly adopting transgenic cotton planting seed (Table 8.2); commercial seed companies focus heavily on integrating the latest transgenic traits into elite lines.

The most common breeding procedure used to develop transgenic cultivars is the backcross method. This technique involves mating a donor parent with the transgenic trait of interest to a recurrent parent with the most agronomic traits of interest. Breeders are provided donor plants with the transgene of interest. To date, all commercial transgenes for cotton are independently segregating, dominantly inherited genes. Breeders are also given tools to identify the

Table 8.2 Global acres (millions of hectares) of transgenic cotton (Bt and Bt/Herbicide Tolerance) grown from 1996 to 2006

Year	Bt	Bt and herbicide	Total
1996	0.8	0.0	0.8
1997	1.1	<0.1	1.1
1998	1.4	0.1	1.5
1999	1.3	0.8	2.1
2000	1.5	1.7	3.2
2001	1.9	2.4	4.3
2002	2.4	2.2	4.6
2003	3.1	2.6	5.7
2004	4.5	3.0	7.5
2005	4.9	3.6	8.5
2006	8.0	4.1	12.1

Source: International Service for the Acquisition of Agri-Biotech Applications (ISAAA 2006). http://www.isaaa.org/RESOURCES/PUBLICATIONS/BRIEFS/35/pptslides/default.html

transgene event in the way of Southern Blot assays, polymerase chain reaction (PCR) technology, and/or lateral flow strips. This enables breeders to identify the transgene throughout the breeding process. With PCR technology, identification of homozygous plants is possible, too. Generally two to three backcrosses are made to a non-transgenic line with superior production potential. Sometimes transgenic sister-lines are performance tested, and the best lines are bulked together to form the new transgenic cultivar. Other times, all available backcrossed plants are bulked together to create the cultivar. In the later scenario, it is unlikely that genetic improvement beyond the recurrent parent can be made. From the initial hybridization with a transgenic donor plant to commercial sales, the process generally takes six years (Table 8.3). Genetic gain during this process is affected by how many plants can be backcrossed and how many sister-lines can be tested during preliminary and advanced trials. Breeding costs are further driven up for strict quality control measures involved with transgenic breeding. Consequently, transgenic breeding is much more expensive and less quantitative genetic gain is accumulated in comparison to strictly conventional cotton breeding procedures.

Table 8.3 Timeline for commercial transgenic cotton variety development using the backcross procedure

Year	Activity
1	Backcrossing three generations per year if greenhouses or counter-season nurseries are used.
2	Final backcross and isolation of homozygous transgenic plants.
3	Seed increase and preliminary yield trials.
4	Seed increase and advanced yield trials.
5	Seed increase and preliminary commercial level testing.
6	Seed sales.

8.8 Integration of New Biotechnologies in Breeding Programs

New biotechnological approaches to transferring genes can complement conventional plant breeding programs (Jauhar 2006). A new era of biotechnology in cotton breeding has emerged since the first transgenic commercial cotton cultivars were released in 1996. Genes conferring resistance to herbicides that kill a wide range of weeds and genes for resistance to *Lepidopteran* insects in cotton are being utilized primarily by private sector cotton breeders. These genes were inserted into cotton cultivars using biotechnologies including tissue culture, *Agrobacterium*-mediated transformation, and/or biolistic transformation. While using these techniques to develop transgenic cultivars, unique molecular sequences of DNA (i.e. RFLPs, AFLPs, RAPDs, SSRs, SNPs, etc.) have been

identified. These molecular markers potentially are useful in marker-assisted selection as tools to increase the efficiency of conventional cotton breeding. Publicly available information about cotton molecular research and other resources is continuously updated by two databases, CMD (Cotton Microsatellite Database; http://www.cottonmarker.org/; Blenda et al. 2006) and CottonDB (http://cottondb.org/; Yu et al. 2006).

Tools and techniques of biotechnology can be aimed at improving cotton in four areas: (1) decrease the time needed to develop cultivars (marker-assisted selection); (2) genetically modify agronomic characteristics (herbicide and insect resistance; abiotic stress tolerance; fiber traits); (3) genetically modify fatty acid profiles (alter type and proportion of fatty acids); and (4) genetically modify cottonseed meal quality (remove gossypol to extend the use of cottonseed to human consumption).

Marker-assisted selection allows breeders to improve efficiency by identifying lines fixed for a given trait early in the line development process. Only plants carrying the trait of interest are carried forward. Plants without the trait of interest can be discarded immediately rather than expending time and resources for phenotypic evaluations. This selection strategy may be useful when introgressing traits from a related species (Lacape et al. 2003). Zhang et al. (2003) tested the efficiency of marker-assisted selection to detect quantitative trait loci (QTL) associated with increased fiber strength. The SSR marker $FSS1_{130}$ efficiently detected the homozygous genotype associated with a major QTL for fiber strength in three segregating populations with the cotton line 7235 as a parent. The potential of large-scale use of SSR technology is being evaluated in a diallel study utilizing additional parental lines (Hinze et al. 2007).

Most new biotechnologies to modify agronomic traits in cotton are being utilized in the breeding programs of private companies. The first transgenic cotton technology, BXN, was sold in 1995. This allowed plants to be tolerant to normally lethal doses of bromoxynil herbicides. The following year more transgenic cotton technologies resistant to the herbicide Roundup® and *Lepidoptera* insects were released by Monsanto. Insecticide and herbicide resistance are still the only two transgenic traits marketed as of 2007. New areas of transgenic research by companies include drought and stress resistance/tolerance, salt tolerance, and improved fiber quality. Public institutions continue to explore biotech solutions to agronomic problems that are less financially lucrative, but important to stakeholders. Cotton plants transformed with an endochitinase gene from the *Trichoderma* fungus have significant resistance to *Rhizoctonia solani* and *Alternaria alternate* (Emani et al. 2003). Tolerance to *Thielaviopsis basicola* has also been identified in transgenic seedlings expressing a synthetic antimicrobial peptide (Rajasekaran et al. 2005).

There is ongoing research into the pathways of lipid synthesis and development of transgenic plants to modify the fatty-acid profile of cottonseed oil. Oleic acid in cottonseed has been increased by suppressing a Δ-12 fatty acid desaturase (FAD2) using a mutant allele and inserting this construct into cotton plants using *Agrobacterium*-mediated transformation (Chapman et al. 2001).

Other groups have attempted to increase seed oleic acid by antisense suppression of FAD2 (Liu et al. 2002; Sunilkumar et al. 2005). Successful research efforts in producing such transformants have not yet been adapted to commercial cultivars.

Pigment glands are located throughout the cotton plant. These glands contain gossypol and gossypol precursors that are the greatest quality detriment to cottonseed products. Cotton breeders have been trying for decades to eliminate the antinutrient gossypol (Muramato 1969; Dilday 1986; Altman et al. 1987; Vroh et al. 1999). In the 1950s, breeders discovered a mutant that completely lacked the compound, but the gossypol-less plant was vulnerable to pests and a commercial failure. To target seeds while keeping defenses intact in vegetative tissue, molecular biologists began to examine metabolic pathways of gossypol formation. Attempts to eliminate gossypol in seeds via antisense suppression of (+) δ-cadinene synthase gene methods have been unsuccessful (Townsend et al. 2005) or have reduced gossypol, but not completely eliminated it (Martin et al. 2003). Most recently, researchers have achieved a reduction of gossypol in cottonseed using RNAi to disrupt the gossypol biosynthesis pathway, specifically by interfering with expression of the δ-cadinene synthase gene during seed development (Sunilkumar et al. 2006). Another approach for eliminating gossypol is removal of glands that store gossypol in seeds. This approach is directed toward the dominant Gl_2^e gene which controls glandless expression (Kohel and Lee 1984). Research has been initiated to clone the Gl_2^e gene and link it in antisense with a seed-specific promoter so that no glands occur in seeds while they remain unaltered in the rest of the cotton plant (Yu et al. 2000a,b; Decanini et al. 2001; Kohel et al. 2001).

8.9 Seed Production

High-quality seed production can only occur when cotton bolls are fully mature and carefully harvested, ginned, and stored. Seeds produced during the early fruiting stage that are fully mature have a higher protein content, a more favorable saturated to non-saturated fatty acid ratio, a higher seed weight, and a greater oil content than seeds from later, less mature fruiting structures (Conkerton et al. 1993; El-Nockrashy et al. 1976). Generally, plants managed for high lint production are more likely to produce high-quality seed. Poor plant nutrition, low-light quality, and drought stress can each reduce seed weight and quality. High rates of nitrogen and excessive soil moisture during late-season also can be detrimental to seed quality by promoting more late maturing bolls. Moreover, delays in harvest that expose open cotton bolls to moisture is very damaging to seed quality. Excessive moisture elevates free fatty acid accumulation which is also associated with seed embryo mortality (Hoffpauir et al. 1947). Therefore, timely harvests are crucial to securing the highest quality seed.

Insect pests, particularly pink bollworms (*Pectinophora gossypiella* Saunders) and stinkbugs (*Euschistus* spp.) can be very damaging to seed quality. In addition, cottonseed can become infected with *Aspergillus flavus* and contain lethal amounts of mycotoxins. Cottonseed infected with *A. flavus* usually coincides with seed damaged by pink bollworms. Users of cottonseed should regularly test for such contamination. Bt cotton offers excellent protection against the pink bollworm, and compositional analysis of Bollgard II® cottonseed, a Bt cotton, showed no significant differences in composition to seed from other non-transgenic cotton cultivars (Hamilton et al. 2004).

Ginning and storage are important in maintaining seed quality. Gin machinery should be adjusted so that minimal damage to seed coats is incurred. Ginning practices that chip seed coats promote disease and premature degradation of the kernel. Storage begins when seed cotton is harvested and packed into modules for transporting to gins. After ginning, seed is stored at the gin and later at oil mills or by whole cottonseed users such as dairies. During all storage steps, airflow is important to maintain low humidity and reduce heat accumulation. These conditions reduce free fatty acid formation and improve the end-use value of cottonseed.

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Chapter 9
Peanut

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9.1 Introduction

Peanut is one of the world's major sources of vegetable oil. According to United States Department of Agriculture (USDA) databases, peanut is fifth worldwide in vegetable oil production among nine major oilseed crops (http://www.fas.usda.gov/psdonline/psdHome.aspx). Although peanut is widely viewed as an oilseed crop, utilization of peanuts varies greatly from country to country. In some countries, the majority of production is crushed for oil, whereas in others such as the United States (US), peanuts are used primarily for food.

Peanut is grown on every continent, but the majority of production occurs in Asia, Africa, South America, and North America. During the five-year period 1996–2000, China, India, and the United States accounted for almost 70% of the total annual peanut production globally (Rovoredo and Fletcher 2002). By country, China accounted for about 39%, India about 25% and the US about 6% of total production. Average pod yield ranged from 0.43 t/ha in Africa to 3.54 t/ha in North America (world average is 1.35 t/ha) (Dwivedi et al. 2007). In the US, pod yield increased dramatically in the early 1970s, but only slowly during more recent years (Fig. 9.1). More than 90% of the crop is grown in developing countries and the majority of the seeds are consumed in the country of origin. This helps to explain why world trade of peanuts is relatively small. Historically, only about 5% of peanuts enter the export market, with only three countries accounting for the majority of these exports.

In the United States, peanut is utilized primarily as a snack food. Industries that process peanuts and manufacture peanut products require that new cultivars deviate from the historical type as little as possible. This constrains genetic gain for seed size and other agronomic traits because corporations that shell and process peanuts for food have developed sophisticated manufacturing techniques and equipment based on a tight range of standards. New cultivars

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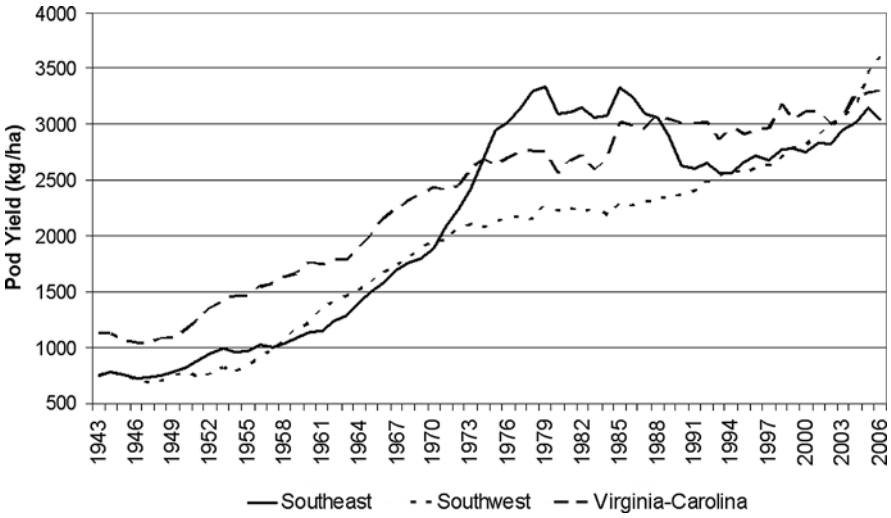

Fig. 9.1 Five year running average of peanut pod yield in the three primary growing regions of the US. Legend details: Southeast – Alabama, Florida and Georgia; Southwest – New Mexico, Oklahoma and Texas; Virginia-Carolina – North Carolina, South Carolina, and Virginia Source: United States Department of Agriculture.

that do not meet seed industry expectations are less likely to be accepted even though they may have significant benefits for farmers.

Peanut breeding goals are largely dictated by crop utilization. Although a majority of world production is crushed for oil, peanut is also heavily utilized as a food crop. In the 5-year period 1996–2000, nine of the 15 major peanut producing countries crushed more than 55% of their crop for oil whereas six used more than 60% for food (Rovoredo and Fletcher 2002). Due to the wide variation in utilization among countries and regions, peanut breeding goals may greatly differ. However, some goals such as increasing disease resistance and yield are common to all breeding programs. Abiotic (drought) and biotic (mostly fungal and viral diseases) stresses are the major constraints on world production, and are the focus of nearly all peanut breeding programs.

The domesticated peanut is plagued by many disease and insect pests, with early leaf spot (*Cercospora arachidicola* Hori), late leaf spot (*Cercosporidium personatum* (Berk. & M.A. Curtis) Deighton), rust (*Puccinia arachidis* Speg.), and viruses being widespread wherever the crop is grown. Many other problems are regional in nature, with Sclerotinia blight (*Sclerotinia minor* Jagger), white mold or stem rot (*Sclerotium rolfsii* Sacc.), Cylindrocladium black rot (*Cylindrocladium crotalariae* (Loos) Bell and Sobers), nematodes (*Meloidogyne* spp.), and tomato spotted wilt virus (TSWV) being among the most important diseases in the US. In addition, aflatoxin (caused by *Aspergillus* spp.) is a major industry problem that is predominately solved by testing seed samples from farmer's fields at buying stations and removing contaminated lots from the

edible market. Allergens also are a major problem for the industry, and because they are found in the major seed storage proteins, all peanut products (except purified oils) will cause allergic reactions to susceptible individuals. Much of the domesticated peanut collection has been screened for resistance to the leaf spots and rust (see Holbrook and Stalker 2003 for review), and although moderate levels of resistance have been identified, extremely high levels of resistance apparently do not exist in the germplasm collection. Variation has also been observed for reaction to viruses, such as TSWV, but the resistance is due to unknown effects of the environment in the field rather than to the virus per se (see Holbrook and Stalker 2003). Resistances to many of the most important disease and insect pathogens of peanut have been identified in other species of the genus, including members of section *Arachis* (to which the domesticated peanut belongs), and thus the potential for genetic improvement of *A. hypogaea* through interspecific hybridization exists (Stalker and Simpson 1995).

Although peanut originated in South America, these countries produce only a small fraction of the world's crop. Argentina is the largest peanut producer in South America, followed by Brazil. South America is rich in peanut genetic resources and both wild and cultivated genotypes from that continent have contributed to improved cultivars in the United States and elsewhere.

9.2 Origin and Domestication

Peanut is a low-growing annual or perennial plant that is distinguished from most other species by producing aerial flowers, but fruiting below the soil level. *Arachis* belongs to the family *Fabaceae*, tribe *Aeschynnomeneae*, subtribe *Stylanthinae*. *Arachis hypogaea* L. is the only domesticated species in the genus, and Krapovickas (1969) concluded that *A. hypogaea* var. *hypogaea* is likely the most ancient type because it has similar branching patterns as wild species, no compound florets, and a prostrate growth habit. The primary center of diversity for the species is the Chaco region between southern Bolivia and northwest Argentina (Gregory and Gregory 1979). The peanut is now grown in most tropical to subtropical regions of the world, with more than 90% of the world's production in Asia and Africa. Peanut seeds contain 36–54% oil, 16–36% protein, and 10–20% carbohydrates (Knauft and Ozias-Akins 1995). It also is a good source of minerals (Ca, Mg, P, and K) and vitamins E, K, and B_1 (Savage and Keenan 1994). At least two additional species have been cultivated for their edible seeds (*A. villosulicarpa* Hoehne and *A. stenosperma* Krapov. and W.C. Gregory), a few species are cultivated as a forage (*A. glabrata* Benth. and *A. pintoi* Krapov. and W.C. Gregory), and *A. repens* Handro is grown as a ground cover for lawns in Central and South America (Stalker and Simpson 1995).

The center of origin for the genus *Arachis* is the Mato Grosso area of Brazil, but species evolved over a wide range of habitats in South America (Gregory et al. 1980). Molecular data indicate that the center of genetic variation also is

the Mato Grosso area of Brazil to eastern Bolivia (Stalker et al. 1994). Because the most ancient species are believed to have tuberoid roots, tuberiform hypocotyls, or rhizomes, the first species are thought to be from highland areas, with subsequent distribution by water to lower elevations. Eighty species have been described (Krapovickas and Gregory 1994; Valls and Simpson 2005) which have been divided in to nine sections based on morphology and cross-compatibility relationships. Based on cross-compatibility data, Smartt and Stalker (1982) and Stalker (1991) concluded that genomic groups have evolved in the genus which mostly follow sectional designations (Am – *Ambinervosae*, T – *Triseminalae*, C – *Caulorhizae*, EX – *Extranervosae*, and E – *Erectoides*, R – *Rhizomatosae*, and A, B and D – *Arachis*). Smartt and Stalker (1982) also proposed that the A and B genomes of section *Arachis* may be an A_1 and A_2 rather than being truly different based on chromosome pairing relationships. The species of different sections have overlapping distributions in many areas. Hybrids between species in different sections are difficult to produce and are usually sterile, while intrasectional hybrids can be fertile if they have similar genomic make-up (Stalker et al. 1991). Most species in the genus are diploid ($2n = 2x = 20$), but tetraploids ($2n = 4x = 40$) exist in sections *Arachis* and *Rhizomatosae*, and several species in section *Arachis* are aneuploid ($2n = 2x = 18$) (Lavia 2000). Polyploidy is believed to have evolved independently in sections *Arachis* and *Rhizomatosae* (Smartt and Stalker 1982), and likely independently more than once in section *Rhizomatosae* (Nelson et al. 2006).

The domesticated peanut (*A. hypogaea*) evolved from two diploid species of section *Arachis* approximately 3500 years ago in the southern Bolivia to northern Argentina region of South America (Gregory et al. 1973). Secondary centers of diversity developed in South America and tertiary centers in Africa (Smartt and Stalker 1982). Domesticated peanut is taxonomically a member of section *Arachis*, along with 25 other diploid ($2n = 2x = 20$), four aneuploid ($2n = 2x = 18$) and one tetraploid ($2n = 4x = 40$) species. Species in this section have evolved into three genomic groups, with most species having an A genome, six species having a B genome, and *A. glandulifera* Stalker a D genome (Holbrook and Stalker 2003; Valls and Simpson 2005). The domesticated peanut is believed to be an allopolyploid with AABB genomes because it has only one small chromosome pair in somatic cells and most meiotic cells have 20 bivalents, although multivalents can occur. Kochert et al. (1991) supported this conclusion because they observed multiple bands on RFLP gels for *A. hypogaea*, but only single bands for diploid species. Many species have been proposed as possible progenitors, but the most likely candidates are *A. duranensis* Krapov. and W.C. Gregory (A-genome) and *A. ipaensis* Krapov. and W.C. Gregory (B-genome) (Kochert et al. 1996). *Arachis duranensis* is believed to be the female parent of the ancestral hybrid based on analyses of cytoplasmic genes (Hilu and Stalker 1995).

As compared to wild species of the genus, domestication of *A. hypogaea* led to an upright growth habit, shorter branches, suppressed hypanthium length, stronger and shorter pegs, and pods with the internode between seeds

suppressed (Stalker and Simpson 1995). The most ancient *A. hypogaea* types have alternating inflorescences, main stems without flowers, and flowers that are simple (vs. compound inflorescences), prostrate growth habits, are late maturing, with hairy leaves, 2-seeded pods with a beak, small seeds with a long dormancy period, and long lateral branches (Stalker and Simpson 1995). The species *A. hypogaea* has two subspecies and six botanical varieties (Table 9.1). The subspecies are separated morphologically based on presence or absence of flowers on the main stem and regularly alternating vegetative and reproductive nodes on branches. Although accessions of *A. hypogaea* are morphologically variable, relatively little molecular variation has been observed. This led Kochert et al. (1996) to conclude that there has been little introgression from related species into the domesticated peanut since its origin. In addition to morphological and genetic variation,

Table 9.1 Subspecies and varieties of *A. hypogaea*[1]

Variety	Market type	S.A. location	Characteristics
Subspecies *hypogaea*			
hypogea		Bolivia, Amazon	No floral axes on main stem; alternating pairs of floral and reproductive axes on branches; branches short; less hairy
	Virgina		Less hairy; large seeded
	Runner		Less hairy; small seeded
hirsuta	Peruvian runner	Peru	More hairy
Subspecis *fastigiata*			
fastigiata			Floral axes on main stem; alternating pairs of floral and vegetative axes on branches
	Valencia	Brazil Guaranian Goias Minas Gerais Paraguay Peru Uruguay	Little branched; curved branches
peruviana		Peru N.W. Bolivia	Less hairy, deep pod reticulation
aequtoriana		Ecuador	Very hairy; deep pod reticulation; purple stems; more branched; erect
vulgaris	Spanish	Brazil Goias Minas Gerais Paraguay Uruguay	More branched; upright branches

[1] After Stalker and Simpson (1995).

Stalker and Dalmacio (1986) reported at least five translocation types in different varieties of *A. hypogaea*.

9.3 Varietal Groups

Two botanical groups of cultivated peanut (*A. hypogaea*) are defined for the presence or absence of flowers on the main stem (Knauft et al. 1987). The sub-species *A. hypogaea* subsp. *hypogaea* flowers only on the lateral branches whereas *A. hypogaea* subsp. *fastigiata* flowers on both the lateral branches and main stem. Two varieties are defined within each of these sub-species; var. *hypogaea* and *hirsuta* are of sub-species *hypogaea* and *fastigiata* and *vulgaris* are of sub-species *fastigiata* (Simpson and Coffelt 1997).

Similar to the botanical classifications, peanut is divided into market types which have economic consequences in the United States. The market types called 'runner' and 'virginia' are within variety *hypogaea*. The 'valencia' market type is in the variety *fastigiata* and the 'spanish' market types are within the *vulgaris* variety. There are no commercial types of the variety *hirsuta* grown in the United States, but plant introductions from Mexico have proven valuable in breeding programs for their resistance to TSWV.

A pedigree analysis of the cultivars grown in the US shows that most of them have ancestors belonging to both *A. hypogaea* subspecies (Isleib and Wynne 1992). Plant introductions have been of great importance in peanut, in large part for resistance to Sclerotinia blight, root-knot nematode, and TSWV (Isleib et al. 2001).

9.3.1 Market Types in the United States

Of the four market types of peanut (runner, virginia, spanish, and valencia) produced in the US, runner and virginia types are the most widely grown. Both usually have two seeds per pod and the USDA has created standards that define these two market types on the basis of pod width. Pods that are wider than 13.5 mm are defined as 'virginia' pods. Genotypes that have less than 40% 'virginia' pods are classified as runner market types and those with at least 40% are defined as virginia market types. The percentage of virginia pods can be greatly influenced by environment. Some genotypes can have more than 40% virginia pods in one environment and less than 40% in another, which would technically classify them as different market types. In reality, the United States industry definition of the acceptable range of pod and seed size for runner and virginia market types is stricter than the USDA definition. In addition to the percentage of virginia-type pods, seed size is important in both runner and virginia market types.

In the US, most commonly grown runner market type cultivars have between 10 and 20% virginia pods although the correlation between virginia pods and seed size is loose. There are three primary seed size classes for runner peanuts. Seed that ride a screen with 8.3 mm by 25.4 mm slots are called jumbo seed, those that fall through, but ride a screen with 7.1 mm by 19.1 mm slots are called medium seed and those that fall through both larger screens but ride a screen with 6.4 mm by 19.1 mm slots are called number one seed. Seed that fall through all three screens are called other kernels. Seed that split, but are otherwise normal, are called sound splits.

In the United States, the price that a farmer receives for a certain mass of runner type peanuts is adjusted based on the percentage of total sound mature kernels or 'grade' (TSMK). The TSMK is total percentage of the jumbo, medium, number one and sound split seeds resulting from shelling a certain mass of peanut pods (Davidson et al. 1982). Above about 74% TSMK, farmers receive a premium price, but the value is discounted below the threshold.

In addition to the runner and virginia types, two other market types are grown in the US. The spanish market type is characterized by small seed and early relative maturity when compared to the runner types. Along with runner types, spanish types are used primarily for peanut butter, oil and candy. The valencia market type peanut has more than two seeds per pod and is used primarily for in-shell roasting and boiling. Virginia types are used for in-shell roasting and salted or cocktail peanuts.

9.4 Genetic Resources

Several review articles have been published that summarize genetic resources of the domesticated peanut and related *Arachis* species (Isleib and Wynne 1992; Singh and Simpson 1994; Stalker and Simpson 1995; Dwivedi et al. 2003; and Holbrook and Stalker 2003), so only a brief review will be presented. The *A. hypogaea* accessions are maintained in large germplasm collections by the USDA (more than 8,000 accessions) (Holbrook 2001) and by the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), which has an international mandate for peanut improvement (14,966 accessions) (Upadhyaya et al. 2001). Duplication of accessions, either within or between the two collections, has not been addressed. Descriptors for peanut have been published by the International Board for Plant Genetic Resources (IBPGR) and ICRISAT (1992) and by the USDA (Pittman 1995). Germplasm preservation of the domesticated peanut is relatively straightforward because plants are self-pollinating (although out-crossing does occur), with the major constraint to long term storage being sufficient low temperature and humidity facilities because peanut is a large-seeded crop. Most of the germplasm collection at ICRISAT has been evaluated for biotic and abiotic stresses commonly found in the semi-arid tropics and the

information has been summarized by Dwivedi et al. (2007). Although large numbers of accessions have been evaluated for agronomically useful traits in the USDA and ICRISAT collections, relatively few accessions have been utilized by breeders for cultivar development in the US (Isleib et al. 2001) or at ICRISAT (Dwivedi et al. 2007).

Core collections were developed by Holbrook et al. (1993) who subdivided the larger US collection into 831 accessions based on six morphological traits. Upadhyaya et al. (2003) developed a second core collection consisting of 1,704 accessions from the ICRISAT collection. Upadhyaya et al. (2002) also developed a minicore consisting of 184 accessions based on agronomic and quality traits among the core accessions; and Holbrook and Dong (2003) later developed a second minicore with 111 entries in the US based upon morphological traits. Germplasm evaluations of the core accessions at ICRISAT identified accessions with early maturity (Upadhyaya et al. 2006), tolerance to low temperatures (Upadhyaya et al. 2001), and drought tolerance (Upadhyaya et al. 2005). Evaluations of US core collections have identified new sources of resistance for Cylindrocladium black rot and early leaf spot (Isleib et al. 1995), TSWV (Anderson et al. 1996), root-knot nematode (*M. arenaria* (Neal) Chitwood) and preharvest aflatoxin contamination (Holbrook et al. 1998), rhizoctonia limb rot (*Rhizoctonia solanii* Kuhn) (Franke et al. 1999), and Sclerotinia blight and pepper spot (*Lfeptosphaerulina crassiasca* (Sechet) Jackson and Bell) (Damicone et al. 2003).

In addition to *A. hypogaea* collections, more than 1,300 *Arachis* species accessions have been collected (Stalker et al. 2002c), with about 800 *Arachis* entries being maintained by the USDA (Stalker and Simpson 1995). Preservation of wild *Arachis* species is difficult for most accessions because the long, fragile pegs break during harvest and soil must be sifted to recover pods. Stalker and Simpson (1995) reported that nearly 25% of the species from which seed can be obtained under nursery conditions have fewer than 50 seeds in storage. Additionally, at least 25% of the *Arachis* species accessions in germplasm nurseries are maintained vegetatively because of their poor seed production under cultivation. A large number of disease, insect, and other agronomically useful traits are present in accessions of *Arachis* species, which makes them potentially valuable genetic resources for crop improvement (Stalker and Moss 1987; Stalker and Simpson 1995).

9.5 Major Breeding Achievements

Peanut breeding in the United States began in the late 1920s (Knauft et al. 1987; Gorbet 1999). Since then, peanut cultivars have changed and improved dramatically. Incremental improvements in cultivars have occurred over time, but several major achievements stand out, including release of a single dominant cultivar, changes in oil chemistry, and improved disease resistance.

9.5.1 Florunner Cultivar

In 1969, the cultivar Florunner was released by the University of Florida (Norden et al. 1969). Tests showed that the pod yield of Florunner was about 18% greater than Early Runner, the dominant runner cultivar of that time. By 1974, Florunner occupied more than 90% of the acreage in the southeastern US, and for almost 20 years it remained the dominant runner type cultivar by a large margin (Gorbet 1999). The seed, pod, and grading characteristics of Florunner were so good that it quickly became the standard, not only for peanut farmers, but also for shellers and manufacturers, and remains the standard against which shellers and manufacturers measure new cultivars. To a large extent, Florunner is the reason that the US industry has maintained high-quality products.

In the mid 1990s, TSWV became endemic in the Southeast and eventually eliminated production of Florunner and other susceptible cultivars. However, the cultivar Flavorunner 458 is a mutation of Florunner with elevated levels of oleic acid (Horn et al. 1997). It is virtually indistinguishable from Florunner otherwise and is widely grown in Texas where TSWV is not problematic.

Florunner was introduced into Argentina during the early 1980s and was grown on about 80% of their acreage for about 10 years. Later, a selection from Florunner called Florman was released as a cultivar in Argentina (Casanoves et al. 2005; J. Baldessari 2007, personal communication) and was grown on about 80% of the acreage by the late 1990s. Florunner has also been used as a parent in China. The cultivar Lu Hua 15 has Florunner in its ancestry and has been widely grown in Shandong Province, China (Xue and Isleib 2002).

Florunner had a dramatic and long lasting impact on the peanut industry in the US and Argentina. Twelve cultivars released in the US are direct descendants of Florunner (Isleib et al. 2001) and no other runner type cultivar has had such a significant impact on the peanut industry.

9.5.2 High Oleic Acid Content

The fatty acid profile of normal peanut is similar to other food nuts such as hazelnut, walnut, almond, and macadamia (Maguire et al. 2004). Compared to soybean, the major oilseed crop in the US, typical peanut oil has more oleic acid, less linoleic acid and no linolenic acid (White 2000). Partly because of its fatty acid profile, peanut oil is considered to be a premium oil and is desirable for cooking and salad oil (McWatters and Cherry 1982). Oleic and linoleic acids make up about 80% of the total fatty acids in peanut oil and are inversely correlated (Worthington and Hammons 1977). Peanut genotypes differ in the amount of each of these fatty acids, but most typical peanut cultivars contain 45–50% oleic acid and 30–40% linoleic acid. Prior to 1987, the highest level of oleic acid found in a peanut genotype was around 69% with a corresponding level of

linoleic acid of about 15% (Worthington and Hammons 1977; Treadwell et al. 1983). In 1987, Norden et al. reported discovery of two experimental peanut lines that contained almost 80% oleic acid and only 2% linoleic acid. Later work showed that the trait is conditioned by two recessive alleles (Moore and Knauft 1989). One of the alleles appears to be relatively common in runner and virginia germplasm, but the other is extremely rare (Knauft et al. 1993). Normal oleic acid content is incompletely dominant to the high oleic trait so that heterozygous genotypes can be distinguished from either of the homozygous genotypes (Isleib et al. 2006c).

The primary benefit of high oleic peanuts is dramatically improved product storage life. This has been demonstrated in whole peanuts as well as for peanut oil. A common measure of product stability is the peroxide value which is associated with the rancidity that causes off-flavors in peanuts and peanut products. In a study of roasted, in-shell virginia type peanuts, normal oleic acid peanuts reached an unacceptable peroxide value in only four weeks compared to 32 weeks for the high oleic peanuts (Mozingo et al. 2004). Similar results were obtained with peanuts that were salted in-shell. High oleic peanut oil also proved to be more resistant to oxidation as measured by peroxide values. Peroxide values of the normal oleic acid peanut oil began to rise rapidly after only 47 h of heating whereas the peroxide value of the high oleic peanut oil did not begin to rise until over 650 h (O'Keefe et al. 1993).

The first high oleic cultivar released in the US was a runner type named SunOleic 95R (Gorbet and Knauft 1997). Since that time, the trait has been incorporated into virginia and spanish types (Isleib et al. 2006b; Simpson et al. 2003a). Although several cultivars with the high oleic trait are in commercial production in the US, as of 2005 they occupied less than 20% of total acreage. Adoption of high oleic cultivars has been slowed partly because yield performance of high oleic cultivars lagged slightly behind the traditional types and because the market has had difficulty determining and/or capturing its economic value. Some manufacturers who have marketed high oleic peanuts exclusively have reported improved product performance and consumer acceptance. If high oleic cultivars become dominant in the marketplace it is likely that manufacturers will realize the benefits on a large scale and high oleic peanuts would become preferred over normal oleic peanuts.

9.5.3 Resistance to Leaf Spots, Root-Knot Nematode, and Spotted Wilt

In the Southeastern US, controlling diseases and nematodes in peanut accounts for about 20% of the total variable costs. In areas where pesticides are not available, or are too expensive for subsistence farmers to purchase, diseases and pests substantially reduce yield. Cultivar resistance has the potential to improve

production in situations of limited crop inputs and reduce costs where pesticides are used. The diseases that breeders target are sometimes localized to a particular environment, but there are diseases and pests that have widespread importance, such as leaf spots and root knot nematodes.

On a worldwide basis, leaf spot diseases caused by *Cercospora arachidicola* Hori and *Cercosporidium personatum* (Burk. & Curt.) are arguably the most widespread, devastating and costly to control of the many diseases that afflict peanut (Shokes and Culbreath 1997). Yield losses of 50% are common when fungicides are not applied, and losses can be 70% or more (Shokes and Culbreath 1997). Historically, control is achieved by application of fungicides because there were no cultivars with sufficient resistance. The first leaf spot resistant cultivar in the United States developed from the University of Florida program was Southern Runner (Gorbet et al. 1987). Its leaf spot resistance was derived from PI 203396. Since D.W. Gorbet at the University of Florida began an intensive effort to breed peanut for resistance to leaf spots, several cultivars with rate limiting, partial resistance have been released (Chiteka et al. 1988a,b). Research has shown that fungicide applications targeting leaf spot can be reduced by half on these cultivars with little or no reduction in pod yield and grade (Gorbet et al. 1990). The same study showed that pod yield of resistant genotypes was double that of susceptible lines when no fungicides were applied. These cultivars offer the potential to significantly reduce fungicide cost, but they have not been widely accepted by the industry. Seed quality has been an overarching problem with leaf spot-resistant lines and commercial success of cultivars with the highest levels of resistance has been limited (Morton 2007; Morton et al. 2006). In the future, utilization of the significantly higher levels of leaf spot resistance in *Arachis* species than found in *A. hypogaea* may aid peanut breeders in developing new cultivars. Germplasm lines have been released with very high levels of leaf spot resistance derived from *A. cardenasii* (Stalker et al. 2002b).

Similar to leaf spots, the root-knot nematode species that attack peanut (*Meloidogyne arenaria* (Neal) Chitwood, *M. hapla* Chitwood, and *M. javanica* (Treub.) Chitwood) are distributed worldwide (Kokalis-Burelle and Rodriguez-Kabana 1997). Yield losses in severely infested fields can be as high as 90%. Since root-knot nematodes are soil borne, rotation to a non-host crop can be an effective control measure. However, economics of rotational crops in some peanut growing areas prevent adequate crop rotation for many farmers. Continuous peanut culture has resulted in fields that are heavily populated by root-knot nematodes and a need for resistant cultivars has emerged. Moderate levels of *M. arenaria* resistance have been identified in *A. hypogaea* (Minton and Hammons 1975; Holbrook and Noe 1992) whereas several *Arachis* spp. have high levels of resistance to root-knot nematodes (Nelson et al. 1989; Holbrook and Noe 1990). Subsequent interspecific hybrids between cultivated peanut and accessions in section *Arachis* were highly resistant to *M. arenaria* and *M. javanica* (Nelson et al. 1990; Starr et al. 1990, 1995; Stalker et al. 2002a). Genetic control is believed to be monogenic and dominant in most germplasm, but a

second dominant gene has been reported (Choi et al. 1999). The cultivars COAN (Simpson and Starr 2001) and NemaTAM (Simpson et al. 2003b) contain the nematode resistance gene, but since they are essentially derived from Florunner, they have limited production because they are highly susceptible to TSWV. Another breeding line (C724-19-15 from the USDA in Georgia) that contains this gene for nematode resistance and has good TSWV resistance has been released as 'Tifguard' (C.C. Holbrook 2007, personal communication; Holbrook et al. 2007). Cultivars with both nematode and TSWV resistance would be beneficial to growers in the Southeastern US.

A third, very destructive disease in the US is spotted wilt caused by tomato spotted wilt virus (genus *Tospovirus*; family *Bunyaviridae*) (Culbreath et al. 2003). While several production factors have been shown to reduce the risk of crop loss from spotted wilt, cultivar resistance is the most important factor (Brown et al. 2005). When spotted wilt severity began to rise significantly in the southeastern US during the middle 1990s, all widely grown cultivars were very susceptible and spotted wilt caused significant crop losses in Texas and the Southeast (Culbreath et al. 2003). Researchers in Texas first discovered that the cultivar Southern Runner had some resistance to spotted wilt (Black 1991), which is thought to have come from PI 203396. The cultivar Georgia Green, a cross between Sunbelt Runner and Southern Runner, was released just before the height of the spotted wilt epidemic in the southeastern US (Branch 1996). It had inherited a moderate level of field resistance to spotted wilt from Southern Runner and quickly became the dominant runner market-type cultivar grown in the southeastern US. Without spotted wilt resistance in Georgia Green, the peanut industry in the Southeast would have been crippled.

9.6 Goals of Peanut Breeding

The goals of peanut breeding revolve largely around the requirements of the various 'customers' of peanuts and peanut products including farmers, shellers/seedsmen, manufacturers, and consumers. Specific goals have changed little since the reviews by Norden (1973), Knauft et al. (1987), Norden et al. (1982), Knauft and Ozias-Akins (1995), and Holbrook and Stalker (2003), so a brief summary will be presented in this chapter. Although some goals are common to most peanut breeding programs, the different market types of peanut have unique problems that must be addressed by peanut breeders at different institutions. Goals that are common to all market types include pod yield, market grade, high oleic oil, and disease resistance.

9.6.1 Goals for the Farmer

Peanut farmers provide much of the operational funding for peanut breeding in the US through state and federal government funding mechanisms and royalties

paid on seed sales. Therefore, much of the work in peanut breeding is focused to their needs. Peanuts are sold on a tonnage basis, so the per acre pod yield and grade (TSMK) are the two primary factors that determine crop value for the farmer. Therefore, all new cultivars must demonstrate improvement in one or both of those areas. Pod yield of peanut has risen significantly since the 1940s (Fig. 9.1). The contribution of breeding to pod yield improvement is significant, but few studies have documented it. The genetic contribution to yield in virginia market types was estimated to be 14.7 kg/ha/year from 1944 to 1985 (Mozingo et al. 1987), but similar studies have not been published for the other market types. However, the sharp increase in yields in the southeastern US can be partly attributed to the Florunner cultivar as described in Section 9.5.1. Tests showed that the yield of Florunner was about 18% greater than the dominant runner market type cultivars at the time of its release in 1969 (Norden et al. 1969).

In addition to pod yield and grade, resistance to diseases and pests is a breeding goal focused to the needs of the peanut farmer. In the US, peanut diseases vary regionally although some are common to all regions. Diseases of regional importance include white mold, Cylindrocladium black rot, spotted wilt, and Sclerotinia blight. Where these diseases are important, breeders are actively screening germplasm and breeding populations for resistance. Diseases and pests of national and international importance include leaf spots, root knot nematode and spotted wilt.

Breeders throughout the US and other parts of the world are working to develop peanut cultivars with resistance to the leaf spot diseases described in Section 9.5.3. Although cultivars with partial resistance to leaf spot are available in the US, production has been limited. The major limiting factor is seed vigor, which has been generally poor when these cultivars are grown and handled in the commercial seed chain (Morton et al. 2006). Unfortunately, the physiological or chemical cause of reduced seed vigor is not known. A second factor that limits production of leaf spot resistant cultivars is relative maturity. As a group, the leaf spot resistant cultivars are about 14 days later maturing than the cultivars that are most commonly grown. Producers prefer earlier maturing cultivars partly because of the logistics of planting and harvesting other crops planted in rotation with peanut and to avoid freeze damage to seeds. Similarly, the cost and risk associated with an additional two weeks that the crop is in the field limits their appeal.

9.6.2 Goals for the Seed Producer/Sheller

Commercial companies that purchase and shell peanuts are also the primary seed producers and marketers in the US. Their customers are both peanut farmers and companies that manufacture peanut products. As a seed producer and marketer, traits that are important to farmers are important to them as well. Cultivars with the best yield and grade are favored by peanut farmers.

Where disease limits either of these components, disease resistant cultivars become very important, for example, with spotted wilt when the cultivar Georgia Green was introduced (see Section 9.5.3).

As a peanut sheller, traits that affect the shelling process as well as those that affect the merchantability of the shelled peanuts are important. Peanut shelling companies want cultivars with uniform pod size that shell easily. Uniformity in pod and seed size minimizes segregation in storage warehouses and minimizes modifications in shelling machinery and processes. Cultivars with uniform seed and/or pod size are also desired by their other customers, the manufacturers of peanut products.

9.6.3 Goals for the Manufacturer and Consumer

In the US and other countries where peanut is used primarily for food, manufacturers and consumers demand high quality, nutritious peanuts and peanut products. In areas where peanut is used primarily for oil, traits needed for food use are not as important. Peanut flavor is cited by manufacturers as the most important attribute for peanut consumers. Traditionally, the runner and spanish market-types have had the best flavor profiles. Flavor is heritable, but estimates of broad sense heritability are relatively low at about 0.01–0.31 (Pattee et al. 1993; Isleib et al. 2006a). Due to the costly and time consuming process of taste panels evaluating flavor attributes, most breeders conduct flavor evaluations on only a few advanced breeding lines each year. This can lead to release of agronomically superior cultivars with slightly inferior flavor profiles (Isleib et al. 2000). However, breeding lines and cultivars with improvements in flavor attributes are being identified within all market types. Recently, researchers from North Carolina State University have identified virginia market-type breeding lines with flavor profiles similar to runner market-types (Pattee et al. 2007). One factor in maintaining flavor of peanut and peanut products is consistency of roasting. Because seed size contributes to roast variability, manufacturers have communicated their desire to have about 50% of the shelled peanut stock from runner market type cultivars in the medium category. If the raw peanut product is consistent, then the variability in the final roasted product should be more consistent as well. Interestingly, this requirement is not shared by all manufacturers. Peanut butter manufacturers are the primary benefactor of an abundance of medium sized seed. Because they utilize about 50% of the peanuts produced in the US, breeders of runner market-type peanuts pay close attention to the percentage of medium seed during the breeding process. Some manufacturers of confectionary products prefer the smaller spanish market-type seeds for candy bars. Others prefer a variable raw product so that the finished product does not look artificial. Still others want large virginia market-type peanuts or the jumbo seeds from runner market-types for whole roasted and salted peanuts. Virginia market-type peanuts are

primarily marketed in-shell. As such, the color and texture of the pod is important.

Manufacturers also want cultivars with high oleic acid content. As described in Section 9.5.2, high oleic peanuts offer several advantages over those with normal oleic acid. Among these advantages is extended shelf life. Since flavor is such an important attribute for peanut consumers, manufacturers realize that high oleic cultivars could help maintain peanut flavor and, therefore, enhance consumer acceptance.

9.7 Breeding Methods and Techniques

Breeding methods used by peanut breeders are those common to other self-pollinated crops and include pedigree selection, single seed descent, and mass selection. In the US, many breeders practice single seed descent whereas others use the pedigree method or a combination of both. Some cultivars have been developed by mutation such as Flavorunner 458 (Horn et al. 1997), but mutation breeding is not common. Breeders have employed backcrossing to introgress nematode resistance (Simpson et al. 2003a, b) and the high oleic trait (Isleib et al. 2006b). Crossing is usually conducted in greenhouses and general methods have been previously described (Norden 1980). Several reviews have been written that thoroughly describe peanut breeding methods (Knauft and Ozias-Akins 1995; Knauft et al. 1987; Norden et al. 1982; Norden 1973). Since the basic methodology has not changed or has changed very little, this section will focus on the unique aspects of peanut breeding that arise from the uniqueness of the peanut plant and peanut as a crop.

Unlike any other common oilseed crops, the peanut plant bears fruit underground. This phenomenon creates several challenges in the breeding process regardless of the breeder's preferred method of selection. Perhaps the most difficult problem is determining maturity. Because peanut has an indeterminate flowering habit and pods are underground, clues about seed maturity are not readily visible as the crop matures. With thousands of plots to harvest and hundreds of genotypes varying in maturity, precise prediction of optimum maturity is very difficult. The most practical technique for determining maturity is to scrape away the outer layer of the pod to reveal inner layers (Sanders et al. 1982; Knauft et al. 1987; Baldwin 1990). As the peanut pod matures, the subcutaneous layers change color from cream to orange, then brown and finally black. Most breeding programs use the pod scrape method to determine maturity of a few known genotypes and then set a harvest schedule accordingly. Testing every genotype is usually not feasible.

Another problem presented by the geocarpic nature of peanut is one of harvest logistics and high moisture levels. Whereas the harvest operation of most oilseed crops is a single step, peanut harvest requires several. First, the peanut plants must be dug from the soil. Since the plant is still living at the time

of digging, the green foliage must be allowed to dry for several days before combining, during which time pods and seeds also begin to lose moisture. Second, the pods must be threshed from the plants in an operation similar to the harvest of other typical oilseed crops, and then the seed is dried by forced, heated air. This harvest process requires considerably more manpower, time, and energy than harvest of other oilseed crops. Similar to other members of the pea (*Fabaceae*) family, peanut seeds are enclosed in a pod, but in the case of peanut, the seed are not separated from the pod in the harvest process. Seed must be separated from the pods during a shelling and cleaning operation after they are sold by the farmer. Because of the uniqueness of the peanut plant, some individuals have suggested that breeding peanut is more similar to breeding a vegetable crop than a traditional row crop like soybean, canola, or sunflower.

Another issue that makes breeding peanut a challenge is the size of the seed and the sowing density. Peanut seed ranges in size from one half gram per seed to over one gram per seed. There are about 1,500 seeds/kg of a typical runner market-type cultivar but this can vary considerably among cultivars. In the southeastern US, farmers plant about 125 kg/ha of runner market types and harvest about 2,100 kg/ha of seed for seed increase ratio of 15:1 to 20:1. In contrast, soybean seed are planted at about 73 kg/ha with a seed yield of around 2,700 kg/ha for a seed increase ratio of 30:1 to 40:1. Thus the seed increase ratio for peanut is about half that of soybean. Because of this, peanut breeders must devote considerable resources to producing seed for planting tests, especially tests planted at several locations.

9.8 Integration of New Biotechnologies into Breeding Programs

Molecular technologies have the potential for greatly increasing breeding efficiency of peanut, but marker systems are needed to enhance selection for desired traits. Because resistance to many of the disease and insect pests of peanut is difficult to select in the field or greenhouse, molecular technologies have the greatest potential for solving these problems. For example, selection for resistance to TSWV, Sclerotinia blight, white mold, Cylindrocladium black rot, and leaf spots could be greatly enhanced by utilizing marker technologies. In particular, molecular markers could allow breeders to select multiple genes when traits have complex inheritance and multiple mechanisms of host-pathogen resistance (for example in the leaf spots). To date, molecular technologies have played a minor role in peanut breeding programs, with nematode resistance being the only trait where it has been effectively applied.

In addition to traits that are important for producers, consumer-oriented characters, such as eliminating toxins and allergens, are of great importance to the peanut industry because most of the US grown peanuts are consumed by humans. Little progress has been made through traditional breeding in this area, and applying molecular technologies has the potential for solving several

highly complex problems. Genetic enhancement of several health-related traits also would expand markets, for example increased folate levels, additional flavanoid production, increased vitamin E, the introduction of Vitamin A into the seed (from other species), and modification of proteins to make them more digestible. To better coordinate molecular genetic research efforts in peanut, a strategic plan was prepared in 2004 by a group of peanut breeders and geneticists in consultation with industry representatives to set priorities in six areas, including (1) genetic tools and breeding methods, (2) plant transformation technology, (3) genomic sequencing and gene discovery, (4) functional genomics and proteomics, (5) immunology of peanut proteins in model systems, and (6) bioinformatics (Wilson 2006).

9.8.1 Marker Development in Peanut

Molecular marker development in peanut has been a slow process in large part because of the low levels of polymorphism identified among *A. hypogaea* accessions (Halward et al. 1991; Kochert et al. 1991). Isozymes, Restriction Fragment Length Polymorphisms (RFLPs), Randomly Amplified Polymorphic DNAs (RAPDs), Amplified Fragment Length Polymorphisms (AFLPs), Cleaved Amplified Polymorphism (CAP), Single Sequence Repeats (SSR), and Reverse Transcriptase Polymerase Chain Reaction (RTPCR) have all proved ineffective for producing sufficient numbers of polymorphisms to create a saturated molecular map in the domesticated peanut (Paterson et al. 2004). For example, He and Prakash (1997) used 28 primer pairs to generate 111 AFLPs for DNA markers in *A. hypogaea*, with about 3% of the primers being polymorphic. AFLPs were the first molecular marker system used in peanut to separate botanical varieties of *A. hypogaea* (He and Prakash 2001) and the marker system was later used to differentiate closely related peanut cultivars (Herselman 2003). Hopkins et al. (1999) isolated the first SSR markers in peanut, and He et al. (2003) reported that microsatellites developed from SSRs are more variable than other types of markers. They identified 19 polymorphic markers among *A. hypogaea* genotypes. Many hundreds of SSR makers have been developed during recent years (Jayashree et al. 2005; Luo et al. 2005; Ma et al. 2007), with less than 30% being polymorphic among *A. hypogaea* lines (Ferguson et al. 2004; Moretzsohn et al. 2004; He et al. 2005). SSRs can be used to separate cultivars (Moretzsohn et al. 2005) and this marker system holds great potential for developing useful markers to improve selection efficiency for traits in peanut breeding.

To date, only a limited number of traits have been associated with markers in *A. hypogaea* populations, including a RAPD marker linked to *Diabrotica undecimpunctata howardi* Barber (corn rootworm) resistance and other RAPD markers were associated with components of *C. arachidicola* resistance and with plant color (Stalker and Mozingo 2001); AFLP markers have been associated

with resistance to aphids (*Aphis craccivora* Koch) by Herselman et al. (2004), CAP markers with the high oleic acid content in oils (Lopez and Burow 2004), a SSR marker with *S. minor* resistance by Chenault and Maas (2006), and a RTPCR marker with drought resistance by Jain et al. (2001). Obviously, the numbers of associations of molecular markers with peanut traits within *A. hypogaea* is small and of limited value for breeding programs that select for multiple resistances and quality characters.

In contrast to the *A. hypogaea* gene pool, molecular markers are highly variable among *Arachis* species for many marker technologies, including isozymes (Lu and Pickersgill 1993; Stalker et al. 1994), RFLPs (Kochert et al. 1991; Paik-Ro et al. 1992), RAPDs (Halward et al. 1992), AFLPs (Gimenes et al. 2002; Milla et al. 2005a) and SSRs (He and Prakash 1997). Further, intraspecific variation was found within the diploid species *A. duranensis* (Stalker et al. 1995) and selected markers are useful for identifying individual accessions. Progenies of interspecific hybrids have been used to associate several traits with molecular markers, including RFLPs with *M. arenaria* resistance (Burow et al. 1996; Garcia et al. 1996) and components of *C. arachidicola* resistance (Stalker and Mozingo 2001); RAPD markers with *Empoasca fabae* Harris (leafhopper) and Cylindrocladium black rot resistances (Stalker and Mozingo 2001); and AFLPs with aflatoxin contamination (Milla et al. 2005b) and TSWV resistance (Milla et al. 2004). In addition to markers being useful for associating with specific traits, they hold promise for following gene introgression from *Arachis* species to *A. hypogaea*.

9.8.2 Molecular Maps of Peanut

High-density molecular mapping in domesticated peanut has thus far not been possible because of the low levels of molecular marker variation and the polyploid nature of the species with homeologous chromosome pairs. To circumvent this problem, hybrid derivatives of species in section *Arachis* have been utilized by several investigators. The first molecular map in peanut was created by using RFLPs to analyze progenies of the cross *A. stenosperma* × *A. cardenasii* (both A-genome species) (Halward et al. 1993). They mapped 117 RFLP markers into 11 linkage groups. Garcia et al. (2005) developed a RAPD-based linkage map of peanut based on a backcross population [*A. stenosperma* × (*A. stenosperma* × *A. cardenasii*)] where 167 RAPD loci and 39 RFLPs also mapped to 11 linkage groups; all common markers mapped into the same linkage groups and mostly in the same order as in the Halward et al. (1993) map. A second map was created by Burow et al. (1996) who grouped 383 RFLP markers into linkage groups by analyzing progenies of a cross between *A. hypogaea* and TxAG-6. They also associated the marker R239 with nematode resistance, which mapped to the same linkage group in the map produced by Halward et al. (1993). Unfortunately, both RFLP maps have low saturation levels and the information does not directly translate into the polyploid species *A. hypogaea*.

A microsatellite map was produced by Moretzsohn et al. (2005) with progenies of the cross *A. duranensis* × *A. stenosperma* (both A-genome species) that clustered markers into 11 linkages groups. A second map with the B-genome species *A. ipaensis* and *A. magna* Krapov., W.C. Gregory, and C.E. Simpson was generated by utilizing the same SSR markers, and a comparison between the A- and B-genome maps indicated that they were generally collinear (Gobbi et al. 2006). Herselman et al. (2004) mapped 12 AFLP markers into five linkage groups by using *A. hypogaea* crosses.

Creating a comprehensive physical map for peanut will require a very large-scale effort with a library of large-insert DNA clones (Paterson et al. 2004). Yüksel and Paterson (2005) produced a bacterial artificial clone (BAC) library with 182,784 clones using the tetraploid peanut cultivar Florunner, and this library should serve as a highly useful resource for developing a physical map. However, it will be difficult to distinguish true homologes vs. homoeologs from related species in BACs by hybridization-based molecular approaches because of duplicate copies of genes from the two ancestral species (Paterson et al. 2004). Lin et al. (2000) developed a method to determine the subgenome-specificity of individual BAC clones in polyploid species, so an alternative method may be to produce BAC libraries for diploid progenitors of *A. hypogaea* and then compare libraries from the diploids and tetraploids (Paterson et al. 2004).

9.8.3 Gene Sequencing in Arachis

Arachis hypogaea has 5.91 pg DNA and *A. duranensis* ranges between 2.49 and 2.87 pg DNA (Temsch and Greilhuber 2000), with variation being observed at different elevations and latitudes (Temsch and Greilhuber 2001). Singh et al. (1996) concluded that the A and B genomes contributed nearly equal amounts of DNA to the domesticated peanut. The large genome size and polyploid nature of the domesticated peanut makes it an unlikely candidate to be completely sequenced. Further, the *Arachis* genome also has a high concentration of repetitive DNA (Dhillon et al. 1980). An alternative to complete genome sequencing is to develop libraries of expressed sequence tags (ESTs) which can provide a significant amount of information about gene function. As of June 2007, 12,832 long-sequence ESTs were deposited in GenBank, with the majority of sequences from seeds. In addition, a large number of ESTs have been produced from *A. hypogaea* seeds and leaves that are yet to be deposited in GenBank (Guo et al. 2004; Chen et al. 2006; Stalker and Nielson, unpublished data); and more than 25,000 unigenes have been identified from existing EST datasets (S. Knapp 2007, personal communication). In addition to the long-read ESTs, Jayashree et al. (2005) identified 1,312 short-read sequences that were isolated from SSR-enriched libraries and S. Knapp (personal communication) is currently sequencing large numbers of short DNA segments with methylation-filtration techniques.

Several genes found in peanut have been sequenced, including the Δ12-fatty acid desaturase gene by Lopez et al. (2000), and several *Ara h* genes (which give rise to proteins causing allergens in humans) by Viquez et al. (2003, 2004). Also, homeologous *Ara h 6* genes are present in diploid progenitor species, one in the A-genome and two in the B-genome. Genomic sequencing and microarray-based screening has been used to identify putative genes that may be associated with resistance to *Aspergillus parasiticus* Speare and drought stress (Luo et al. 2005) and for aflatoxin contamination (Guo et al. 2003). In addition, Yüksel et al. (2005) evaluated bacterial artificial clones from the BAC library and found 250 putative resistance gene loci in peanut.

9.8.4 Reverse Genetic Technologies

Gene discovery from Targeting Induced Local Lesions IN Genomes (TILLING) populations is a method developed to find genes of interest in a mutant population of a species. Because allergens in peanut cause a significant health problem in the human population, a high priority of the peanut industry is to eliminate (or suppress) the proteins that cause allergens. There are multiple seed storage proteins that give rise to allergens, with the major ones being *Ara h 1*, *Ara h 2*, and *Ara h 3* (Burks et al. 1998); *Ara h 2* is the most important for causing human allergies (Koppelman et al. 2004). A TILLING population in peanut is being created with the goal of eliminating *Ara h 2* and possibly other allergen genes (Ozias-Akins et al. 2006).

9.8.5 Peanut Transformation

Ozias-Akins et al. (1993) reported the first successful transformation and accompanying plant regeneration in peanut by utilizing the micro-bombardment technique. Micro-bombardment has since been completed in peanut with a number of genes conferring disease resistance (Magbanua et al. 2000; Ozias-Akins and Gill 2001; Higgins et al. 2004; Yang et al. 2004; Livingstone et al. 2003; Dar et al. 2006). Unfortunately, efficiency levels are low and the process takes many months before plants mature (Egnin et al. 1998), and a high-efficiency, rapid technique to transform peanut is greatly needed.

Chen et al. (1996) used an Agrobacterium-mediated transformation system with a Valencia-type peanut. Sharma and Anjaiah (2000) published different methodologies to transform peanut with *Agrobacterium tumefaciens* which apparently works with a wider range of genotypes. Bhatnagar-Mathur et al. (2007) utilized this methodology to develop transgenic lines with increased transpiration efficiency to overcome drought stresses. To date, genetically modified (GM) peanuts have not been sold in the marketplace. Before this occurs, transgenic peanuts must be approved by governmental agencies [US

Department of Agriculture's Animal and Plant Health Inspection Service (APHIS), the Food and Drug Administration (FDA), and the Environmental Protection Agency (EPA)] and licensing agreements will need to be obtained for the incorporated traits.

9.9 Seed Production

In the US, production of peanut seed follows the traditional seed certification chain: breeder, foundation, registered, and certified. Specific purity standards are required to meet certification. These include the time since peanut was grown in the field, the distance from other peanut cultivars within the same field, the percentage of contamination with other cultivars, crop or weed seeds and in some cases, rules about seed storage and conditioning. Other than seed saved by farmers under the US. Plant Variety Protection Act (PVP), nearly all seed sold to farmers is certified by a state seed certification agency. The vast majority of cultivars are protected by PVP which specifies that seed can be sold only as a class of certified seed.

Producing high quality peanut seed requires somewhat more effort than producing eatable peanuts. Most peanut seed are grown with irrigation and optimum inputs of pesticides and fertilizers. One unique aspect of producing high quality peanut seed is the fact that calcium content in the soil is critical to producing seed with good germination. Most seed producers apply 625–900 kg/ha of gypsum (calcium sulfate) when producing peanut for seed. Adams et al. (1993) showed that calcium content of runner seed is vital for optimum germination. The amount of calcium to be applied varies depending on soil tests and the cultivar being grown. Research demonstrates that cultivars with smaller seed require less calcium than cultivars with larger seed, but there is also substantial variation in calcium required by genotypes with similar seed size (Cox et al. 1982; Walker et al. 1976). In fact, germination of some runner market-type cultivars may not respond to gypsum application (Gomillion et al. 2007).

Most seed is grown by farmers under contract with peanut shelling companies. Each year, about 10% of the peanut acreage is devoted to seed production. This may seem excessive, but as described in Section 9.7, peanut seeds are large and the seeding density is relatively high. Peanut seed is about 10% of the variable cost of producing the crop.

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Chapter 10
Castor

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10.1 Introduction

Castor (*Ricinus communis* L.) has the potential to become the premier vegetable oil crop for industrial oil production across the globe (Roetheli et al. 1990). Castor is an ideal candidate for production of high value, industrial oil feedstocks because of the very high oil content (48–60%) of the seed, the extremely high levels of potential oil production (500–1,000 l of oil/acre), and this plants unique ability to produce oils with extremely high levels (80–90%) of ricinoleic acid (Brigham 1993). Additionally, the high potential yield and unique fatty acid composition of castor allows this oil to provide economically competitive feedstocks needed for the production of premium quality biodiesel, short chain aviation fuels, fuel lubrication additives, and very high value biopolymers (Geller and Goodrum 2004; Goodrum and Geller 2005; Roetheli et al. 1990). Because castor is not used for food and can be grown productively on marginal lands this crop represents a unique opportunity to expand industrial vegetable oil production on a global basis. Historically, commercial production of castor has been limited by concerns about the toxins found in castor seed, unstable global markets for the oil and the lack of efficient technologies to produce and process the crop (Brigham 1993). Development of improved production and genetic technologies will help ensure rural regions across the world can participate in the economic potential of this crop.

Most castor seed contains approximately 50% oil which is composed of 80–90% ricinoleic acid (12-hydroxyl-cis-9-octadecenoic acid) (Atsmon 1989). This unique hydroxy fatty acid is used in a number of processes to create unique chemicals and polymers. Ricinoleic acid can be used in several bio-based fuels and industrial products. Pyrolysis of methyl ricinoleate generates methyl 10-undecylenate which can be processed to make Nylon 11 and a seven carbon product (heptaldehyde) that can be used as an octane enhancer for combustion engine fuels. Both of these products are highly valued industrial

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chemicals. A large variety of other reactions have been described that produce other high value products with great potential as biofuels and industrial polymers.

10.2 Origin and Domestication

Castor was probably one of the first crops cultivated by early man who used the oil extracted from the seed for a wide variety of uses including lamp oil (Weiss 2000). Castor is a member of the *Euphorbiaceae* family that is thought to have originated in Eastern Africa but has spread through out the tropical regions of the world. Castor is a diploid ($x = 10$, $2n = 20$) with few if any natural polyploids (Moshkin 1986). Early taxonomists tried to classify castor (*Ricinus communis* L.) into several subspecies based on phenotypic differences but most botanists now believe all castor belong in the same species. Castor accessions display significant differences in height, branching, color or growth habit, and many of these phenotypic traits are simply inherited (Peat 1928; Fig. 10.1) and most accessions will readily intercross (Atsmon 1989).

Fig. 10.1 Phenotypic variation for capsule spines in castor (*Ricinus communis* L.) showing spineless capsules (ss), reduced spines (Ss), and normal spines (SS; Peat 1928). Photos: A.D. Limmer, Texas Tech University

Most castor accessions produce monoecious flowers with the male (staminate) flowers on the base of the inflorescence and the female (pistillate) flowers located on the terminal end of inflorescence (Atsmon 1989). However, the relative proportion and the location on the inflorescence of the male and female

flowers vary between different genotypes. Castor is highly cross pollinated with estimates on the High Plains of Texas ranging from 70 to 90% (Brigham 1967a). Consequently, self pollinated seed can only be produced by sacking individual inflorescences prior to flowering (Atsmon 1989).

Historical summaries of castor production have been published by both Atsmon (1989) and Weiss (2000). In 2005 and 2006, India, China and Brazil produced the majority of the world's castor oil with Ethiopia, Thailand and Paraguay contributing relatively minor amounts (Table 10.1). Annual total world production of castor seed exceeded one million tons during this period but average seed yields were never more than 1,200 kg/ha during this period. The volatility of castor oil prices and variability in production has made the international market for castor oil very unstable (Roetheli et al. 1990). Increasing demand for vegetable oils for biodiesel and other industrial applications have increased interest in improving the genetics and production of castor worldwide.

Table 10.1 2006 and 2007 average castor seed production area, seed yield and total seed production in six major producing countries and world wide (FAO-STAT 2008)

Country	Production area (ha)	Seed yield (kg/ha)	Total production (MT)
India	805,000	1,063	861,000
China	255,000	961	245,000
Brazil	184,231	701	130,565
Ethiopia	14,500	1,034	15,000
Thailand	13,430	781	10,492
Paraguay	10,000	1,100	11,000
World	1369,720	956	1314,193

10.3 Varietal Groups

Castor has not been divided into varietial groups in recent times but most breeders recognize tall, late flowering types as tropical in origin. Those types which flower and mature quickly are usually adapted to either high altitude environments or more temperate latitudes in either the northern or southern hemisphere. There have been no reported barriers to intercrossing the two types and obtaining segregating populations.

10.4 Genetic Resources

A search of International Germplasm collections on the Bioversity web site combined with the USDA-ARS castor germplasm at Griffin, GA (USA) identified 12 major sources of germplasm and a total of 6,588 accessions (Table 10.2). Extensive germplasm collections are held in Brazil, China, Ethiopia, India, Kenya and the former USSR, but availability of these germplasm resources is not known. Additional castor germplasm can be obtained from

Table 10.2 Major germplasm collections of castor (*Ricinus communis* L.) as listed by the Bioversity International Directory (October 14, 2008)

Country	Collection agency	Accessions reported
Brazil	CENERGEN/EMBRAPA	360
Brazil	Centro Nacional de Pesquisa de Algodao (CNPA)	199
Brazil	Empresa Baiana de Desenvolvimento Agricola S.A.	528
Brazil	Instituto Agronomico de Campinas (I.A.C.)	200
China	Institute of Crop Science (CAAS)	1,689
China	Institute of Oil Crops Research (CAAS)	1,652
Ethiopia	Biodiversity Conservation and Research Institute	232
India	Region Station Akola, National Bureau of Plant Genetic Resources (NBPGR)	290
Kenya	National Dryland Farming Research Station, Kenya	130
Kenya	National Genebank of Kenya, Crop Plant Genetic Resources Centre, KARI	43
Romania	Agricultural Research Station Teleorman	66
Russia	N.I. Vavilov All-Russian Scientific Research Institute of Plant Industry	423
Serbia	Maize Research Institute	69
Serbia	Institute of Field and Vegetable Crops	43
Ukraine	Institute for Oil Crops	255
United States	USDA-ARS-PGRCU	364
World	39 Institutes	6,588

public breeders in South America including Brazil and Columbia. In tropical climates world wide, castor can be found as an introduced plant species surviving as a weed in roadsides and non-cultivated areas. This feral castor can be a valuable source of germplasm especially for adaptation to localized diseases, pests and environmental conditions.

10.5 Major Breeding Achievements

10.5.1 Fatty Acid Composition

In 2004, a natural mutant of castor was isolated from USDA Plant Introduction PI 179.729 that had increased concentrations of oleic acid and reduced levels of ricinoleic acid (Rojas-Barros et al. 2004). This mutant produced seed oils with approximately 78% oleic acid and from 10.1 to 18.8% of ricinoleic acid. The increased levels of oleic acid appeared to be controlled by two independent genes (*ol*, *Ml*) with epistatic interaction (Rojas-Barros et al. 2005). Incorporation of this mutant and other high oleic acid mutants in improved varieties may enhance the use of castor oil as a biodiesel feedstock.

Castor oil biosynthesis is a matter of considerable biochemical interest, due to the unusual chemical nature of the ricinoleate hydroxylation – a stereospecific, geometrically specific hydroxylation of a hydrocarbon chain, still an unrealized dream for the synthetic organic chemist. Although the cloning of the gene for this enzyme, oleoyl-12-hydroxylase, provided interesting insights into the production of ricinoleate and other uncommon fatty acids, transgenic plants that expressed the hydroxylase gene produced oils containing less than 20% hydroxy fatty acid (Broun and Somerville 1997).

Since the hydroxylase gene alone was not sufficient to elicit high levels of hydroxy fatty acid production, it seemed that there must be other enzymes that are required in order to achieve high ricinoleate levels in oil. Based on metabolomic studies of castor oil biosynthesis carried out by castor seed microsomes, there are several enzymatic steps that appear to be important for high ricinoleate levels (McKeon and Lin 2002). Further studies (Lin et al. 2002) indicated that 6-fold more ricinoleate is incorporated into triacylglycerol (TG) than oleate. This result indicated the final step in oil biosynthesis, diacylglycerol acyltransferase (DGAT), as the reaction that led to high ricinoleate content and minimal oleate in the oil. The diacylglycerol acyltransferase (DGAT) is a transmembrane enzyme that catalyzes the acylation of diacylglycerol (DG) to TG, using acylCoA as the source for the final acyl group. The DGAT reaction is widely considered to be the limiting step in oil biosynthesis, with considerable evidence indicating that altered DGAT activity levels dramatically affect the yield of oil (He et al. 2005). With the cloning of the DGAT1 from developing castor seed, it has been demonstrated that the activity and protein level of the cloned DGAT is closely correlated with the onset of oil biosynthesis in the seed (He et al. 2004). The castor DGAT enzyme displayed a 2-fold preference for using diricinolein vs. other non-hydroxylated fatty acyl DG. The acyl-donor for the DGAT reaction is produced by the acylCoA synthetase (ACS). One of several ACS cloned from castor displays a threefold preference for condensing ricinoleate vs. oleate with CoASH (He et al. 2007) suggesting the combined effect of the enzymes from the two cloned genes could result in a significantly higher incorporation of ricinoleate vs. oleate into oil. The question of how the castor seed produces an oil with such a high proportion of ricinoleate and a high oil content, approaching 60% continues to provide a challenge. Understanding this process may ultimately lead to increased oil content and the ability to engineer the production of other uncommon and industrially useful fatty acids in the castor seed.

10.5.2 Castor Toxins

Ricin is a protein toxin found in the endosperm of mature castor seed that is capable of inhibiting protein synthesis by enzymatically destroying the ribosomes of eukaryotes (Khvostova 1986). The presence of ricin in the high protein

meal of castor remaining after oil extraction has historically reduced its value as an animal feed (Roetheli et al. 1990). Since ricin has the potential to be extracted and used as a chemical warfare and bioterrorism agent, the production and processing of castor has undergone increased scrutiny by international law enforcement agencies since the terrorist attacks of September 11, 2001 (Lowery et al. 2007; Franz and Jaax 1997). Development of castor cultivars with reduced levels of ricin would improve the economics of castor oil production, reduce the potential for accidental poisoning and eliminate the potential of ricin being used by terrorists.

Ricin has both an A and a B chain linked together by a disulfide bond. Both the A and B proteins have been DNA sequenced and appear to be initially produced by a single gene in castor (Halling et al. 1985; Tregear and Roberts 1992). Ribosome-inactivating proteins such as the ricin A chain typically contain a N-glycosylated, 32 kDa monomer (Olsnes and Pihl 1973). The A chain is attached to the cell surface by a the 34 kDa protein (B chain) (Roberts et al. 1985; Frankel et al. 1989). The ricin A chain has also been used in the production of immunotoxins which target specific diseases in humans (Ghetie and Vitetta 1994). Castor meal when applied as an amendment to greenhouse potting media has been shown to improve growth of okra (*Hibiscus esculentus*) and suppress root-knot nematode (*Meloidogyne arenaria*) (Ritzinger and McSorley 1998). Ricin has historically been degraded by exposure to high temperature for two or more hours (Roetheli et al. 1990). Ricin can also be degraded by exposure to concentrations of 3 mM sodium hypochlorite (Mackinnon and Alderton 2000).

Castor seeds also contain a second toxin, *Ricinus communis* agglutinin (RCA_{120}) (Hartley and Lord 2004). RCA_{120} is very similar in both amino acid sequence and structure to ricin (Hartley and Lord 2004). RCA_{120} is composed of two A-chains and two B-chains linked together by disulfide bonds. This compound is much less toxic to mammals than ricin (Lowery et al. 2007).

Ricin makes an ideal target for genetic manipulation since this toxin appears to be controlled by a single gene that encodes both the A and B chains of castor (Halling et al. 1985). Conventional genetics were used to reduce the levels of ricin in dwarf-internode castor using crosses with two accessions from the Soviet Union, PI 258 368 and PI 257 654, which had been previously selected for reduced levels of ricin (Khvostova 1986). In subsequent segregating generations, individual plants were selected for dwarf-internode growth habit and reduced levels of ricin and RCA_{120} using a radial immunodiffusion (RID) assay (Auld et al. 2003; Auld et al. 2001; Pinkerton et al. 1999). In 2003, twelve F_8 lines where intercrossed to develop a synthetic population adapted to mechanical harvest. In 2004 and 2005, this population was screened for semi-dwarf internode growth habit and lack of shattering. This process developed a new experimental castor variety, 'Brigham' which has a ten fold reduction in the level of ricin.

A third toxic substance found primarily in the capsules and seed hulls of castor is the alkaloid, ricinine (Bukhatchenko 1986). Ricinine is thought to be product of specific nitrogen synthesis and consists of a monocyclic derivative of

pyridine which carries a cyanide group. It appears to be a naturally occurring insecticide in castor that has a relatively low human toxicity. Russian researchers reported a negative correlation between the concentration of ricinine and oil in castor seeds. It also appears that drought conditions during seed maturation enhance the concentration of ricinine.

10.5.3 Castor Allergens

The allergy caused by exposure to the plant tissue and residue of castor has historically caused human health problems to those individuals working around or in close proximity to castor fields or plants processing castor seed across the globe (Panzani and Layton 1963). Most of those individuals expressing an allergic reaction have asthma like symptoms (Mercier and Panzani 1988). Cross-reactivity can occur with other species of plants within *Euphorbiaceae* (Layton et al. 1970). There has not been sufficient research to describe the chemicals produced by castor that cause these allergic reactions or germplasm screening to see if castor genotypes differ in their relative ability to cause allergic reactions in humans. However, the development of castor cultivars which do not cause allergic reactions would enhance production and processing of this crop.

10.5.4 Qualitative Traits

Capsule drop resistance caused by pathogens such as *Botryotinia ricini* (Godfrey) Whet. in humid areas of the USA is controlled by one or possibly two genes (Culp 1966). At least one of these genes appeared to be closely linked to the short pedicel trait. Researchers in both Russia and Trinidad have conducted exhaustive genetic studies that show the color of stems, leaves and capsules (Fig. 10.2), waxy coat on the stem and petiole, color of the hypocotyl and stigma of flowers, spines of the capsules, dehiscense of capsules, pedicel length, color and form of the seeds (Fig. 10.3), female sterility, flowering period, seed hull color, plant height and numerous other phenotypic traits are fairly simply inherited (Moshkin 1986; Peat 1928).

10.5.5 Quantitative Traits

In castor as well as in the majority of cultivated plants, the agronomic characteristics of primary economic importance are inherited in a quantitative manner including seed and total biomass production.

Fig. 10.2 Phenotypic variation in pod color of castor (*Ricinus communis* L.) caused by different combinations of the M (Mahogany) and G (Green) genes (Peat 1928). Photos: A.D. Limmer, Texas Tech University

Fig. 10.3 Phenotypic variation in seed shape, size and color of several different accessions of castor (*Ricinus communis* L.). Photo: A.D. Limmer, Texas Tech University

Hooks et al. (1971) evaluated the behavior of inbred lines of castor in a diallele cross using the method of Gardner and Eberhart (1966) and the procedures of Hayman (1954 and 1958). They observed that additive genetic effects were important in the initiation of bloom, the number of racemes per plant, and seed oil content. However, the number of nodes prior to flowering showed significance of additive genetic effects which agreed to the estimates derived by Swarnlata et al. (1984). Giriraj et al. (1974) evaluated a diallele cross of six geographically diverse cultivars and evaluated the length of the primary raceme, the number of capsules per primary raceme and 100-seed-weight. They showed that these traits also had very significant additive genetic effects. Solanki et al. (2003) evaluated the genetic effects of eight agronomic characteristics with similar results.

Solanki and Joshi (2000) evaluated a diallele cross between monoecious and female plants and found that additive effects were primarily responsible for the number of nodes, the length of the primary raceme, number of racemes per plant, number of capsules per primary raceme, 100-seed-weight, and total weight of seeds produced 120 and 240 days after the sowing. This study also

showed that the characteristics of days to 50% of bloom of the main cluster, 100-seed-weight and height of plants had high heritabilities indicating rapid selection efficiency.

Russian researchers have conducted extensive investigations on the inheritance of the components of seed yield and oil content in castor with similar results (Moshkin 1986).

10.6 Breeding Methods and Techniques

Castor is an often cross pollinated species with 14–45% self pollination under tropical conditions. In castor, the procedures for making artificial crossings and self-fertilizations are relatively easy and result in several seeds. Because of the reproductive biology of this species, the methods used to improve self pollinated plants as well as the process of recurrent selection that is most often used on cross pollinated crops are feasible.

10.6.1 Mass Selection

Mass selection is most effective for characteristics with high heritabilities in populations with high levels of natural genetic variability such as heterogeneous land races. Two procedures are useful in increasing the efficiency of the mass selection in populations of castor: The self-fertilization of the selected plants to prevent cross pollination, and the use of controlled selection techniques to reduce environmental variation. Moshkin (1967) cites examples of application of the mass selection for enhancement of female flowers, in the selection for resistance to *Fusarium*, and reduction of plant height. Savy Filho (2005) used mass selection to develop IAC-38, an important dwarf castor cultivar in Brazil.

10.6.2 Individual Plant Selection with Progeny Tests

As in the mass selection, the selection of individual plants based on progeny tests is highly effective for the improvement of populations of castor with high levels of natural genetic variability. In the case of simultaneous selection for several characteristics, selection for the characteristics must be made using the highest possible number of self pollinated lines and be preceded first by the traits with high heritability that can be identified without the use of replicated trials. Subsequent selection and the final evaluation should be done on about 200 inbred lines arranged in a lattice experimental design with 3–4 replications in two or three locations and years. This type of testing allows selection for high seed and oil yield. In all phases of selection, the use of appropriate commercial check varieties will enhance selection

efficiency. Amaral (2003) successfully used the individual plant selection followed by progeny tests in developing the cultivar 'Guarany' of castor with increased seed yield.

10.6.3 Methods Involving Sexual Hybridization

When populations of castor with sufficient natural genetic variation for agronomic characteristics are not available, it is necessary to produce sexual hybrids between different lines or cultivars to generate sufficient genetic variability to support a selection program. The choice of the parents of these populations must be based on their agronomic performance within the targeted production region. In the case where there are several promising parents or cultivars it may be necessary to use a diallele cross design to identify those potential crosses with the greatest potential for creating highly performing sexual hybrid populations.

10.6.3.1 Pedigree Method

In the F_2, F_3, and F_4 generations, self pollinated individual plants are selected to derive uniform lines. During this process, selection is practiced both between lines and within lines by selecting only the best plants within the best lines. By the F_5 or F_6 generation the inbred lines should have a high degree of homozygosity. These lines are then subject to a final evaluation using an experimental design and statistical interpretation of data taken over multiple locations and production years. Those inbred lines with superior performance that out-yield the check cultivars can be increased to create a new commercial cultivar.

The pedigree method works best when it is necessary to select simultaneously for several characteristics. Selection for the characteristics with the highest heritabilities should be made in the initial generations (F_3 and F_4), and the selection of quantitative characteristics in later generations. The pedigree method is limited by the selection of initial plants in the F_2 generation needed to produce F_3 inbred lines. This early generation selection process can restrict the full expression of individual plants genetic variability since F_2 plants are highly heterozygous.

10.6.3.2 Bulk Method

The bulk method allows the hybrid population to segregate without artificial selection until the F_5 or later generations (F_6 or F_7). The bulk method is most effective when the main objective of the program is to improve the adaptation of castor to stress conditions such as drought, acid soils, high levels of salt and resistance to diseases. After selection elite lines undergo a final evaluation as described for the pedigree method.

10.6.3.3 Single Seed Descent Method

The use of the single seed descent (SSD) method in the improvement of castor has not been frequently practiced but it offers two interesting aspects. It does not allow either natural nor artificial selection during the segregating generations but it does allows the increase of up to two generations per year using off season increases in either winter nurseries or greenhouses. In addition this techniques does not have as drastic reduction in the genetic variability in the F_2 or F_3 generations as the pedigree method.

10.6.3.4 Backcross Method

The backcross method of selection is most effective when there is a need to improve some simply inherited, qualitative characteristic in a commercial cultivar or promising elite line. The non-recurrent parent must have the characteristic absent from the recurrent parent. The method of backcrossing is especially effective in castor for the improvement of characteristics such as seed shattering, flower height, and disease resistance.

10.6.3.5 Recurrent Selection

Recurrent selection can be defined as successive cycles of selection and recombination of selected lines or individual plants. Recurrent selection has been more extensively used in species such as maize rather than in castor; Zanotto et al. (2004) had considered recurrent selection with use of inbred lines for the reduction of plant height in the cultivar 'Guarani'. The selection cycles occurred in two stages. In the first stage short plants were selected and self pollinated from the cultivar 'Guarani'. In the second stage, 180 self pollinated lines were evaluated for plant height in isolation and racemes of five plants of each one of the 30 selected lines were self pollinated by paper bags as described by Savy Filho (1999). After the selected lines had gone through at least five cycles of self pollination, the 30 selected lines were intercrossed and the harvested seed mixed to generate the cycle 1 seed. This procedure was repeated for four additional cycles of selection. According to Oliveira and Zanotto (2008) plant height was reduced by 28 cm, 13 cm, 19.9 cm, 11.7 cm and 3.4 cm, respectively, for the five cycles of selection. This process demonstrated the effectiveness of using recurrent selection with self pollination in castor.

10.7 Integration of New Biotechnologies in Breeding Programs

Allergen and ricin content are major issues that affect interest in production and processing of castor seed for castor oil. Since castor is not a food crop, one potentially fruitful approach to eliminating these noxious proteins is genetic engineering, to silence their expression during seed development. Both the primary allergen, 2S albumin, and ricin are expressed at a very high level during

seed development (Chen et al. 2004; Chen and McKeon 2005). With the proper choice of promoter and application of gene silencing techniques, gene expression can be suppressed up to 10,000 fold, which would be adequate for safe exposure to the seed meal.

However, genetic transformation of castor has proven to be highly challenging, as it is recalcitrant to efficient regeneration of stably transformed plants. The first report of transformed castor (McKeon and Chen 2003) described a vacuum infiltration technique using *Agrobacterium* carrying marker genes. Since then, an apparently more efficient method has been developed (Sujatha and Sailaja 2005). However, given the need to reduce ricin content by a factor of 10,000 and a similar reduction of allergen, there remains an urgent need to improve the efficiency of castor transformation. With such a method, not only could the noxious proteins be virtually eliminated, improvements that would benefit the growth and production of castor such as herbicide resistance, pest resistance, or monoracemic fruit-bearing could be made available. These traits would enhance the productivity of castor and simplify its harvesting. As a monospecific genus, *Ricinus communis* is somewhat limited in germplasm availability. The ability to introduce foreign genes into this non-food crop would have great potential to expand its growth habit and productivity.

10.8 Seed Production

Heterosis was shown to have a significant impact on days to flowering, racemes per plant, volume weight, oil content and seed yield indicating that increased seed and oil yields could be expected from the production of hybrids of castor (Hooks et al. 1971). Hybrids also appeared to have faster seedling emergence than open-pollinated lines (Brigham 1965). Composites have been used to capture a portion of this heterosis and increase genetic variability in castor (Brigham 1973). The production of hybrid seed in castor has been achieved using the *f* N-pistillate gene for female racemes and environmentally sensitive genes of interspersed-staminate flower by producing seed in locations with cooler temperatures (75–83°F) (Zimmerman and Smith 1966). In addition, two female-sterile characters (fs_1 and fs_2) were identified by Brigham (Brigham 1967b). Commercial hybrids are now used in commercial production in Brazil, India and other parts of the world.

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Chapter 11
Oil Palm

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11.1 Introduction

The oil palm is the world's most important oil crop producing 24.9% of total vegetable oils and fats surpassing soybean at 23.9% (Mielke, http://www.oilworld.bz, 31 March 2007). It produces two types of oil from its fruits, mesocarp oil and kernel oil known as crude palm oil (CPO) and palm kernel oil (PKO), respectively, in international trade. Total world production of CPO stands at about 38 million tons worth around US$ 20 billion. The oils are produced from some 13 million ha of plantations in the humid tropical countries of Asia, Africa and Latin America: Indonesia (5.3 million ha), Malaysia (4.2 million ha), Papua New Guinea, Colombia, Ivory Coast, Nigeria and Thailand with the first two countries having the bulk of the plantings. Palm oil is the largest internationally traded vegetable oil with its main markets in China, European Union, Pakistan, India, Japan and Bangladesh. Palm oil is mainly used in food (80%), e.g. as cooking oil, margarine, vanaspati or vegetable ghee and shortenings, and the remaining 20% are used as oleochemicals replacing mineral oil to feed the detergents, cosmetics, pharmaceutical/nutraceutical, plastics and lubricants industries. With the recent high rise in petroleum prices and that the deadline for meeting the requirements of the Cartagena Protocol on Biosafety in terms of 'green' or renewable energy substitution is approaching, there has been a tremendous demand for palm oil as a source of biofuel (biodiesel). Also, responding to consumer health and environmental concerns, secondary and by-products from the palm oil industry have spawned new industries, e.g. vitamins A and E and other antioxidant health supplements from the oil, animal feed and organic fertilizers from the kernel, and sludge cakes and wastes from oil extraction mills have served as value additions.

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11.2 Origin and Domestication

The oil palm has been postulated to originate in Gondwanaland which disappeared when the American and African continents drifted apart in prehistoric times (Zeven 1965) giving rise to the evolution of African oil palm (*Elaeis guineensis* Jacq.) and American oil palm (*E. oleifera* or *E. melanoccoca* previously). The African oil palm is endemic to the equatorial belt of Africa stretching eastwards from Guinea at the Atlantic coast to Madagascar Island in the Indian Ocean and from Senegal in the sub-Sahara to the Angola-Namibia border in the south. However, the centres of origin and diversity appear to be concentrated in the tropical forests of the west (Ivory Coast, Nigeria, Ghana, Cameroon) and central (Congo, Zaire, Angola) African countries where they occur as semi-wild forest groves fringing rivers in the lowlands usually close to settlements, although they can thrive in drier and higher areas (Hartley 1988; Latiff 2000). The American counterpart is endemic to the tropical countries of Central and South America, from Mexico in the north to the Amazon in the south and from the Pacific to the Atlantic coasts. Its distribution appeared to be more discontinuous, it is also found in open grasslands and river banks and associated with native Indian migratory trails (Santos et al. 1986). The oil palm fruit forms an important part of both the native West African and South American diet as a source of fat, carbohydrate, protein and vitamins.

Interest in oil palm as an industrial crop arose from the need to substitute animal fat in the production of candle wax, soap and margarine. The European colonists started oil palm plantations in Indonesia and Malaysia to ensure a steady supply of oil. Four seedlings planted in Bogor Botanic Gardens in Indonesia derived apparently from the same fruit bunch in West Africa and obtained via Amsterdam and Mauritius/Reunion, gave rise to the current industry. Hybridizations and selections among the progenies of these four progenitors, which had thick shell fruits or Dura (D) fruit form, were distributed to the plantations in Deli province in Sumatra and thence to Malaysia (Rosenquist 1986). These Deli Ds were the commercial planting material for the rapidly developing plantation industry in Malaysia and Indonesia from 1911 till the early 1960s. Beirnart and Vanderweyen (1941) elucidated the monogenic inheritance of the shell gene: the cross between the thick shell D parent palm and the shell-less (usually female sterile) pisifera (P) parent would give rise to 100% thin-shell tenera (T) palms (Fig. 11.1) exhibiting incomplete dominance of the shell gene; the T $\times$ T, the T $\times$ P and D $\times$ T crosses would give rise to segregating progenies in the classical Mendelian ratios of 1D:2T:1P for the first (Fig. 11.2), 1T:1P for the second and 1D:1T for the last cross, respectively. Teneras from West Africa have been brought in by breeders and with this revelation the switch to the T hybrid as the commercial material was very rapid (Hartley 1988). This process was spurred on by private plantation companies becoming interested in commercial hybrid seed production and investing in oil palm breeding. Mixed T or D $\times$ P hybrids have been the dominant commercial

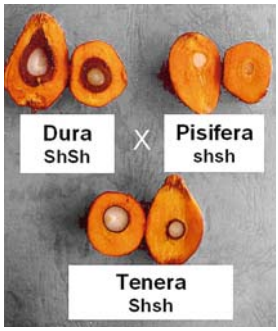

Fig. 11.1 Commercial dura × pisifera hybrid palm seed production

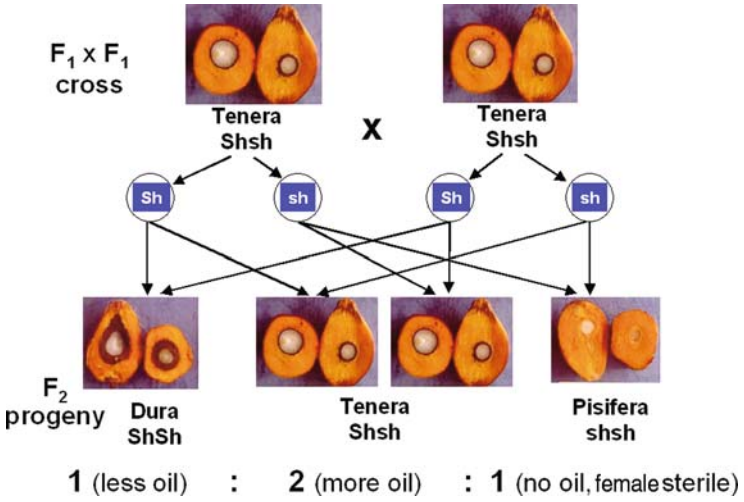

Fig. 11.2 Monogenic inheritance of shell thickness: F_2-segregation

planting materials till today. Recently, oil palm clones from tissue culture have become commercially available although still in limited quantities as compared to the total demand for oil palm seeds (Soh et al. 2006).

11.3 Varietal Groups

In oil palm, there are no varieties in the strict sense as the commercial materials are mixed hybrids from non-fully inbred and sometimes out-crossed parents (Soh 1999). They can therefore be considered as inter-population hybrids. The parent populations e.g. Deli, were usually derived from very few progenitors

and have being accordingly referred as BPROs (breeding populations of restricted origins) by Rosenquist (1986). Only a handful of BPROs are featured in commercial hybrid breeding programmes:

Deli. This BPRO is featured almost exclusively as the female parent in all commercial hybrid seed production programmes. The distribution of this BPRO to various countries followed by local selection led to the development of a number of subpopulations, e.g. Ulu Remis Deli, Elmina Deli, Serdang Deli (Malaysia), La Me Deli, Dabou Deli (Ivory Coast). The Dumpy and Gunung Malayu are dwarf mutants of the Deli.

AVROS. The ancestral palm was the 'Djongo' or 'Best' palm found at Zaire's Eala Botanical Garden. Seeds were planted in Sungai Pancur, Sumatra by AVROS (Algemeene Vereniging van Rubberplantera ter Oostkust van Sumatra) in 1923 giving rise to the well-known SP540T parent. Subsequent crosses with other T palms in Bangun Bandar Experimental Station were followed by backcrosses to SP540T selfs and became the AVROS BPRO. AVROS seeds were brought into Malaysia and their subsequent progenies distributed widely in Malaysia, Indonesia and thence to Papua New Guinea, Colombia, Costa Rica and Thailand. The major oil breeding and seed production programmes in these countries are based on the AVROS Ps or its derivatives. Vigorous trunk growth and high oil yield from big fruits with thick mesocarp are characteristic features of this BPRO. Pure descendents of SP540T gave rise to the RISPA BPRO in Indonesia.

Yangambi. The breeding programme of INEAC (Institut National pour l'Étude Agronomique du Congo) at Yangambi station involving open-pollinated progenies of the Djongo palm and other Ts from Yawenda, Isangi and N'gazi gave rise to this BPRO. This lineage is also featured in a number of major breeding programs as a source of Ps in D $\times$ P hybrid seed production. Yangambi BPRO shares similar features of vigorous palm growth, high oil yield from big fruits with thick mesocarp as the AVROS. A short variant (16R) was found and developed in this population.

La Me. This BPRO was bred by IRHO (Institut de Recherches pour les Huiles et Oleagineux) currently known as CIRAD (Centre International Recherche Agricola et Développement) from the 21 T palms, particularly the Bret 10 palm, derived from seeds collected from wild groves in Ivory Coast. This BPRO, characterized by its smaller palm stature, production of many smaller bunches with small fruits and generally tolerant of less favourable growing conditions, forms the genetic base of the breeding and seed production programmes in West Africa and Indonesia advised by CIRAD.

Ekona. Using wild palms found in the Ekona area of Cameroon, breeding by the Unilever plantations group at the Cowan, Ndian and Lobe estates led to the development of this BPRO. Largely through the Unilever plantations group network, the Ekona BPRO, particularly progenies from the Fusarium wilt resistant and high oil yielding palm, CAM 2/2311, were distributed to Malaysia,

Costa Rica and Thailand. This BPRO confers smaller fruits but with high oil content to their progenies.

Calabar. The genetic base of the Nigerian Institute for Oil Palm Research is much broader from collections made in Aba, Calabar, Ufuma and Umuaibi. Progenies from its Calabar selections, particularly palm NF 32.3005 have been distributed to Ghana, Costa Rica, Indonesia and Malaysia.

Derived and recombinant BPROs. A number programmes have initiated the recombinant phase of breeding having interbred or introgressed parents from various traditional BPROs to form new BPROs with mixed lineages or recombinant BPROs, e.g. URT (Ulu Remis teneras), Dumpy.AVROS, Dumpy. Yangambi.AVROS, La Me x Yangambi, La Me x Dumpy.AVROS (Soh et al. 2006).

11.4 Genetic Resources

Recognizing the very narrow genetic base (Deli, AVROS, Yangambi, La Me) of the existing oil palm breeding programmes and considering that Malaysia had a very high stake in the rapidly expanding industry, MARDI (Malaysian Agricultural Research and Development Institute) and subsequently PORIM (Palm Oil Research Institute of Malaysia), currently known as MPOB (Malaysian Palm Oil Board), launched a series of expeditions to Africa and Latin America to prospect for new *E. guineensis* and *E. oleifera* germplasm for genetic base broadening, breeding and conservation in its *ex situ* genebank (Ooi et al. 1973; Rajanaidu et al. 1979; Obasola et al. 1983; Rajanaidu and Rao 1988). The first prospection was systematically done in Nigeria in collaboration with NIFOR and the then International Board of Genetic Resources with the objectives of genetic conservation and studying its population genetic structure. Genetic studies done on the prospected progenies revealed that genetic variability was higher within families than between families and populations. Subsequent collections in Angola, Cameroon, Gambia, Guinea, Madagascar, Sierra Leone, Tanzania and Zaire were guided by this finding and also targeted towards accessions with prospective economic and agronomic traits. Prospections for *E. oleifera* in Latin America were carried out in Brazil, Colombia, Costa Rica, Ecuador, Honduras, Panama, Peru and Suriname and included also other oil bearing palms (*Bactris gossipaes* or Pejibaye, *Jessenia-Oenocarpus*, *Orbignya martiana* or Babassu) with unusual fatty acids and other uses. The accessions have been planted and maintained as living collections in Malaysia with a sample retained by the host country (Rajanaidu 1990; Rajanaidu and Jalani 1994). These prospections proved to be scientifically successful in that a number of extremely desirable traits and traits absent in existing breeding populations have been discovered (Table 11.1) and introgressed into advanced breeding populations or developed into new populations (Sharma 1999; Rajanaidu et al. 2000).

Table 11.1 Oil palm collections from various countries and their special attributes

Accessions	Useful traits
A. *E. guineensis*	
Nigeria	High in unsaturated fatty acids, dwarfness, high stearic acid, low lipase
Angola	Large fruits, high carotene content
Zaire	Tolerance to Ganoderma disease
Cameroon	Tolerance to Ganoderma disease
Tanzania	Thin shell
B. *E. oleifera*	
Colombia	Very high unsaturated fatty acids content
Brazil	Thicker oil bearing mesocarp
Costa Rica	Compact stature, good oil yielding
Ecuador	Thicker oil bearing mesocarp, very small palms
Suriname	Very compact palm tree stature

11.5 Major Breeding Achievements

11.5.1 Tenera Hybrid Improvement

Davidson (1993) attributed 70% of the oil palm yield improvement in Malaysia for the previous 50 years to breeding improvement and 30% to improved agronomic practices. Undeniably, the mere switch-over from the thick shell, thinner oil-bearing mesocarp (ca. 60% mesocarp to fruit content) D to the thin shell, thicker mesocarp T (ca. 80%) variety would account for at least 30% of the yield increase, notwithstanding improved FFB yield. There have been at least two generations (ca. 20 years) of improved T materials since and oil yields have improved from about 5 t/ha/yr to about 10 t/ha/yr based on trial results then. Hardon et al. (1987) estimated that there had been an average yield improvement of about 15% per generation over two generations of breeding in the D, but they did not translate this into T hybrid improvement. Subsequently, Lee et al. (1990) and Rajanaidu et al. (1990) estimated a resultant T improvement of only 6–7% when they progeny tested the same Ps on two successive generations of selected Ds. Lee and Yeow (1985) reported that selecting the best P (top 15%) from the progeny-test of seven Ps would give 12% improvement. In commercial seed production, at least 3–4 progeny tested Ps (top 30–50% selection) need to be used reducing the improvement to about 5%. Reconciling the estimated improvements in the two parental populations, the estimated improvement of 10–15% per generation for the T hybrids was not unreasonable (Soh et al. 2003a). The grossness of the estimate is inevitable as there were no common standard crosses linking the different trials of different generation materials and the difficulty of identifying a standard (single hybrid, sample of mixed hybrids) treatment to represent a particular generation of commercial mixed hybrids. Breeding programmes based on the MRRS (modified reciprocal recurrent selection) system (Soh et al. 1999) were better placed

to compute breeding progress more objectively. The original progeny tested hybrid selected for reproduction as commercial hybrid (using the selfs of the parents) was used consistently as the standard cross. Likewise, the superior hybrid selected for reproduction as the second cycle commercial hybrid can be used as a standard for comparing subsequent cycle hybrids. Oil yield improvements of 18% (Gascon et al. 1988) and 25% (Lubis et al. 1990) in the first cycle of MRRS, and 10–15% in the second cycle (Cochard et al. 1993) were purportedly achieved in Ivory Coast and Indonesia.

11.5.2 Cloning Improvement

The projected 30% yield increase by cloning the best individual palms from commercial mixed hybrids provided the impetus in the development of the tissue culture clonal propagation technique of the oil palm (Jones 1974; Hardon et al. 1987; Meunier et al. 1988). Soh (1986), however, contended that the likely increase would be about 13% with the first cloning, based on general theory because of the low heritability for yield in advanced commercial hybrids. The results from the CIRAD group comparing the improvements made by their clones and improved hybrids from the MRRS programme were in general agreement with Soh's estimate (Nouy et al. 2006). However, the mean yield advantage of clones over commercial hybrids summarized from five trials testing 68 clones by AAR (Applied Agricultural Resources) was about 18%. There were clones exceeding hybrids by 30% which could be recloned, but at the same time hybrid improvement would have caught up by 10–15% (Soh et al. 2003a,b, 2006). Yields of up to 11–12 t/ha/yr oil have been reported in trial and commercial clonal plantings (Mohd Isa et al. 2005; Soh et al. 2006).

11.5.3 Improvement in Other Traits

Besides breeding for yield per se, there are programmes which also emphasize other desirable agronomic/economic traits, e.g. dwarfness or improved oil quality. The semi-dwarf Dumpy.AVROS variety which was about 20% shorter than the popular but tall AVROS variety was available since the early 1980s and represented about 10–20% of Malaysia's annual oil palm plantings till recently (Soh et al. 2006; Mohd Isa et al. 2005). An improved version of the Dumpy.AVROS variety, the Dumpy.Yangambi.AVROS (Fig. 11.3) with better physiological traits and thus higher yield potential has been developed (Soh et al. 2006). The development of dwarf palms high in unsaturated fatty acids derived from their Nigerian *E. guineensis* accessions by MPOB and *E. oleifera* × *E. guineensis* hybrid-derived compact statured clones by ASD have been announced (Rajanaidu et al. 2000; Escobar and Alvarado 2003). Few groups have also stated to produce limited numbers of

Fig. 11.3 Breeding semi-dwarf high oil yielding palms: Dy.Ybi.AVROS vs. Dy.AVROS vs. AVROS varieties

biclonal (clonal parents on both sides) and semi-clonal (clonal parent on one side, usually the dura) hybrid seeds from proven parents. The impact from the commercial planting of these newly developed special materials remains to be seen.

11.6 Current Goals of Breeding

Owing to the versatility of the crop in terms of the myriad uses of its oil, many suggestions for various desirable traits to be improved in the palm and its oil have been made. To reconcile opinions and lobbies, MPOB in 2003 organized a workshop comprising breeders, agronomists, biotechnologists, oleochemists and technologists, palm oil end-users and traders for prioritizing traits for improvement in the oil palm, as it is inefficient if not impossible for breeders to consider all the useful traits in a breeding program. The four top priority traits in ranking order were: high oil yield, dwarf stature, resistance to Ganoderma disease and high oleic acid oil (Table 11.2). The first three are agronomic traits related to yield. This is not surprising as palm oil is still essentially a commodity crop mainly used as food where high yield ensures lower cost of production and competitiveness against other vegetable oils, e.g. soybean oil. Except for high oleic acid content, other oil quality components and value addition traits such as stearic acid, carotene or tocotrienols were given lower

Table 11.2 Priority list (top four traits) of desirable traits for genetic improvement of oil palm in Malaysia

Priority	Trait	Rationale
1	High palm oil yield	Commodity crop, mainly used as food. Lower cost of production. Oil already versatile in its uses.
2	Dwarf stature	Current palm varieties grow too tall too fast. Inefficient harvesting. Scarcity and high cost of workers.
3	Ganoderma resistance	Ganoderma basal stem rot is becoming a serious problem in young second cycle plantings, decimating the stand. Cultural and chemical controls are ineffective.
4	High oleic acid	Healthy monounsaturated oil. Liquid oil in temperate countries for use in salad dressing and cooking. More suitable for production of oleochemicals and biodiesel.

rankings. This priority list of traits is perhaps most relevant to Malaysia. In African countries, drought resistance and Fusarium wilt resistance would have higher priority, as with resistance to bud rot in some Latin American countries. Difficulty of harvesting tall palms is currently not a constraint in Indonesia with abundance of cheap labour.

11.6.1 Oil Yield

High palm oil yield is still the prime goal of most if not all breeding programmes. However, there are many facets in terms of achieving a high yield.

Potential yield. This is the maximum yield achieved by a variety when grown under stress-free conditions to which it is adapted (Evans and Fischer 1999). Modern high-yielding varieties, resulting from a plant ideotype breeding approach are typically smaller statured plants that can be planted at higher density, are consequently capable of high biomass production and possess a high harvest index or better conversion of dry matter directed to yield rather than to vegetative growth. The combination of high biomass production and high harvest index results in a super high yield (Soh 2005). Such varieties are single cross hybrids maximizing heterosis and stand uniformity. Early oil palm breeding efforts were biased toward high early individual palm yield resulting in aggressive plant types, and their use in mixed hybrid planting would not fully exploit the potential yield of the crop. The ideotype approach in oil palm breeding has been advocated since the early 1980s and some early results from these efforts are available (Breure and Corley 1983; Squire 1984; Breure 1986; Henson 1998; Soh et al. 2006).

Harvestable/recoverable/realizeable yield. Harvesting oil palm fruit bunches particularly from older and taller (5–12 m) palms is still a tough and tedious manual operation (man with sickle on long pole) with no imminent prospective

mechanized alternative resulting in inefficient harvesting and crop loss due to poor loose fruit recovery and low quality of unripe, over-ripe and rotten fruits. Dwarf palms with thinner and longer bunch stalks would facilitate harvesting manually or mechanically. Non-shedding fruits would reduce loss of loose fruits (Osborne et al. 1992), while low lipase fruits would have longer 'shelf-life' (inhibiting the production of free fatty acids) thus reducing the need for more frequent harvesting rounds. Virescent fruits (green unripe, bright yellow ripe) would facilitate identification of ripe bunches especially in tall palms as compared to the common nigrescens fruits (dark purple unripe, orange red ripe).

Adaptability Genotype by environment interaction (G×E) is a function of both differential genotypes and differential environments. If either component is not differential, then a G×E effect cannot be detected. This would explain its non-detection in earlier oil palm studies (Rosenquist 1982; Cochard et al. 1993). With current hybrid progenies and clones being more genetically uniform and discrete, and as such materials are being planted across national borders and agri-ecological zones, G×E interaction will assume more importance, necessitating the breeding of genotypes with local adaptation (Donough et al. 1996).

Abiotic and biotic stress tolerance. Breeding for drought tolerance is integral in the oil palm breeding programmes in West Africa, as there is a long drought period annually (Houssou et al. 1987; Okwuagu and Ataga 1999). In Papua New Guinea, oil palms planted on sandy soils tended to exhibit Mg defiency and hence tolerance to Mg deficiency has been an important breeding objective (Breure et al. 1986). Basal stem rot caused by the *Ganoderma boninense* basidiomycete fungus has become a serious disease in the major oil palm growing countries particularly in young oil palm replantings from oil palm and coconut where it has become more prevalent (Ariffin 2000; De Franqueville et al. 2001). It is no longer a disease confined to high water table areas and affecting only older palms (>15 years old). Cultural and chemical control measures are cumbersome and ineffective. Disease tolerance is the long term solution requiring an important separate breeding programme sometimes involving multipartite collaborative research efforts (CAB International 1988; Breton et al. 2005). Sources of tolerance genes have been found in MPOB's Zaire × Cameroon accessions (Idris et al. 2005) and in advanced breeding populations by Durand-Gasselin et al. (2005). Nursery screening techniques have just been developed requiring refinement (Ariffin et al. 1995; Breton et al. 2005). Oil palm planted in West Africa not only needs to be drought tolerant but also tolerant to vascular wilt disease caused by *Fusarium oxysporum* f.sp. *elaedis*. Disease tolerance is available and its nursery screening is an integral part of the breeding procedure (De Franqueville and Renard 1990). Lethal bud rot decimates oil palm plantings in many parts of Latin America. O×G hybrids appear to be tolerant although the pathogenic cause of the disease is still contentious (Ariffin 2000).

11.6.2 Oil Quality

As palm oil's principal use is in food production, its dietary quality is under close scrutiny by the current health conscious consumers. Palm oil is often regarded mistakenly as a saturated tropical fat with the connotation that its consumption will lead to elevated levels of blood cholesterol and risk of cardiovascular heart disease (CVD). Although it contains about 42% saturated fatty acids, it is in the form of palmitic acid which is not known to be cholestrolemic; unsaturated fatty acids such as the monounsaturated oleic acid (40%) and polyunsaturates (linoleic and linolenic acids, ca. 15%) mainly comprise the rest. Oleic acid has been identified as a top priority trait for improvement because it confers a healthy liquid oil similar to olive oil that is marketed in temperate countries for salad dressing and as cooking oil. It also serves as a very useful feed stock for other oleochemical and biofuel industries. Palm oil also contains other useful organic components such as carotene (vitamin A), tocopherols and tocotrienols (vitamin E) as well as other antioxidants which not only confer health (anti-CVD, anti-cancer) properties to palm oil, but can also be isolated and spun-off in the health-care and cosmetics industries (Yusoff 2000; Khosla and Sundram 1996; Sundram and Chandrasekharan 2000).

11.7 Breeding Methods and Techniques

11.7.1 Breeding Methods

The oil palm is a cross-pollinated perennial tree crop. Not surprisingly, it has adapted breeding methodologies developed in maize, e.g. recurrent selection, and animal breeding, e.g. sire (pisifera) testing, index and BLUP selection, and a close temporal and genetic correspondence of commercial hybrid production with each cycle of breeding (Soh 1999).

The major oil palm breeding programmes adopt either the modified recurrent selection method (MRS; Soh 1999) or the modified reciprocal recurrent method (MRRS; Meunier and Gascon 1972). The former is practiced by programmes particularly in the Far East linked to or influenced by the programmes of the Unilever plantations group, while the latter is adopted by countries in West Africa and Indonesia advised by CIRAD.

In the *modified recurrent selection* scheme (Fig. 11.4), selection of Deli D parents for further breeding and for mother palms in commercial hybrid seed production is based on family and individual palm performances, hence Rosenquist (1990) chose to name the method as FIPS (family and individual selection). Tenera parent selection for further P breeding is also based on FIPS. Being female sterile, P is selected as the male parent for D×P hybrid seed production based initially on its T sib performance in the T×T family followed by a D×P progeny test by crossing it with a sample of usually 3–5 of the selected

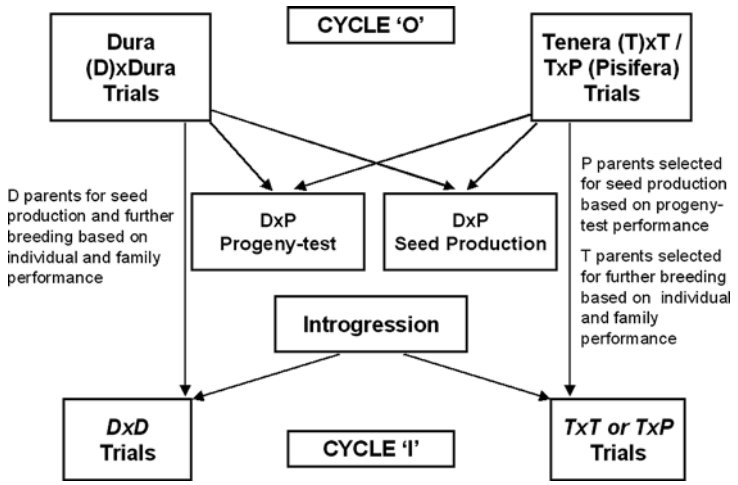

Fig. 11.4 Modified recurrent selection scheme in oil palm

D female parents in a nested mating design (NCM 1), i.e. top-cross or sire testing. The resultant commercial mixed hybrid is akin to a synthetic in the plant breeding literature (Simmonds 1979; Allard 1960) except that its subsequent progeny seeds should not be saved for commercial planting. The variability within the commercial mixed hybrids varies with the genetic diversity and inbreeding status of the parents used. The main advantages of this scheme are that more recombinant crosses and genotypes can be turned over within shorter time and smaller space without the need for extensive progeny tests, and that the genetic variability of the commercial hybrids would ensure a good adaptability and reduced risk of genetic vulnerability. This breeding scheme exploits only general combining ability (GCA), but not specific combining ability (SCA). Also, the assumption that the additive or GCA effects expressed within the parental D×D and T×T crosses will be reflected in the D×T/P inter-population hybrid yield performance may be untenable, especially when parents become more restricted and inbred (Soh 1999; Soh and Hor 2000).

In *modified reciprocal recurrent selection* (Fig. 11.5), parents selected for further breeding and for commercial hybrid seed production have been progeny tested. Instead of D×P progeny testing, a D×T progeny test of the selected D and T parents from the D×D and T×T families is done. To save time, selfs and sibs of the parents undergoing progeny testing are made and planted simultaneously as the progeny test crosses. The best hybrid crosses are identified from the D×T progeny test. The best hybrid is then readily reproduced as commercial D×P hybrid using the Ds from the selfs of the D parent and the Ps from the selfs of the T parent. This scheme exploits both GCA and SCA, and the

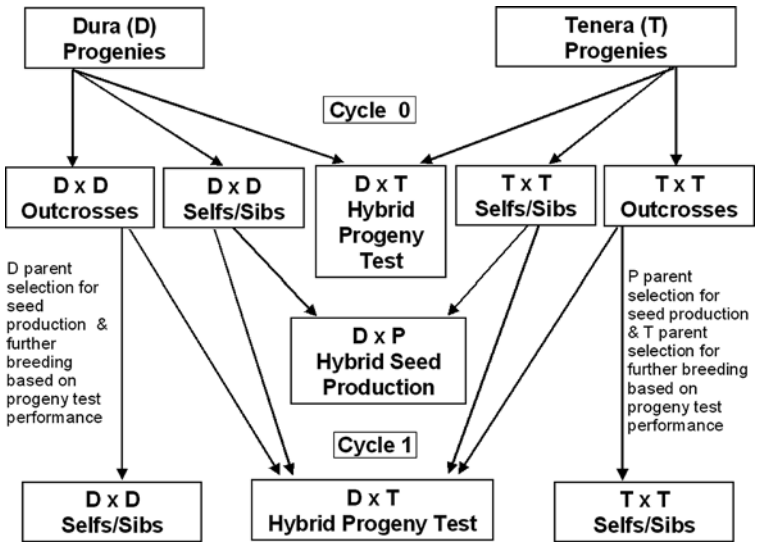

Fig. 11.5 Modified reciprocal recurrent selection in oil palm

commercial hybrid material is a reproduction of the progeny tested hybrid. Depending on the inbreeding status of the parents the commercial hybrids are generally more uniform or near true F_1 hybrids. The scheme encompasses a within-hybrid improvement phase to further refine a particular hybrid combination and a recombination phase involving outcrosses to maintain genetic variability for long term genetic improvement. The main disadvantage is the large programme size requiring 500 crosses and 180 selfs to be planted over 600 ha and evaluated over 15–25 years in order to select the top 15% crosses to be reproduced as 3–4 million commercial hybrid seeds (Soh 1999); the world's requirement of oil palm seeds is about 250–300 million seeds per annum. The other purported disadvantage is the severe inbreeding depression limiting selfing of the parents to only one generation and thus inhibiting expanded production of a particular hybrid. Hybrid maize breeding in the early years also faced this problem but has since circumvented it by selecting segregants more tolerant of inbreeding.

The *backcross breeding* method is usually applied in introgression programmes in which desirable traits from a relatively unimproved genotype are incorporated into an otherwise recurrent host genotype. The AVROS and the Dumpy.AVROS were developed in this manner, in the former the cross of SP540T with Bangun was backcrossed to SP540T and in the latter, the dwarf trait in the Dumpy Deli was introgressed into the AVROS by backcrossing (Soh et al. 2006). A similar approach is adopted in the introgression of desirable traits (oil quality traits, dwarfness, disease resistance) from the relatively wild or

unimproved recent *E. guineensis* and *E. oleifera* introductions to advanced breeding populations (Soh et al. 1999; Sharma 2000). It should be borne in mind that for a perennial tree crop backcrossing (in the strict sense) to the original recurrent tree is seldom possible but rather to the recurrent parental progeny or population.

In *recombinant inbred breeding*, which follows an earlier proposal based on the dominance theory of heterosis rather than on overdominance and epistasis (Pooni et al. 1989), programmes to develop recombinant inbred varieties have been attempted. In this approach, there is no necessity to separate the breeding parents into heterotic groups, and any superior recombinant genotype can be developed by single seed descent into an inbred variety. In theory, at least, one could achieve an inbred D variety superior to the T hybrid which could be propagated indefinitely by selfing. Others would rather pursue D and T recombinant inbred line development to produce second or advanced cycle hybrids as in maize breeding (Bernado 2002), perhaps based on faster commercial exploitation of every hybrid generation and proprietary right considerations (Soh 1987, 1999).

With *breeding for clonal propagation* in tree crops where vegetative propagation is possible, maximum genetic segregation is generated by crossing unrelated trees with complementary desirable traits. This is then followed by identifying the superior segregant progeny tree or individual genotype and confirming and fixing it through cycles of field testing and selection (Simmonds 1979; Tan 1987; Brown et al. 1988; Kawano et al. 1998). With the ability to clonally propagate oil palm via tissue culture, it is tempting to adopt this approach. That would require repeated cycles of cloning (from clones), the feasibility of which is still uncertain in the oil palm (Soh et al. 2003a). This approach would better be used as an adjunct to the main recurrent breeding programme, especially at the early D×P testing stage of the recombinant phase where the T progeny palms are still genetically segregating appreciably.

Index and BLUP selection. Tree crop breeding as cattle breeding has to contend seriously with space and time constraints and hence with the critical choice of the few highly selected parents to go into the next cycle of breeding and also to hybrid production. This would apply more so in the choice of parents or ortets for clonal propagation. Ascertaining the breeding values of the parents and the combination of multiple traits to select are thus important selection techniques (Falconer and Mackay 1996). The selection index method for multiple trait selection and for incorporating plot and family information was found to be useful for selecting ortets in oil palm and could be extended to breeding parents which are usually selected based on GCA for individual traits (Soh and Chow 1989, 1993; Baudouin et al. 1994; Soh et al. 1994). The selection index is constrained by the inability to obtain accurate estimates of genetic variance and heritability. The BLUP (best linear unbiased prediction) technique developed in cattle breeding (Henderson 1984) can integrate unbalanced data from mating and experimental designs and even production data and was used to obtain sire (P parent) breeding values in oil palm (Soh 1994). BLUP has also been used to

predict hybrid performance in oil palm (Purba et al. 2001), as in maize (Bernardo 1994, 1995, 1996) and sugarcane (Chang and Milligan 1992a,b).

11.7.2 Breeding Techniques

Breeding system. The oil palm is monecious bearing male and female inflorescences on the same palm occurring in different but overlapping cycles. Stress conditions such as drought, malnutrition or plant competition favour male inflorescence production (Corley and Tinker 2003). Pisifera palms do produce female inflorescences especially under good growing conditions, but they usually are aborted. Cross pollination in the oil palm is effected efficiently by the pollinating weevil, *Elaidobius kcameroonicus*, in its natural home in Africa. Prior to the weevil's introduction to the Far East, pollination occurred by wind and thrips (*Thrips hawaiiensis*) which was less efficient. Stringent controlled-pollination procedures had to be adopted after discovering that serious illegitimate pollination had occurred in the commercial hybrid seeds resulting in high frequency of D contaminants in young commercial T fields soon after the introduction of the weevils to the plantations in the Far East. In male and female inflorescences, anthesis and receptivity occur about 2 months after emergence or appearance. The pollen viability and receptivity periods of the male and female inflorescences are about 3–5 days. The set fruit ripens, changing colour from dark purple or black to reddish orange, in about 5 months.

Controlled pollination. The formalin surface-cleaned male inflorescence is isolated with a permeable terelene/canvas/paper bag about 7–10 days prior to anthesis. The anthesized male inflorescence is harvested and air-dried for about 3 h in an oven, chamber (38–39°C) or air-conditioned chamber; the pollen is shaken out, sieved and further dried in filter paper envelopes at 38–39°C in an oven or desiccator till 6% moisture content and stored in test or specimen tubes in a freezer. Properly processed pollen can retain its viability for 6 months up to a year; freeze-drying is recommended for longer storage. Isolation of the female inflorescence is done in exactly the same manner. Controlled pollination is carried out when the female inflorescence is observed to be receptive, by puffing a 1:10–1:20 pollen: talcum powder mixture through a hole made in one or more plastic windows sown onto the isolation bag (Fig. 11.6). The bag is removed after a month and the fertilized bunch allowed to ripen in about 5 months. As a stringent quality control measure against illegitimate pollination, the inflorescence is discarded if there is any hole or tear in the bag or the presence of weevil spotted inside the bag.

Seed germination. The oil palm seed is recalcitrant and requires heat treatment at 37–38°C and 17–19% moisture content for D seed (20–21% for tenera seed) for 40–60 days for germination at ambient conditions after rehydrating to 21–23% moisture for D (27–28% for T seeds). Germination of very thin-shelled T and shell-less fertile P seeds is erratic (Arasu 1970) and in vitro germination (embryo rescue) is advisable.

Fig. 11.6 Controlled pollination in oil palm

11.7.3 Field Experimental Techniques

11.7.3.1 Mating Designs

Biparental (BIP), nested (NCM1 or North Carolina Model 1), factorial (NCM2) and diallel are the usual mating designs adopted in the parental D×D and T×T/P within population crosses as well as the D×T/P interpopulation progeny test crosses (Soh and Tan 1983; Soh 1999). BIP is more favoured in the parental crosses as it would allow more cross combinations to be tested when estimates of combining ability are not critical. NCM1 and NCM2 matings are commonly used in the progeny test crosses, the former favoured if GCAs of the parents are of primary interest and the latter favoured if SCAs and specific combinations are sought. BIP would also allow the most cross combinations to be tested if the objective is to seek the best combination irrespective of whether it resulted from GCA or SCA effects. Owing to the difficulty to obtain a complete set of the desired matings (NCM1, NCM2) with a large number of parents, balanced incomplete and inadvertent unbalanced designs are made. General least squares (GLM) and maximum likelihood (REML or restricted maximum likelihood, BLUP) methods are used to analyze such experimental data.

11.7.3.2 Field Experimentation

Experimental layout. The randomized complete block design (RCBD) is most commonly used in field trials. Owing to poor seed germination in some crosses, incomplete blocks commonly result and missing plot or GLM analysis is

needed. Augmented designs (Federer et al. 2001) would also be useful in such situations. Incomplete block designs, e.g. the balanced lattice (Cochran and Cox 1957) to circumvent soil heterogeneity effects within large blocks associated with large sets of crosses have been attempted in oil palm but were found to be cumbersome and did not improve trial efficiency much (Soh et al. 1990). Plot sizes of 10–20 palms (without border trees) planted in 3–6 replicates are commonly used with the large plots preferred if between-cross differences especially in growth habits are suspected. When the trial is replicated over locations, 2–3 replicates per location would suffice. Soh et al. (1989, 1990) found that a trial size of 6 replicates of 16 palm plots or 5 replicates of 20 palm plots is suitable to detect treatment differences of 15% with a trial coefficient of variation of 10% under Malaysian conditions. Single palm plots have been found to be not efficient unless highly replicated to about 100 times, and they are also not experimenter-friendly for field visual appraisal. Single palm plots in completely randomized design (CRD) would be useful in field screening for disease resistance and for estimating between palm within progeny variability in genetic studies.

Data collection. The following data are collected on an individual palm basis: For vegetative growth measurements (Corley et al. 1971; Corley and Breure 1981), height, girth, leaf production, leaf area and weight measurements are made annually. For fresh fruit bunch yield (FFB), number and weight of ripe bunches are recorded in each 10 day harvesting round. Yield data is collected from start of harvesting at about 3 years after field planting till about 9 years. For bunch analysis (Blaak et al. 1963), bunches are sampled over the yield recording period to determine bunch and fruit quality and oil content. Fruit sub-samplings are made to estimate the ratio of fresh fruit weight in the bunch (F/B%), the ratio of mesocarp weight in the fruit (M/F%), the ratio of kernel weight in the fruit (K/F%) and the ratio of oil weight in the mesocarp (O/M%). The product F/B × M/F × O/M gives O/B% (oil to bunch) and F/B × K/F gives K/B (kernel to bunch). For the determination of a progeny mean for O/B% about 160 bunches from about 40 palms are sampled over the yield recording period. For individual palm means usually more than 5 analyses are needed. The following trait data can be generated from the above measurements:

Vegetative growth: girth, height and height increment, total leaf production, leaf area index, leaf area ratio, vegetative dry matter production (VDM).
Yield: bunch dry matter yield, BDM = FFB × dry matter/fresh matter.
Bunch analysis: oil yield, OY = FFB × O/B; kernel yield, KY = FFB × K/B; kernel oil yield, KOY = FFB × K/B × 0.5. These are reflective of the commercial oil extraction, kernel extraction and kernel oil extraction rates achieved in the mills. Total dry matter production, TDM = BDM + VDM; bunch index, BI = BDM/TDM; harvest index, HI = BI × 0.4.

All these primary yield, yield component and morpho-physiological traits, either formally based on measured data or visually/intuitively scored, are taken

in consideration in most oil palm breeding programs, besides oil quality and stress resistance in others.

11.8 Integration of New Biotechnologies in Breeding Programmes

11.8.1 Tissue Culture for Clonal Propagation of Oil Palm

The commercial oil palm planting material being a heterogeneous mixture of non-uniform hybrid progenies is genetically variable. Individual palms within a commercial planting can yield considerably more than the field average. Reproducing these superior individual palms by conventional hybrid breeding would take more than 20 years. Thus the original objective of clonal propagation in the oil palm is to short-cut this process by capturing elite individual palms from the genetically variable planting material as clones and mass-propagate them for large scale commercial planting.

Despite the early success in developing the tissue culture clonal propagation technique and the subsequent expanded efforts in clonal propagation for trial and pilot field tests, large-scale commercial propagation and planting of proven elite clones until very recently have yet to take-off. To do this successfully, a number of critical issues including somaclonal variation, cloning efficiency, ortet (parent palm of clone) selection efficiency, recloning and the suspension culture system need to be resolved or circumvented (Soh et al. 2003a).

Somaclonal variation in the oil palm occurs as mantled parthenocarpic fruits resulting in bunch abortion and sterility (Corley et al. 1986). This has been the main stumbling block for commercial in vitro propagation of oil palm for the past 20 years and continues to be so for expanded scale commercial production (Fig. 11.7). Susceptibility varies between and within clones, and the risk tends to increase with extended culture, recloning or liquid culture. The current explanation of the causal mechanism is that of an epigenetic change involving methylation of the homeotic or flowering MADS box genes (Van der Linden et al. 2005; Auyong et al. 2005). Putative markers were found to be clone specific and thus of no general applicability.

AAR (Applied Agricultural Resources Sdn Bhd) pioneered the development of the commercial gel culture propagation method through research into protocol development and refinement to improve the cloning efficiency with minimal risk of the mantled fruit clonal abnormality and by cloning a package of ortets. This method, although adopted by other laboratories for commercial propagation, is still an inefficient process being hampered by the low percentage of palms amenable to mass propagation, low plantlet production capability and high labour and space requirement. Commercial propagation is achieved by putting a large number of ortets into culture each time to circumvent the cloning inefficiency and to hedge on clonal abnormality risk. This approach requires a

Fig. 11.7 Somaclonal variation in oil palm: mantled parthenocarpic fruits (*left*), bunch abortion and sterility (*right*)

continuous supply of superior ortets and compromises on the maximum yield potential of clones achievable.

Ortet or individual palm selection is inefficient for oil yield improvement because of its low heritability. Good clonal testing is mandatory to identify outstanding clones. To exploit these outstanding clones as proven clones recloning is needed. AAR pioneered in the feasibility of recloning contrary to other earlier experiences with higher clonal abnormality risk. Recloning is still constrained by the inefficiency of the gel culture system. AAR subsequently developed and demonstrated the feasibility of the liquid suspension culture system for both clones and reclones again contrary to the high abnormality risk experienced by others. The advantages of the liquid system are its very high capacity for uniform plantlet production and its amenability to automation including the use of bioreactors. With these advances the technology for commercial production and planting of proven superior clones is in place.

Nevertheless, the ability to scale up clonal production with minimal somaclonal variation to capture a significant proportion of the world's large hybrid seed market (ca. 250 million) poses the biggest challenge (Soh et al. 2006).

11.8.2 Tissue Culture Process

The tissue culture cloning process in oil palm (Wong et al. 1997; Soh et al. 2003a, 2006) involves the following stages (Fig. 11.8) in a commercial laboratory (Fig. 11.9):

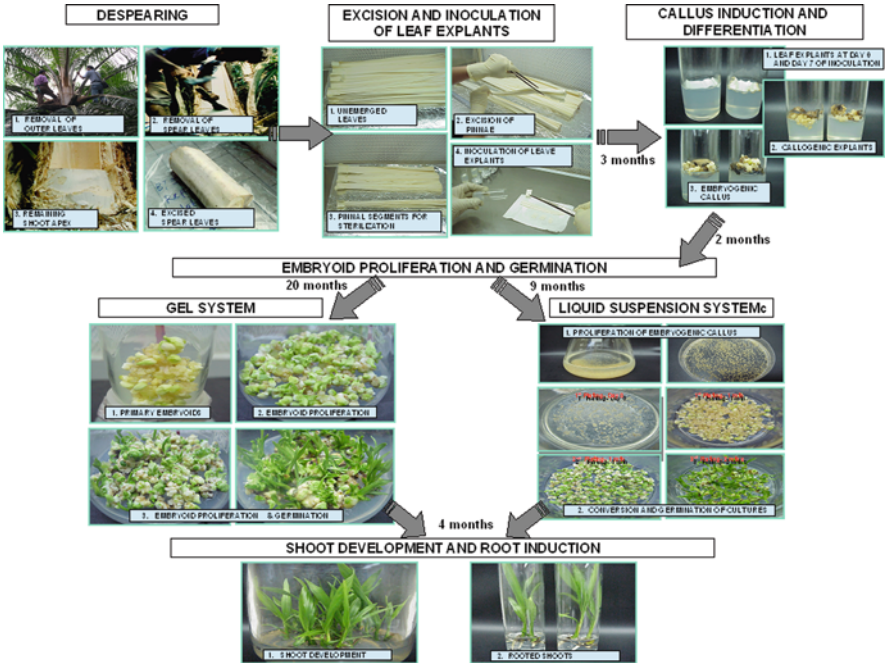

Fig. 11.8 Oil palm tissue culture system (gel and liquid systems)

Fig. 11.9 Commercial oil palm tissue culture laboratory with two million plantlet production capacity (Applied Agricultural Resources, S.B., Malaysia)

Explant sampling. The explants are the young leaf pinnae of the youngest non-emerged spear leaves of the selected palm. The harvested spear is surface-sterilized before the leaves are unraveled in the laminar flow chamber and 1,000–2,000 explants of 1 cm^2 pinnal segments are inoculated onto agar medium in test tubes.

Callogenesis. Callus growth is induced on the explant using MS (Murashige and Skoog 1962) medium which may be supplemented with additional nutrients, variable levels and proportions of phytohormones, typically 2,4-D and/or NAA and with or without the addition of charcoal. Some laboratories also apply explant pretreatments. The explant cultures are incubated at ambient conditions (26–28°C, 90% relative humidity) in the dark or in lighted rooms. Calli begin to appear on the leaf explants at about 3 months and continue proliferation up to a year or more.

Embryogenesis. Callus differentiates into somatic embryos or embryoids when nodular callus on explants is sub-cultured on medium with reduced levels of hormones to stimulate callus differentiation into embryoids or somatic embryos in air-conditioned culture rooms maintained at 26–28°C and 50–60% relative humidity.

Embryoid proliferation and germination. Good embryoids are transferred onto embryoid proliferation medium and sub-cultured usually at bimonthly intervals. In the case of liquid suspension culture, embryogenic calli are transferred to liquid medium in a conical flask on an orbital shaker for proliferation. At each monthly sub-culture, embryoids of the desired size are sieved out and transferred to fresh medium.

Plantlet regeneration. In the gel system, shoots begin to germinate at about the 5th sub-culture on the polyembryogenic mass consisting of secondary embryoids, primary embryoids and callus. The shoots are harvested from the polyembryogenic mass and put onto a liquid medium in a test tube for shoot development and root induction. The polyembryogenic mass is put back onto fresh medium for further proliferation. In the liquid suspension system, mature embryoids are inoculated onto plated medium for germination. The shoots are subsequently transferred individually into tubes for growth and root induction as for the gel system.

Acclimatization. Rooted plantlets of about 8–12 cm height are selected and planted out in trays containing inert potting mixture, e.g. sand, peat fibre, vermiculite in a plastic incubation chamber in the conditioning nursery with 75% shade, 90% relative humidity and 27–30°C temperature. Shade and humidity are gradually reduced by opening the flaps of the chamber for increasing lengths of time to condition the plantlets. After 3 weeks the trays of plantlets are taken out from the plastic chamber and put under 50% shade to be hardened and foliar fertilized. Hardened 3–4 leaved plantlets are harvested, cleaned of its potting medium and treated with a fungicide before dispatching as 'bare root seedlings' contained in moistened plastic bags packed in carton boxes to the plantation nurseries, where they are raised as for normal seedlings.

11.8.3 Commercial Planting of Oil Palm Clones

The commercial planting of oil palm clones, totaling less than 20,000 ha out of the world's total oil palm planting area of 14 million ha, can be considered in its infancy or at only a larger scale pilot commercial testing state (Fig. 11.10). The clones are produced by a handful of commercial tissue culture laboratories with

Fig. 11.10 Nursery and field plantings of oil palm clones

only a couple dominating the production, although more laboratories have been set up lately. The clones are usually derived from ortets selected from D × P/T progeny test trials or mass selected from commercial fields with the former preferred. Depending on the laboratory, reclones (reproduced from proven clones) and clones from liquid suspension cultures may also be included. Preferably, a package of 5 different clones is to be planted at the same time to hedge against abnormality (somaclonal variation) and inefficient yield selection risks. To ensure adequate pollination for plantings on good areas, inclusion of low sex ratio (lower female inflorescence production) clonal or hybrid palms in the clonal package is usually practised.

11.8.4 Clonal Fidelity and Performance Tests

As evident from the above there are still a number of unresolved issues in both the commercial production and planting of clones, especially related to cloning efficiency, clonal fidelity (abnormality risk) and yield selection efficiency. Hence research is still ongoing to develop and fine-tune protocols in cloning, recloning, gel or liquid cultures, and techniques in selecting and testing ortets and clones as well as planting system configurations to maximize the yield potential of clones in the field (Soh et al. 2003a). For fidelity testing, from each clone/protocol treatment about 40–100 plantlets may be sampled representing different levels of plantlet production and planted in the nursery and the field at closer spacing to observe for clonal fidelity, i.e. for normal or abnormal behaviour (e.g. mantled abortive bunches, sterile palms, abnormal vegetative growth, terminal inflorescence or premature flowering in the nursery). Plantlets from proliferating cultures from good ortets and treatments are also planted in clone test trials and commercial test plantings (which may include different planting arrangement treatments) for observing yield performance and field management efficiency besides clonal fidelity.

11.8.5 Molecular Breeding

Molecular markers are now widely used in plant breeding, and oil palm breeding is no exception since the oil palm is a perennial tree crop where savings in breeding time, space and effort are much sought after. DNA from oil palm is obtained mainly from leaves, although it is also possible to isolate it from other tissues (Lim and Rao 2005). The detection and determination of markers in oil palm is possible through molecular biology techniques such as isozyme (Rajanaidu et al. 1993; Choong et al. 1996), RFLP (Cheah 1990; Mayes et al. 1996), RAPD (Shah et al. 1994), AFLP (Cheah 2000; Kulratne et al. 2000) and SSR-PCR (Billotte et al. 2001, 2005; Chua et al. 2005; Phoon et al. 2005). RFLP, AFLP and SSR markers are particularly robust, reliable and were

mainly used to saturate the majority of oil palm genetic maps (Mayes et al. 1997; Billotte et al. 2005; Ting et al. 2005).

Some of these polymorphic markers are used for oil palm DNA fingerprinting, particularly useful for genotype identification and genetic diversity assessment (Cheah and Wooi 1995; Cheah et al. 1999). As concerns about breeder's rights on planting materials increase, Malaysia has gazetted the Plant Variety Protection Act in 2004. The Malaysian Department of Agriculture aided by MPOB and industry members is in the process of drafting test guidelines for the conduct of tests for distinctness, uniformity and stability in compliance with UPOV (Union for the Protection of New Varieties of Plants). DNA fingerprinting for genotype identification is identified as one of the elements to complement morphological markers. Of particular interest are elite clones and inbred parents for hybrid seed production that need protection from piracy and subsequently ensure profit return to the rightful breeder. Discriminating commercial 'varieties' of oil palm is envisaged to be more difficult as they are mixed hybrids from parents with very similar genetic backgrounds.

In advanced breeding programmes, it is becoming increasingly difficult to select for extreme outliers because of the highly selected nature of the genetic materials. Genetic diversity studies with the estimation of genetic distance or relatedness between oil palms can help identify suitable divergent parents for base broadening breeding or for crossing to enhance hybrid vigour in the progeny or seed produced. The same can be applied to inbreeding programmes to assess homozygosity or inbreeding level of the resulting materials and in backcrossing programmes to recover the recurrent genotype. Genetic diversity studies can assist in hastening the prediction of parents with good combining ability in that a pre-selection of materials can be included in a breeding trial to expedite creation of homozygous inbred parents.

The ability of molecular markers to assess relatedness also introduces the possibility of assessing illegitimacy of a cross or a commercial hybrid (Corley 2005). With increasing efforts and expenditure invested in oil palm breeding programmes and commercial field planting of hybrids, illegitimacy in the breeding and commercial hybrid materials cannot be tolerated. With molecular marker technology, crossing programmes can be quality-checked and breeding as well as commercial hybrid seed production will become more efficient.

With the construction of genetic maps, it is possible to identify the genomic location of specific traits by using genetic markers. Marker based selection can be used as an early screening technique for specific traits of interest such as desirable fatty acids, amenability to tissue culture, fruit colour, shell thickness, stem height and mature frond length, even before the palms mature or before the trait is expressed. Marker assisted selection can help reduce the breeding trial size thus increasing the chances of selecting desirable extreme outliers for a given trait and thereby expedite the improvement process. Single genes control fruit colour and shell thickness, therefore single markers can be developed for their selection (Mayes et al. 1996; Moretzsohn et al. 2000; Billotte et al. 2005). The issue at hand is whether the marker is close enough to the target gene to

prevent recombination and thus is reliable. For a marker for virescent fruit it appeared to be so, but not for the shell gene. Other traits mentioned above are likely to be controlled by multiple genes or quantitative traits and several markers are needed for their selection. Markers controlling quantitative traits can be found in the same or across different linkage groups. The location of these quantitative trait loci (QTL) can be identified using fine mapping techniques, such as the bulked segregant analysis (BSA) as described in Weising et al. (2005). Currently, the interest is to analyze single nucleotide polymorphisms (SNPs) to further saturate the genetic map so that more precise markers may be located (Rajinder and Cheah 2005).

Fluorescent *in situ* hybridisation (FISH) was used to detect collectively the amount of *E. oleifera*'s DNA in interspecific crosses of *E. oleifera* (O) with *E. guineensis* (G). The whole genome of O can be labelled, hybridised to the O × G genome and detected with fluorescent dye. Chromosomes that contain genome from O will show fluoresce under fluorescent microscope revealing the composition of genetic material from both parents. The inheritance of genetic material in the F_1 hybrid is 50% from each parent. However, as reintroduction of desirable G characteristics by backcrossing with G continues, percentages of genetic content from O would gradually reduce from 50 to 0%. Monitoring with FISH therefore enables targeting of progeny with the desirable genetic composition and reduces the number of backcrosses needed (Maria et al. 1998a,b; Cheah et al. 1999) prior to screening for other desirable traits (e.g. oil quality), which is essential in long life cycle crops.

As alluded to earlier, the current thought on the cause of the floral abnormality somaclonal variant is that of methylation (Jaligot et al. 2005) and alteration in the expression of MADS box gene (Van der Linden et al. 2005). In order to detect methylation in the MADS box gene, use of methylation sensitive MADS-box directed profiling is currently under investigation (Auyong et al. 2005).

There has been also an increasing interest in the expression marker system for the detection of complicated biological events such as embryogenesis and floral abnormality. These markers should detect expression level changes coinciding with a biological event, making it possible to detect an event of interest. Genes involved in embryogenesis have been isolated from cDNA libraries (Ong-Abdullah et al. 2005). Currently, more genes are to be analysed and identified using the microarray technology (Low et al. 2005), which can screen through thousands of expressed genes in one single analysis. Several genes involved in floral abnormality and embryogenesis have been identified and are undergoing expression studies and functional analysis.

Genetic transformation of oil palm via biolistics is now a reality with BASTA-resistant oil palm produced (Ghulam Kadir et al. 2005) and used as a selection means for transformation. Of particular interest is the creation of oil palms that produce desirable fatty acids. This can be done by enhancing and/or suppressing key genes that produce certain fatty acids. Some of these key enzymes are: β-ketoacyl-ACP synthase II (KASII) which catalyses conversion of palmitic acid to stearic acid, stearoyl-ACP desaturase which

catalyses conversion of strearic acid to oleic acid, palmitoyl-ACP thioesterase and oleoyl-ACP thioesterase which cleaves palmitic and oleic acid respectively from acyl carrier protein (ACP). Other novel genes of interest to be inserted into oil palm are bioplastics producing genes. These genes' expression can be targeted to leaves (e.g. bioplastic genes) or mesocarp (fatty acid synthesis genes) using suitable promoters. Nursery plants of such putative transformants are available awaiting field testing for stable trait incorporation and expression as well as in biosafety requirements. Research in *Agrobacterium* transformation has also been initiated in view of the inefficient transformation process associated with the biolistics approach yielding chimaeras and partial or multiple copies of the transgene.

11.9 Commercial Seed Processing

The various steps of oil palm seed processing are described according to Periasamy et al. (2002):

Depericarping. The ripe bunch from controlled pollination harvested from the D mother palm is chopped to separate the fruit spikelets which are then allowed to ret for days in a basket or gunny/raffia sack to detach and soften the fruit. The mesocarp of the fruits is then stripped off using a depericarping machine to give the fresh seeds. Fresh seeds are then further cleaned of the adhering fibres, washed with a detergent and treated with a fungicide.

Fresh seed quality checking. A sample of the seeds is taken from each bunch, the shells cracked and the kernels and embryos scored for quality. The kernels must be present, fresh looking and not diseased or rotting. The embryos must be well-formed and not shrivelled, cylindrical in shape and white in colour with a pale yellow green tinge. Bunches with more than 80% good quality seeds are acceptable for further processing into commercial seeds.

Seed moisture adjustment and storage. A sample of seeds from each bunch is checked for its moisture content. The seeds from the bunch are then soaked or surface air-dried to achieve 18–19% moisture content for storage in an air-conditioned room at 21°C.

Heat treatment. Stored fresh seeds (at about 18–19% moisture) when required to be germinated are sent to the germinator (hot room) to be heat treated at 37–39°C for about 50 days. The heat treated seed can be directly processed for seed germination or cold stored again as 'preheated' seed.

Resoaking and germination. Preheated seeds need to be remoistened to 21–22% moisture before setting them to germination at ambient conditions. Germination begins after a week with weekly flushes for about 6 weeks. With good quality seeds, more than 90% germination rates would be achieved by the third flush.

Seed selection and dispatch. Germinated seeds/seedlings with pearly white plump and balanced plumules and radicles of <5 cm length are selected and

collected in plastic bags with 200–250 seeds in each. These are then packed in polystyrene-lined and bead buffered carton boxes for dispatch. With current ease of air and surface transport systems, commercial oil palm seeds are now exclusively dispatched as germinated seeds all over the world instead of pre-heated seeds.

11.10 Oil Palm Seed Market

The world trade in oil palm seed is about 250–300 million seeds worth US$ 100 million at around 50 US cents per seed. The main supply and demand is in Indonesia with about 117 million seeds, Malaysia with 85 million seeds and all others with 85 million seeds as well. The break-down of the oil palm seed suppliers, many of which are plantation-based companies and their estimated capacities are given in Table 11.3.

The high crude palm oil prices boosted by oil demands for biofuel have prompted the opening of more land in Indonesia and other countries for oil palm cultivation resulting in increased demand for oil palm seeds, and consequently many seed companies have stepped-up production. The supply of oil palm clonal planting material from about 6–8 tissue culture laboratories is still

Table 11.3 Oil palm seed production companies and their estimated annual seed supply

Country	Company	Estimated supply (in million seeds)
Indonesia	Indonesian Oil Palm Research Institute (IOPRI)	35
	PT. Socfm Indonesia	20
	PT. London Sumatera Indo	20
	PT. Bina Sawit Makmur	20
	PT. Tunggal Yunus Estate	6
	PT. Dami Mas Sejahtera	10
	PT. Tania Selatan	6
Malaysia	Felda Agricultural Services S.B.	27
	Guthrie Research Chemara S.B.	20
	Golden Hope Research S.B.	10
	United Plantations Bhd.	8
	Applied Agricultural Resources S.B.	6
	Ebor Research, Sime Darby Plantations Bhd.	3
	EPA	3
	Sawit Kinabalu	3
	Others	8
Costa Rica	ASD	20
Papua New Guinea	Dami	20
Africa	Ivory Coast, Zaire, Nigeria	17
Thailand	Univanvic	5
South America	Colombia, Brazil, Ecuador	14

very small constituting less than 1% (ca. 2 million) of plants and may rise to 5–10 million in 5 years time; the high price and limited supply of cloned plantlets is attributed to the still inefficient commercial tissue culture process.

11.11 Concluding Remarks

At a still rapidly increasing population particularly in the developing countries, food oil consumption is expected to rise both due to increased population and rising affluence. Additionally, there is an increasing demand for vegetable oils to produce biofuels in response to high fossil fuel prices and environmental concerns on the continuing dependence on mineral oil. Palm oil being the cheapest oil that can be produced sustainably is likely to meet the bulk of this need. Oil palm plantations are likely to increase appreciably and existing old plantings will be continuously replaced with new improved varieties. As such, oil palm breeding and its research will continue to expand utilizing both conventional and biotechnological approaches.

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Chapter 12
Coconut

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12.1 Introduction

The coconut palm, *Cocos nucifera* L. is now grown mainly by smallholders but was once the major perennial plantation oil crop widely cultivated in the humid tropics. It has a pan-tropical distribution, occurring in coastal areas between the latitudes 20° north and south of the equator and at altitudes between sea level and 1,200 m. Coconut grows best under conditions of high humidity, at temperatures of 27–30°C and on moderately to well-aerated soils.

Coconut is the most extensively grown and used palm in the world and about 10 million families in over 80 countries rely on coconut as their main source of food and income. Coconut is a smallholders' crop and a major proportion of the production is usually consumed locally. The actual percentage of domestic consumption of coconut in Asian and Pacific Coconut Community (APCC) countries was around 64% in 2001. Coconut is mainly an oil crop, particularly rich (48%) in lauric acid (Jones 1991). Virgin coconut oil (Bawalan and Chapman 2006), expelled under low heat from fresh coconut meat to preserve its natural vitamins and enzymes (Marikkar et al. 2007) is now becoming popular in the pharmaceutical industry and is gaining a significant international market. Coconut as a bio-fuel is also a newly emerging product which would reinstate coconut palm as a valuable oil crop in the not too distant future. Already people on the island of Bougainville in Papua New Guinea are powering up their vehicles and generators with environmentally friendly coconut bio-fuel. In addition, the coconut industry offers a wide range of coconut products such as desiccated coconut, coconut cream, coconut milk powder, defatted coconut, coconut fibre and fibre products, shell charcoal and activated carbon, coconut vinegar and coconut arrack (liquor) etc. for the local and the international markets. Tender coconut as a natural beverage is presently gaining popularity and currently there is also a growing international

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market for fresh coconut water. Fresh coconut water is already widely used as a natural beverage in some coconut producing countries, India being the largest nut water consuming country whilst in Brazil, in the state of Sao Paulo alone, a daily consumption of 100,000 nuts has been estimated. Accordingly there is a vast interest in growing dwarf coconut varieties for water utilization. Coconut oil is also the principal raw material used in the manufacture of soap, glycerine and margarine. The lauric acid from coconut oil is used to manufacture detergents, cosmetics and pharmaceuticals. Locally the trunk is made into furniture, handicrafts and building materials, the fibre from the nuts for mattresses, doormats and ropes. Coconut as a whole plant, particularly the colour forms of dwarf varieties, are a tropical ornamental, much in demand as a signature tropical landscape element (Meerow and Ayala-Silva 2006). Because of the multiplicity of uses of the coconut palm, it is termed 'one of Nature's greatest gifts to man' (Burkill 1966) and also as 'The Tree of Life'.

The estimated total area under coconut in the world in 2001 was 11.8 million ha. The contribution of the major coconut growing countries in terms of land area cultivated with coconut are Indonesia (31%), the Philippines (26%), India (16%), Sri Lanka (4%), Thailand (3%), Tanzania (3%), Malaysia (2%), Brazil (2%), Papua New Guinea (2%), Vietnam (1%), Mexico (1%), and Mozambique (1%). Samoa, Micronesia, Fiji, Solomon Islands, Vanuatu, Palau, Bangladesh, China, Myanmar, Cocos Island, French Polynesia, Guam, Kiribati, Tonga, Comoros, Ghana, Ivory Coast, Madagascar, Nigeria, Tanzania, Colombia, Dominican Republic and Jamaica contribute the other 8% of cultivated coconut lands. More than one half of the coconut production however comes from Southeast Asia, mainly from the Philippines and Indonesia while almost one quarter comes from Asia, mainly from India and Sri Lanka. The remainder comes almost equally from American, African and Pacific regions (Asian and Pacific Coconut Community 1997).

12.2 Origin and Domestication

Cocos nucifera L. is a member of the monocotyledon family Arecaceae (Palmaceae) in the subfamily Cocoideae that includes 27 genera and 600 species and is the only species of the genus *Cocos*. Coconut possesses a diploid genome with 32 chromosomes ($2n = 2x = 32$).

There are conflicting theories regarding the origin and domestication of coconut. A number of theories supported a New World origin of coconut with subsequent dispersal to Asia and Polynesia (Guppy 1906; Cook 1910; Ridley 1930). For example, the centre of origin of Cocoid palms, the closest relative of coconut, is north–western South America. However, a theory for Polynesian and Asian origin has been postulated (Child 1964, 1974; Burkill 1966; Purseglove 1985; Dennis and Gunn 1971) and both Fremond et al. (1966) and Purseglove (1985) have provided convincing evidence of a Southeast Asian

origin and Indo-Pacific domestication for coconut based on ethnological and entomological evidence. Evidence from fossils and archaeological studies argue for a South–West Pacific origin of coconut (Child 1974; Purseglove 1985) while Indian fossils and the Madagascar forest coconut support an Indian Ocean origin (Chiovenda 1921; Mahabale 1976; both cited in Harries 1995). However, at present the origin of coconut is still not fully resolved but reports are available that wild specimens of coconut have been found growing in natural coastal forests in the Philippines and Australia supporting the theory that coconut originated in the Western Pacific. A possible region for coconut domestication was considered to be Melanesia, on the coasts and islands between South East Asia and the Western Pacific approximately between New Guinea and Fiji (Child 1964; Purseglove 1985) but more recently a submerged continental region of South East Asia (Malesia) has been suggested (Harries 1990, 2002). In an effort to explain the presence of close relatives of coconut in the New World, Purseglove (1985) suggests that the ancestral palm, which is likely to have had a fibrous mesocarp and be able to float and to establish itself under suitable conditions, could have been carried by ocean currents from South America to Polynesia in a similar route to that suggested for sweet potato (Purseglove 1965, 1968), and coconut may have evolved from that ancestor as a separate event in the Melanesian region. Harries (1978) describes a possible evolutionary process for coconut based on natural selection and evolution of large-fruited coconut from a small fruited progenitor. Gunn (2004) provides the most recent review on the phylogeny of the Cocoeae (Arecaceae).

Swaminathan and Nambiar (1961) suggested that dwarf coconuts (coconut palm with short stature, see varietal groups) may have originated as a result of inbreeding among tall coconuts as these show limited self-pollination, but this was not supported by subsequent cytological studies by Raveendranath and Ninan (1974). Purseglove (1985) states that dwarfs are probably mutations of tall types. However, Harries (1978) suggests that some characters of dwarfs (i.e. early germination, precocity, nut shape, different and bright fruit colours, the proportion of husk and for some dwarf forms the high resistance to lethal yellowing disease) indicate that domestication is more likely since dwarf populations could never survive in the wild (Harries 1995).

Coconut has been distributed to all parts of the tropical world including Central and South America, East and West Africa, South and Southeast Asia and the Pacific islands. From its putative centre of origin, coconut has been disseminated both east and west by floating in the sea and by human dissemination (Ohler 1984), particularly by the ancient Polynesian sea voyagers, who carried coconut as a source of food and drink on long sea voyages (Harries 1978). Whitehead (1976) also suggests that the spread of coconut from its putative centre of origin to Central and South America was the end result of the dissemination process and once established in a new vicinity, human activity accounted for further dissemination beyond the initial distribution range.

It is accepted that the coconut palm has been present on the Atlantic coast of Africa and South America and around the Caribbean for less than 500 years (Child 1974; Purseglove 1972) as there were no coconuts in those regions at the time of Columbus's voyages. There is, however, evidence that coconuts were on the Pacific Coast of Panama in pre-Columbian time (i.e. before 1492), either by early Polynesians carrying them or by ocean currents naturally distributing them. Harries (1978) observed a great similarity in West and East African coconuts pointing to a common source of coconuts for those regions. Coconuts in the East African coast were once thought to have either been brought there by ancient Arab traders or to have floated from India in the tenth century where they had grown for 2000 years, but according to more recent studies (Schuiling and Harries 1992; Krain et al. 1993), the presence of coconut palms could pre-date human activity. It is also possible that Malaysian sea-rovers, who reached Madagascar in the first century AD, took coconut with them to Madagascar and the Comoro islands (Lebrun et al. 1998). From there coconut subsequently reached the coast of East Africa. Coconut reached Cape Verde, West Africa only after 1500 AD through the Portuguese (Harries 1977; Purseglove 1985), probably from East Africa or India, and was then taken to the Caribbean and Atlantic Coast of America. European explorers after 1492 contributed to further dissemination of coconut by transporting them from Asia and East Africa to West Africa, the Atlantic coast of South America and the Caribbean region (Purseglove 1972). Zizumbo-Villarreal (1996), reviewing the history of coconut in Mexico, reported that the first introductions of coconut to the Atlantic Coast of Mexico were from West Africa and the Caribbean islands around 1549. He further reports that the introductions to the West Coast of Mexico originated from Panama around 1539, from the Solomon Islands around 1569 and from the Philippines from 1571 onwards by the Spanish during the Spanish colonial period. Plantations were developed throughout the tropics by the end of nineteenth century. Coconut populations that are now considered endemic to the Atlantic Coast of Africa, America and around the Caribbean region are basically the same as the coconuts in East Africa, India and Sri Lanka (Harries 1977) while coconuts in the Pacific Coast of America are related to the Pacific islands and Southeast Asian coconuts based on fruit component analysis data (Harries 1978; Vargas and Blanco 2000; Zizumbo-Villarreal et al. 2005).

12.3 Varietal Groups

The classification of coconut has been highly non-standardized, resulting in different authors in different countries using different terminology of coconut. However the major classification of coconut is based on stature and breeding behaviour which groups coconut broadly into two groups or types: tall (also termed *typica*) and dwarf (also termed *nana*), but Menon and Pandalai

(1958) were quoting Narayana and John (1949) who included *javanica* as another type of dwarf in India. Liyanage (1958), working in Sri Lanka, used similar *typica-nana* terminology, but discarded *javanica* and described *aurantiaca* as a new group. Neither of these South Asian classifications includes the Niu leka dwarf from the South Pacific. Tall types are the most commonly grown commercially exploited group and grow to a maximum height of about 20–30 m. They are predominantly allogamous (cross-pollinating), although a limited degree of autogamy (self pollination) has been reported for some tall groups (Bourdeix 1988; Bourdeix et al. 1990). The dwarf types in contrast attain a maximum height of about 10–15 m and are predominantly autogamous. Despite this higher degree of autogamy, dwarfs cross-pollinate with one another and with talls. The major differences between tall and dwarf coconuts are given in Table 12.1.

Table 12.1 Contrasting features of tall and dwarf coconuts

Character	Tall (*typica*)	Dwarf (*nana*)
Stature	Tall (about 20–30 m)	Short (about 10–15 m)
Bole formation at base of stem	Yes	No
Time till flowering	6–8 years	3–4 years
Economic life span	Long (about 80–100 years)	Short (about 40 years)
Bearing nature	Continuous	Seasonal
Nuts/palm/year	Average 40	Average 80–100
Copra amount and quality	200 g/nut, good quality	80–100 g/nut, poor quality
Growing conditions	Variable	Sensitive to climate changes
Breeding habit	Out-breeding	In-breeding

In addition to the tall and dwarf groups, a few intermediate groups, sometimes referred to as semi-talls or semi-dwarfs are also found. King coconut in Sri Lanka (Liyanage 1958), and to the understanding of the authors, Gangabondom in India (Menon and Pandalai 1958) and Niu Leka Dwarf in Fiji (Powell 1868; Bourdeix et al. 2005a) are examples of such intermediate groups. However the King coconut which has been classified as *aurantiaca* by Liyanage (1958) is totally different from the Niu Leka, and they cannot be classified in the same group.

As tall coconuts are predominantly cross-pollinated, they are highly heterogeneous and consist of unique individual genotypes. However, many coconut varieties (or cultivars) within the main group of talls exist that show certain general morphological resemblance within the variety but very slight dissimilarity or, in certain cases, contrasting dissimilarity between them. When there is dissimilarity it is generally attributed to fruit traits (size, shape and colour) and the proportions of the components of fruit (percentage weight of husk, shell, nut water, meat etc.) though in certain instances traits such as soft husk, sweet nut water, resistance to particular diseases (e.g. Vanuatu Tall

resistant to foliar decay virus and Sri Lanka Green Dwarf resistant to lethal yellowing disease in Ghana) also contribute to the differences. These cultivars often have different geographical origins and carry a prefix to the cultivar name of the country of origin, or the region of origin within a country, where they are originally and naturally grown. Earlier there had been no proper standardized nomenclature for coconut, whereas now each variety is given a unique international name, usually consisting of the group, whether it is a tall or dwarf, to which a geographical or cultural reference is added; for varieties of uniform colour, for instance, the different colour forms of the dwarf group, that colour information is also added. Examples of cultivars are West African Tall, Sri Lanka Tall, Mozambique Tall, Malayan Tall that originated from different countries and San Ramon, Tagnanan Tall, Markham Valley Tall that originated from various regions within a particular country. Examples of such cultivars in the dwarf group are Cameroon Red Dwarf, Sri Lanka Green Dwarf, Malayan Yellow Dwarf and Madang Brown Dwarf (Madang is a village in PNG). Furthermore, germplasm collections, field gene-banks and the Coconut Genetic Resources Network (COGENT) database have certain other entries termed as populations or accessions for the collections of coconut populations within a particular coconut cultivar, identified in different geographic locations. Examples of such populations are Panama Tall Monagre and Panama Tall Aguadulce, and West African Tall Mensah and West African Tall Akabo, Sri Lanka Tall Ambakelle and Sri Lanka Tall Kasagala. They are also sometimes referred to as ecotypes if the particular accession or population was a result of an environmental selection over several generations. In these populations or accessions the morphological differences are either not noticeable or very slightly detectable, but their adaptability for particular ecological niches or for a particular pest or disease resistance cannot be predicted from morphology.

Within the main varieties, numerous groups of coconut do exist which Liyanage (1958) referred to as forms of coconut and Bourdeix et al. (2005a) referred to as variants that are phenotypically highly distinctive. Some examples are Bodiri, Nawasi, Dikiri pol and Pora pol in Sri Lanka (Liyanage 1958), Laccadive Micro Tall in India (Bourdeix et al. 2005b), Spicata in different countries, Makapuno in the Philippines and Nim in Thailand.

Instead of the above varietal groups, Harries (1978) proposed two main types of tall coconuts; one that has large, long, angular, thick-husked and slow-germinating nuts with less free water content, the 'Niu kafa type' or wild coconut, that evolved naturally and was disseminated by ocean currents, and another that has more spherical nuts with an increased proportion of endosperm, reduced husk thickness, early germination and resistance to disease, the 'Niu vai type' or domestic coconut, that was selected under cultivation for increased nut water content and was disseminated by humans. Harries (1978) further suggests that introgression of these two types and further selection and dissemination by man gave the wide range of varieties and pan-tropical distribution of coconut seen today.

Whichever terminology is adopted, the authors are of the view that many of the named varieties, cultivars or populations reported in coconut literature were the result of either the vernaculars being used by local people and differences in the regions where they come from or because of slight morphological differences. For example, Whitehead (1966) has shown that coconut palms in Pacific islands are designated by several local names which do not always refer to distinct cultivars, but to small morphological differences. It is also possible that there could be some duplication of varieties in different coconut growing areas that are known by different names and hence classified as different varieties or populations. Therefore, until they are studied in detail by systematic morphological and molecular investigations, the true differences between varieties and populations remain unresolved.

12.4 Genetic Resources

In coconut, *Cocos nucifera* L., being the only species in the genus *Cocos*, there are no closely related wild relatives known. Although it is now generally assumed that truly wild type coconuts do not exist any longer, their characters are present in modern populations. Buckley and Harries (1984), Gruezo and Harries (1984) and Leach et al. (2003) reported wild types of coconut on uninhabited coral atolls, on small isolated islands or on remote mainland beaches. The domestic coconut is found associated with isolated or previously isolated human settlements and dwarf coconuts can be included in the domestic group, as they cannot survive in the wild. Present day coconut consists of a mixed complex of wild and domestic characteristics, depending on which of these forms predominated when cultivation began in each region.

Many different coconut genetic resources have been described by different authors (Narayana and John 1949; Gangolly et al. 1957; Menon and Pandalai 1958 and references therein; Liyanage 1958, Bourdeix et al. 2005a). There is a great diversity in fruit characters within and between populations for size, shape and the colour of the fruit and proportions by weight of fruit components viz. husk, shell, endosperm and water. Ashburner et al. (1997a) conducted a survey on diversity in fruit components of South Pacific coconut, and reported great diversity for fruit morphology in a range from populations exhibiting wild type characters to populations displaying domestication characters. Variation in fruit shape varies from near-spherical to short and long angular with different degrees of expression of a pear shape (Foale 1991). It is also observed that there is a great deal of variation in frond morphology, orientation of the crown, number and length of bunch, number of female flowers and number of nuts per palm even within populations (personal observations of the authors). It has been reported that there is more morphological diversity in Southeast Asia than there is in South Asia, Africa or South America (Benbadis 1992; Whitehead 1976). As a consequence, more named varieties are found in Southeast Asia relative to other areas.

Coconut genetic resources are currently threatened by a high rate of genetic erosion all over the world, mainly due to industrialization, urbanization, infra-structure development, changing use of agricultural land for high-value cash crops, and natural disasters such as cyclones, tsunamis, droughts, pests and diseases. Moreover, coconut development programmes involving the replanting of existing areas in regions of greatest diversity with fewer numbers of high yielding hybrids or improved varieties with a narrow genetic base threatens to displace older populations resulting in a further reduction of the genetic base. Therefore, collection and conservation of coconut biodiversity was nationally and internationally recognized as an important objective, as future breeding is based on collected material. As a result, the Coconut Genetic Resources Network (COGENT) was established in 1992 as a global network under the auspices of International Plant Genetic Resources Institute (IPGRI) with the objective of strengthening national programmes to conserve and utilize coconut genetic resources (http://www.cogentnetwork.org). Under this programme, COGENT, with funding from the Asian Development Bank (ADB) assisted by 13 Asia-Pacific countries in 1996 initiated the collection of coconut genetic resources and in 1997 extended support to Mauritius, Madagascar, Seychelles, Sri Lanka and Vietnam to collect and conserve threatened or useful coconut biodiversity. The COGENT participants also set up an international Coconut Genetic Resources Database (CGRD) in 1992 with the objective of construction of a computerized catalogue of accessions representing a large number of coconut cultivars spread throughout the coconut growing regions in order to gain an understanding of coconut diversity and thereby promote coconut germplasm exchange (Hamelin et al. 2005). The CGRD database was designed to provide passport, characterization and evaluation data for the coconut accessions in the database, and the number of accessions in the database by the year 2003 increased to 1426 compared to 500 in 1994. These 1426 entries included 599 tall cultivars, 111 dwarf cultivars and 1 semi tall cultivar, some cross-pollinating dwarfs and populations within cultivars. However, results obtained by molecular studies on 33 coconut populations in Sri Lanka, revealed a very low level of population differentiation (2%) indicating very close relationships between those populations (Perera et al. 2001). This poses the question whether same genotypes carry different accessions/codes in the collection, and this has become an issue to be resolved urgently. The recent book on 'Coconut Genetic Resources' (Batugal et al. 2005) published by IPGRI and the CGRD database provide a comprehensive coverage on the coconut genetic resources available throughout the world. COGENT has also initiated the establishment of four large multi-site international coconut gene-banks in Indonesia, India, Papua New Guinea and Ivory Coast as well as 28 national genebanks in 24 countries, in addition to the existing small international collections in Tanzania, Ivory Coast, the Philippines and India.

12.5 Major Breeding Achievements

As in many crops since early agricultural times, the coconuts grown all over the world were derived by mass selection and open pollination, using criteria determined informally by farmers themselves. The earliest selection of coconut dates back 8,000–14,000 years (Harries 2002) during which time coconut had been selected and domesticated for large round fruits rich in water as a source of sweet uncontaminated water for seafarers travelling from island to island. However, after commercialization of coconut during the nineteenth and twentieth centuries, the yield of copra per palm or one of its correlates have been the major criteria for selection of seed palms by farmers. The efficiency of mass selection of mother palms based on desirable characters has been studied extensively, and the progeny trials established in Sri Lanka occupied the most prominent place in early coconut breeding research and generated much information for developing criteria for selection of seed coconut palms. Most prominent of these is the estimation of heritability values for a number of useful characters in coconut (Liyanage and Sakai 1960) as effective criteria for selection of seed palms (Liyanage et al. 1988). The progenies resulting from open pollinated seeds are the basis of an improved population. For instance, the response to selection for de-husked nut weight by open pollination was a yield gain of 14.4% by selecting the best 5% of the population (Liyanage, 1972). Current seed palm selection criteria in Sri Lanka and elsewhere are developed from such observations. Early studies on coconut also provided information on some useful correlates between seed characters, period to sprouting and flowering, and initial yield and copra outturn. Seeds that sprout early promote seedling height, leaf and root number leading to a shorter flowering period and higher production of copra (Liyanage and Abeywardena 1957; Liyanage et al. 1988). Coconut nursery management practices all over the world adopt this concept for culling weak seedlings from nursery beds, i.e. rejection of late germinated seeds at a given period of time depending on the cultivar and removal of weak seedlings again after keeping in the nursery for a fixed period of time.

From the same progeny trials it was found that open pollinated progenies of certain coconut palms are uniformly high yielding giving a mean yield of about 35–40% more copra than the population mean. That phenomenon was explained as possessing of sufficient dominant yield traits in those palms to pass on to their offspring despite having been indiscriminately pollinated by unknown palms. Such palms were described as prepotent palms (Harland 1957). However, their identification is laborious and time consuming and relatively few palms prove to be prepotent from a large number tested, thus the quantity of seed nuts collected from them for the industry is negligible. With the assumption that progenies arising from artificial pollination using pollen of a prepotent palm as the male parent will be equally high yielding as the natural progenies of prepotent palms, seed production through artificial pollination of selected high

yielding mother palms from pollen of prepotent palms had been practised for the genetic improvement of coconut. Raising an enormous quantity of improved seeds demanded by farmers either by artificial pollination or stringent mass selection is impossible, so seed gardens were designed specifically for mass production of improved coconut genotypes. Since improved genotypes are produced by controlled natural pollination the concept of isolated seed gardens was well appreciated for mass production of superior genotypes (Liyanage 1954, 1961a). The Sri Lanka Tall × Sri Lanka Tall named as Ambakelle Tall or CRIC60 from the Isolated Seed Garden (ISG) at Ambakelle is an excellent example of such improvement attempts, which surpassed the yield of ordinary Sri Lanka Tall coconut (Liyanage et al. 1988).

Despite inherent constraints, the inter-varietal hybridization has shown the greatest gains in coconut breeding, demonstrating the usefulness of heterosis in coconut. The beginning of scientific coconut breeding came when the first controlled hybridisation was made in Fiji in 1926 between Malayan Red Dwarf and Niu Leka Dwarf (Marechal 1928). In India, the first hybridisation between tall and dwarf (West Coast Tall × Chowghat Green Dwarf) was attempted in 1930, with the intention of combining the quality of copra from the tall parent and the high productivity as well as early flowering from the dwarf parent. Sri Lanka initiated studies to test coconut hybrids in 1949 by assessing the cross between Sri Lanka Tall and Sri Lanka Green Dwarf (Liyanage 1954, 1972; Liyanage et al. 1988) and the productivity of this hybrid was remarkable – recording over 20,000 nuts per ha after 12 years from transplanting (Manthriratne 1971, 1972, 1978). Most of the hybrid tests were conducted between 1940–1960 and involved dwarf × tall (inter-varietal) and tall × tall (intra-varietal) crosses and in these studies the superiority of dwarf × tall over local tall cultivars was well established. But it was not until the mid-1970s that coconut F_1 hybrids became widely available in commercial quantities. After the first successful attempt (Harries and Romney 1974), many dwarf × tall hybrids have been produced utilizing different tall and dwarf cultivars originating from different geographical regions. The crosses Malayan Dwarf × Panama Tall (Maypan), Malayan Yellow Dwarf × West African Tall (PB121 or MAWA), Cameroon Red Dwarf × Rennell Island Tall (Maren), Malayan Red Dwarf × Tagnanan Tall (Matag or PCA15-2), Sri Lanka Green Dwarf × Sri Lanka Tall (CRIC65), Sri Lanka Green Dwarf × San Ramon Tall (Kapruwana) are examples of some of the most promising present day hybrids between dwarf and tall used in a wide range of environments. More details on recommended and preferred hybrids in different countries are described by Batugal (2005) and Bourdeix et al. (2005b).

Recently it has been shown that tall × tall hybrids, crosses between talls of different origins are high yielding though they are not promising in terms of early flowering (Bourdeix et al. 2005b). It must be noted that these crosses are different to Sri Lanka Tall × Sri Lanka Tall crosses which are combinations between superior palms of the same variety. The Sri Lankan experience between tall × tall hybrids using Sri Lanka Tall × San Ramon Tall was that they were

highly promising in terms of copra per nut as well as total copra production per unit area (Everard 2002) though they produced equally in terms of nut number as local tall selection CRIC60, but less than 40% of nut number as the dwarf × tall hybrid CRIC65. Some of the other famous tall × tall hybrids are West African Tall × Rennell Island Tall (PB213 or Waren) and West Afrian Tall × Vanuatu Tall (PB 214 or Wavan).

Although the first coconut hybrid tested in the world was a dwarf × dwarf hybrid (Marechal 1928), they are still the least exploited hybrids. However, the authors strongly believe that dwarf × dwarf hybrids are ideal trees for home gardens in urban areas to grow them as ornamental palms while meeting the daily coconut requirement if they show hybrid vigour for meat content per nut and the quality of copra. In Sri Lanka currently there is a demand for coconut varieties with short stature from people living in urban areas as palm height is a problem in small home gardens and picking is difficult in tall varieties. Though no follow up of Marechal's work has been documented, a success story of a cross between Malayan Yellow Dwarf and Malayan Red Dwarf varieties (PB332) produced in 1971 at the 'Marc Delorme' centre in Ivory Coast is reported by Bourdeix et al. (2005b), but no dwarf × dwarf hybrids have yet been widely distributed to farmers.

In terms of breeding for resistance to biotic and abiotic stresses, the inter-crossing of resistant/tolerant germplasm with adapted high yielding materials has been the strategy. All colour forms of Malayan Dwarf have been identified as lethal yellowing disease resistant cultivars and the hybrid Malayan Dwarf × Panama Tall (Maypan) is therefore in particular demanded for areas where the lethal yellowing disease phytoplasma occurs. Further, two Pacific coast tall cultivars have been identified as highly resistant to this disease known as amarilliamento letal in Mexico (Zizumbo-Villarreal et al. 1999). Other disease resistant types include Vanuatu Tall, identified for tolerance to the coconut foliar decay virus, and Sri Lanka Green Dwarf, for Cape St. Paul wilt tolerance (also caused by phytoplasma) in Ghana. In India breeding for root wilt disease is of high priority in the coconut breeding programme. Chowghat Green Dwarf and Malayan Green Dwarf have been identified by the Central Plantation Crop Research Institute (CPCRI), India as resistant varieties, showing a higher level of resistance to root wilt disease compared to other coconut varieties. The cross between Sri Lanka Yellow Dwarf × Sri Lanka Tall has been identified as a tolerant cultivar for *Aceria* mite by evaluating five commercially cultivated coconut cultivars in Sri Lanka in a severely mite affected area (Perera 2005, 2006). Sri Lanka Yellow Dwarf and Gon thembili cultivars have also recently been identified as tolerant cultivars to coconut *Aceria* mite (Perera 2006).

Drought is a serious constraint to coconut production in many countries as coconut is mainly a rain fed plantation crop. Hence breeding for drought tolerance has been given high priority in many coconut breeding programmes. In Sri Lanka, a selection based on mean yield and genotypic adaptation to changes in climate of the Sri Lanka Tall cultivar, correlating 15 years of individual palm yield data with 15 years rainfall data has identified a new

cultivar released under the name of Ambakelle Special (Wickramaratne 1987a,b). In Ivory Coast PB-121 was identified as a drought-tolerant hybrid (Bourdeix et al. 2005b), while much work has been done in India to assess and measure the degree of drought tolerance, resulting in the identification of several tolerant varieties (Rajagopal et al. 2005).

Studies on clonal propagation of coconut have been in progress in many coconut growing countries and also in European laboratories since the 1970s, but have not generated a protocol which can be applied to coconut breeding yet (Oropeza et al. 2005). Successful in vitro culture of coconut embryos has been developed as a tool for safe exchange of germplasm and to rescue embryos of Makapuno coconut, a coconut variety which has high commercial value in the confectionary industry but does not germinate when intact in the nut (Carandang 2002; Rillo 2004).

12.6 Current Goals of Breeding

Coconut breeding objectives are still primarily focused on high yield. The definition of yield in terms of yield improvement in coconut is complex. It is yield in terms of nut number that is attractive to the grower who sells coconut on numbers basis whereas it is the size of the nut or the weight of meat or copra that is demanded by the manufacturer or the processing sector. However, as the number of nuts per bunch and size of the nuts is negatively correlated in coconut, one cannot enhance both traits simultaneously by improving naturally occurring coconut varieties by selection alone. On the other hand, in terms of national production targets of a country it is the total meat or copra content per unit planted area that is important. The breeding programmes aiming at a higher nut number have succeeded through the production of dwarf × tall hybrids that surpass the yield of tall coconut cultivars by over 45% (Perera 2005) when hybrids are agronomically well followed up. The low copra content in the hybrid is more than compensated for by the number of nuts produced. As copra content per hybrid nut is generally low compared to tall nuts, at least in the context of Sri Lanka and India according to the experience of the authors, hybrid nuts are less in demand by manufacturers due to greater labour requirement during processing. In the contrary, the tall × tall coconut hybrid (CRISL98) in Sri Lanka is less demanded by the growers though it produces about 40% more copra per nut, but is slightly lower in number of nuts per palm. This is despite its producing more copra per unit area than the dwarf × tall hybrid compensating through the high copra content per nut. It is a fancy hybrid for manufactures as well as for large scale coconut growers who sell their nuts on a weight basis. Hence, the current breeding goal is the development of hybrids that produce a large number of nuts carrying thick kernel by carefully manipulating the parent palms in the breeding programme. This has been achieved by the hybrid Kapruwana in which parents are large

fruited San Ramon that originated in the Philippines and Sri Lanka Dwarf Green which is a prolific bearer. Kapruwana produces as equally well as CRIC65 in nut number and with high copra content per nut comparable with the hybrid CRISL98.

As the vegetative phase of the commercially grown tall coconut varieties is long taking about 8–10 years, precocity in flowering is still a current goal in coconut breeding. Significant progress has been achieved in precocity in the coconut breeding programme by combining early flowering behaviour of dwarfs with commercially grown tall coconut cultivars, but breeders still see scope of shortening the time till flowering in hybrids by incorporating some dwarf germplasm such as Salak Dwarf from Indonesia, which is exceptionally early in flowering. Breeding for oil content or for an oil rich in a particular fatty acid is in the early phase as an objective in coconut breeding.

With the global climate change, droughts have become frequent in many coconut growing countries and hence they become a constraint in coconut production. Therefore, in the recent past, breeding for drought tolerance has become a breeding goal in the coconut breeding programmes of many coconut growing countries. Selection of cultivars and individual genotypes in the fields prone to droughts has been given high priority with the objective of selecting parents for breeding programmes or improvement of local varieties through selection.

Though many major and minor coconut pests that damage coconut palms exist, efficient and effective chemical and biological control methods are in place to control them. However, all chemical and biological control measures field tested so far under experimental conditions have either been unsuccessful or not practical to adopt to control the coconut mite; *Aceria guerreronis*, a microscopic coconut pest that lives beneath the perianth of the nut causing damage to developing nuts. Hence breeding for tolerance to *Aceria* mite is a current breeding goal in the coconut breeding programme in Sri Lanka and India (Perera 2005, 2006). Screening of coconut varieties for tolerance to *Aceria* mite is in progress and some initiatives have been taken to develop hybrids involving crosses between tolerant parents (Perera 2006). Similarly breeding for disease resistance has also been a current breeding objective given the situation that in certain countries lethal disease of coconut destroys millions of coconut palms. Among them, breeding for lethal yellowing disease in the Caribbean and Latin American countries and for root wilt disease in India has been given priority, where these phytoplasma diseases occur and spread.

Nowadays coconut farmers prefer to cultivate short stature palms because of the unavailability of trained pickers or climbers and hence breeding for short stature is also a current breeding objective.

12.7 Breeding Methods and Techniques

The coconut is the sole species of genus *Cocos* and as such present and past breeding work of this crop is limited to the intra-specific level. Furthermore the long generation interval, high heterozygosity, the lack of a reliable method of

vegetative propagation and the limited number of seeds produced per year, all limit the use of many traditional breeding methods employed in other crops. Obtaining a pure line from heterozygous coconut remains an unrealistic expectation because of the long vegetative phase. Thus coconut breeding is confined to mass selection of phenotypically superior parent palms, and to inter-varietal hybridization.

Mass selection is the most fundamental method for coconut breeding (Liyanage 1955). Palms displaying superior agronomic traits such as stout straight trunk with even growth with closely spaced leaf scars, well spread crown with 25–30 healthy fronds, well packed with all stages of inflorescences and developing bunches and palms with short bunch stalks are initially selected from high yielding populations. Then the number of nuts produced per year, average weight of husked nut and tolerance to adverse weather conditions and to pests and diseases of the selected palms are assessed over a period of 3–5 years. Based on these data only the best 5–10% of the palms are selected as seed palms and seeds collected from these palms are distributed as mother palm seeds (Liyanage 1966).

However, as the number of seed nuts collected from mother palms was insufficient to meet the growers demand, a programme called plus palm selection was introduced in Sri Lanka in 1980 which was very much a stop-gap measure to produce a seed that was, in quality, similar to a mother palm seed, but was not exactly so. In adopting this programme, the actual genetic quality of the seed may have been compromised to a degree due to the extent of the seed requirement at that time.

Plus palms are superior palms selected from high yielding blocks of selected estates based on good agronomic features, but quantitative data are assessed just for a single harvest. The plus palms are selected in two stages: (a) Selection of high yielding blocks from suitable estates based on yield figures for the past five consecutive years, and (b) selection of plus palms within the high yielding blocks. The high yielding blocks must satisfy the criteria that the minimum size of the block should be 2 ha, mean block yield should be at least 8,400 nuts/ha/year and the mean yield per palm should be at least 60 nuts/year, the block must have at least 132 bearing palms/ha, the palms must be in the age range of 15–45 years, and the block must be free of pest and diseases. During the selection of plus palms, 100 palms distributed randomly over the block are harvested, and the mean number of nuts/palm of the block is estimated. Then the number of nuts harvested from each palm is recorded and only the palms that record yield above the estimated mean for the block are selected. Palms selected on the above criteria and with satisfactory agronomic characters are then tested for nut weight. Three ripe nuts are taken at random from each palm, husked and weighed, and if the total husked nut weight of 3 nuts is more than 2.1 kg, they are selected as plus palms. The harvesting of nuts of the marked plus palms is done separately to avoid mixing of nuts in handling. The percentage of selection following this method averages to about 20–24%. In order to obtain steady improvement in the quantitative traits it is important to repeat the method

adopted in mother palm selection and plus palm selection to their progenies from generation to generation.

Since the gain from mass selection through open pollination is limited, seed production by artificial cross pollination between high yielding parent palms has been another breeding method in coconut (Liyanage 1954, 1966). Parents are either selected based on phenotype or based on the progeny performance in order to increase the response to selection. However, seed production through this method is limited and therefore setting up of isolated seed gardens was the answer (Liyanage 1961b). In these seed gardens natural assisted pollination occurs between selected elite palms in current mass production of improved coconut seeds. The isolation of the seed garden is achieved either by a forest barrier around the seed garden that is thick enough to prevent pollen arriving from outside (Liyanage 1955) or through 12 or more guard rows of the same coconut variety. The latter is another technique widely practiced currently in the establishment of seed gardens (Liyanage and Azis 1983). This technique was designed after studying the movements of the honey bee, which is also a carrier for the pollination mites.

For inter-varietal hybridization, different crosses between genetically diverse varieties are made using hand pollination. Then the crosses are evaluated in the field in comparison with recommended cultivars as controls, preferably in different agro-ecological zones and in different soil types in order to select new coconut hybrids and hybrids suitable for particular environments or soil types. Once a suitable cross is identified, parent palms are multiplied in a seed garden and seeds are produced by natural controlled pollination. The coconut palm is monoecious and its inflorescence (a compound flower termed a spadix) contains both male and female flowers. The inflorescence protected by a thick sheath is called a spathe. The spathe naturally splits open to expose the emerging inflorescence which consists of a central axis or rachis with up to 40 lateral branches. Each lateral branch is densely set with numerous (200–300) male flowers and fewer (<50) female flowers. The principle of hand pollination focuses on emasculation of the inflorescence followed by isolation of the female flowers by means of a cotton bag (Liyanage 1954). When the female flowers become receptive, selected pollen is artificially introduced. The close supervision of emasculation determines the rate of success and the legitimacy of the hybrid seed nuts.

Since production of seeds by inter-varietal crosses is laborious and time consuming and generates only a very few seeds in relation to the general demand for improved seeds, the isolated seed garden concept is also adopted for production of inter-varietal hybrids. The two parent varieties, usually one tall and one dwarf type or sometimes two tall types are planted in a given proportion based on which variety is emasculated to serve as the maternal parent. The mother parent is then emasculated and the female flowers of this variety are allowed to be pollinated naturally by the other variety. Sometimes only a single variety is planted in the seed garden where pollen collected from another outside parent is blown on to the receptive emasculated inflorescences

of the palms in the seed garden. This method allows to change the hybrids produced within the same seed garden by changing the pollen source.

12.8 Integration of New Biotechnologies in Breeding Programmes

Although conventional coconut breeding programmes using standard breeding techniques based on phenotypic selection have been relatively successful, the inherent constraints in coconut breeding as described previously make the potential use of new biotechnologies highly attractive. Application of molecular genetics in coconut breeding, particularly molecular markers began in the early 1990s and their application in coconut have been diverse, ranging from assessing genetic diversity to creating genetic linkage maps. Initially the studies were aimed at assessment of coconut genetic diversity and genetic relatedness at the DNA level using universal marker techniques such as RAPD (Ashburner et al. 1997b; Duran et al. 1997; Everard 1996; Dasanayake 2003; Dasanayake et al. 2003), RFLP (Lebrun et al. 1998, 1999), AFLP (Perera et al. 1998; Teulat et al. 2000) and ISTR (Duran et al. 1997; Rohde et al. 2000). Later on the need for coconut specific markers was felt and accordingly in 1999 two sets of microsatellite markers were isolated by two groups of scientists independently using the cultivars Sri Lanka Tall (Perera 1999; Perera et al. 1999) and Tagnanan Tall (Rivera et al. 1999). Microsatellites as co-dominant markers have been particularly useful in analyzing highly heterozygous coconut for genetic diversity and genetic relatedness estimates, germplasm characterization and development of collections (Perera et al. 2000, 2001, 2003; Teulat et al. 2000; Dasanayake et al. 2003; Meerow et al. 2003), hybridity testing (Perera et al. 2004), detecting somaclonal variation in coconut tissue cultures, and for construction of genetic linkage maps (Herran et al. 2000; Lebrun et al. 2001; Baudouin et al. 2006). A microsatellite kit comprising 14 primers and an associated software for data analysis has also been developed (Baudouin and Lebrun 2002) standardizing the techniques across laboratories for comparable results, efficient detection of diversity and identification of varieties. More recently DArT markers for coconut have been developed and used for diversity studies (Perera 2005). Among these markers, SSRs have been the most widely and extensively used in analyzing coconut.

12.8.1 Genetic Diversity Analysis

A high level of genetic diversity in coconut has been observed by Perera et al. (2000, 2003) using microsatellites in a collection of 130 coconut individuals representing 51 tall coconut varieties and 49 dwarf coconut varieties sampled across the entire geographic range in the world. The mean genetic diversity values based on Nei's (1987) unbiased statistic observed by Perera (1999) was

0.647 (±0.139). Perera (1999) also found a reduced number of alleles in dwarf coconut group compared to tall coconut group, comparable with a reduction in the amount of genetic diversity in dwarfs. The results of Rivera et al. (1999), who analyzed 20 coconut varieties, mainly from Southeast Asia and the Pacific and Teulat et al. (2000), who studied 31 individuals of coconut comprising 14 coconut varieties were comparable with those of Perera (1999). Perera (2005) also reported that heterozygous loci were evident not only in cross pollinating talls (30%), but at lower frequency (2.5%) in dwarfs as well. The distribution of genetic diversity between varieties within the tall group was observed to be higher than that within the dwarf group. This finding has since changed the germplasm collection strategies for dwarf and tall groups. The genetic diversity study conducted for coconuts in Sri Lanka (Perera 1999) led to the finding that the genetic base of Sri Lanka coconut is narrow. This has resulted in the change of the breeding strategies of the Sri Lanka coconut breeding programme and led to the importation of exotic coconut varieties and their incorporation in the country's breeding programme. Moreover, these studies identified redundancies in the germplasm collection. Ashburner et al. (1997b) reported the use of RAPDs to study the genetic diversity of 17 distinct South Pacific coconut populations. They observed approximately 60% within population diversity in general and noted two geographically cohesive groups and two single populations in a dendrogram. From the results, they concluded that there had been a low but variable rate of gene migration between South Pacific populations with possible founder effects and subsequent human selection. They proposed that germplasm collection in the South Pacific region should focus on populations rather than individuals because of the highly significant level of variation observed between populations. Perera et al. (2001), who analyzed 33 coconut populations belonging to Sri Lanka Tall variety using SSRs across the island representing different geographical regions concluded that there was no population differentiation (between population variation was only 5%) within Sri Lanka Tall.

12.8.2 Genetic Relatedness

Perera et al. (2003) have constructed a phenetic tree diagram showing the genetic relationships among 51 tall coconut varieties and 49 dwarf coconut varieties across the world. Instantly this phenetic tree divided all tall coconuts into two main groups, the first group comprising all the tall varieties from Southeast Asia, the Pacific and the west coast of Panama and all dwarfs in a sub-cluster within the tall cluster. The second group consisted of talls from South Asia, East Africa and West Africa. Interestingly, none of the dwarf coconuts grouped with the second main tall group. The results of Teulat et al. (2000), based on cluster analysis according to UPGMA using the similarity matrix based on the proportion of shared alleles, confirmed those of Perera

(1999). Results on genetic relationships based on microsatellite markers generally agree with the results using other molecular techniques such as ISTR markers (Rohde et al. 1995) or RFLP markers (Lebrun et al. 1998). Rohde et al. (1995) studied 17 different coconut varieties from different geographical regions and identified groupings of African coconut with Indian Ocean coconuts and Panama Tall coconut from the west coast of Panama with Pacific and Southeast Asian coconuts. Lebrun et al. (1998) reported the use of RFLPs in coconut from various geographical regions. Lebrun and co-workers studied nine cDNA clones and one mitochondrial DNA (mtDNA) clone (*Cox*I) from rice and one ribosomal DNA (rDNA) clone from wheat that were hybridized to genomic DNA from 100 individuals of coconut palms representing 10 tall and 7 dwarf coconut varieties. Two main groups of coconuts were identified, one comprising varieties from the Far East and the Pacific and the other comprising varieties from India, Sri Lanka and West Africa. Higher levels of diversity in Far East and Pacific coconut varieties were observed compared with the other material. Clustering of Panama Tall originating from the Pacific Coast of Panama with coconuts originating from the Pacific and the clustering of West African tall with coconuts originating from the South Asia and Indian Ocean group were also observed. These results were in good agreement with the theory of Harries (1978) on the evolution and dissemination of coconuts. Results from all these studies finally led to the conclusion that *Cocos nucifera* is divided into two large genetic groups, the Southeast Asia and Pacific group and the Indo-Atlantic group.

According to Harries (1978) naturally evolved coconuts, characterized as Niu Kafa type, predominate in South Asia, West and East Africa, the Caribbean and the Atlantic coast of Central America while coconuts selected under cultivation, characterized as Niu Vai type, predominate in Southeast Asia, some Pacific islands and the west coast of Central America. It is generally accepted that the coconut palm has existed on the Atlantic coast of Africa, South America and around the Caribbean region for only about 500 years (Child 1974; Purseglove 1972), and that there is a great similarity between these coconuts and those coconuts in East Africa, India and Sri Lanka (Harries 1978). The grouping of Mozambique Tall, which Harries (1977) suggested as the main original source of coconuts to West Africa and to the Atlantic coast of America, with Cameroon Kribi Tall, West African Tall, Sri Lankan Tall and Andaman Ordinary Tall from the Indian Ocean, appears in a single cluster in the phenetic tree of Perera et al. (1999, 2003) and others (Teulat et al. 2000) thus supporting the validity of Harries's (1977) theory of Portuguese-assisted coconut germplasm dissemination from the Indian Ocean to the Atlantic after 1499. Interestingly, the variety Comoro Tall, from East Africa falls in with the Southeast Asia/Pacific main tall group and it seems that this variety originated from Southeast Asia coconuts, the Niu Vai type. Lebrun et al. (1998) noted that the variety Comoro Tall took an intermediate position between Southeast Asian coconut populations and the Indian Ocean populations, based on RFLP markers. The Thailand Tall varieties, Thai Tall,

Pak Chok, Talai Roi mainly group with the South Asia/Africa Tall group while Kalok variety groups with the Southeast Asia/Pacific tall group. The results of a detailed study on varieties of coconuts in Thailand by Harries et al. (1982) based on fruit component analysis of coconut suggested that both Niu Kafa type and Niu Vai type of coconuts are present in Thailand, with the Niu Kafa type present on the Indian Ocean coast of the country. Harries included the variety Kalok with other large fruited forms such as varieties San Ramon and Tagnanan Tall in the Philippines, Bali Tall in Indonesia, Rennell Tall in the Solomon Islands and Panama Tall and Peru Tall from the Pacific coast of America. He also suggests that variety Malayan Tall probably shares common ancestry with variety Kalok. In contrast, he further observed that variety Pak Chok could be compared with other talls that have Niu Kafa type characteristics, for example the talls from Sri Lanka, India, Mozambique, West Africa, and the Caribbean and Atlantic coasts of America. Rattanapruk (1970) also stated that the variety Pak Chok found growing along the coast of Indian Ocean resembles coconut from Sri Lanka. Interestingly, the variety ecotype Kalok in Perera et al. (1999, 2003) is grouped with varieties San Ramon Tall, Tagnanan Tall, Bali Tall, Rennell Tall and Malayan Tall in the Southeast Asia/Pacific group of coconuts. Similarly, the variety Pak Chok is grouped with those from Sri Lanka, Andaman Islands in the Indian Ocean and Mozambique.

The grouping of Panama Tall (varieties Panama Manarge and Panama Aguadulce, both from the Pacific coast of Panama) with Southeast Asian and Pacific talls is in agreement with Whitehead's (1976) observation of an eastward movement of coconuts from Southeast Asia to the Pacific region and subsequently from there to the Pacific coast of America. These results are largely in agreement with the results from ISTR analysis (Rohde et al. 1995), which grouped Panama Tall with Polynesian varieties/populations of coconuts. Results of all these studies finally support the conclusion that *Cocos nucifera* is divided into two large genetic groups, the Southeast Asia and Pacific group and the Indo-Atlantic group.

The grouping of all dwarf forms from different geographical regions in a single cluster within the main South Asia and Pacific group and the Niu Vai type of coconuts and the loss of allelic richness observed in dwarfs suggest that dwarfs have a common origin and evolved from the Southeast Asia/Pacific group of talls in the Southeast Asia/Pacific region. The results of Teulat et al. (2000) strongly support a common origin of dwarf varieties.

Attempts to investigate the genetic lineages in coconuts using three coconut specific chloroplast microsatellite primers developed by sequencing and isolating mononucleotide microsatellite motifs observed in the PCR amplified chloroplast regions of intergenic spacers between *trn*T and *trn*L, *trn*S and *trn*T, and *trn*C and *trn*D, on a set of 130 coconut genotypes from all over the world, failed to show any chloroplast variation, thus indicating a very close ancestral relationship between coconut groups (Perera 1999, 2002).

12.8.3 Hybridity Testing and Variety Identification

Perera et al. (2004) reported the successful use of microsatellite markers to uniquely identify seed parents in the Sri Lanka coconut breeding programme and the resulting hybrids. This is very important for confirming the identity of varieties and their hybrids in the most likely events of mixing of seed-nut lots, mislabeling of seeds in nurseries and checking the legitimacy of hybrid seedlings. Two SSR primers have exhibited the potential for distinguishing between coconut varieties Sri Lanka Tall, Sri Lanka Green Dwarf and Sri Lanka Yellow Dwarf used as parents in Sri Lanka. This has led to the unique capability of conformity and hybridity testing of two commercially grown coconut hybrids, Sri Lanka Green Dwarf × Sri Lanka Tall and Sri Lanka Yellow Dwarf × Sri Lanka Tall. Similarly Bandaranayake et al. (2005) have developed markers specific to the San Ramon variety for identification of uncontaminated materials for multiplication in a seed garden.

12.8.4 Somoclonal Variation in Coconut Plants

Application of microsatellite markers to study any possible somaclonal variation in a limited number of clonal plants of coconut regenerated from various explants through somatic embryogenesis and their successful field planting in Sri Lanka have been reported by Fernando et al. (2004). The microsatellite markers have confirmed the absence of genetic variants among plants within clones (Fernando et al. 2004).

12.8.5 Linkage Mapping and QTL Identification

Studies on coconut genome mapping have commenced only recently. The first genome map for coconut was developed for an East African Tall × Laguna Tall F_1 population based on ISTR markers (Rohde et al. 1999). This work was extended with a mapping population developed in the Philippines from a cross between Malayan Yellow Dwarf × Laguna Tall using AFLP, ISTR, RAPD and ISSR markers. Three hundred and eighty two markers have been placed in the map resulting in 16 linkage groups and leading to the identification of six QTLs for early germination (Herran et al. 2000). Genetic correlations have been established between early germination and early flowering, and early germination and high yield. Thus, this has become the first report of the opportunity for marker assisted selection in coconut. Further, QTL for other traits such as leaf production, girth and height have also been identified for the same mapping population (Ritter et al. 2000). In addition to this, another mapping population in Ivory Coast derived from Cameroon Red Dwarfs and Rennell Island Tall has been used to map 280 markers on 16 linkage groups resulting in several QTLs

related to nut number, number of bunches and traits related to fruit components (Lebrun et al. 2001; Baudouin et al. 2006).

The size of the mapping population and selection of parents for the maximum segregation of traits are critical when producing a genome map of any crop (Bandaranayake and Kearsey 2005). The size of the mapping population is particularly a crucial issue in coconut because obtaining a reasonable number of seed nuts from a particular cross is a difficult task as a result of very low seed production in coconut, i.e. about 100 seed nuts per palm per year. From a simulation study, Bandaranayake (2006) concluded that about 400 individuals represent an effective size of a mapping population in coconut for a consistent map resolution. However both linkage maps described in Herran et al. (2000) and Lebrun et al. (2001) were based on the genotypic and phenotypic scores of less than 65 individuals in their mapping populations. The size of these mapping populations thus would not be sufficient for constructing a reliable map because any mapping population with less than 100 meioses is unlikely to be useful for generating a map (Young 1994). Furthermore, genetic relationship studies in coconut as described above suggests that maximum segregation of traits in coconut can only be obtained by crossing varieties of Southeast Asia and the Pacific group with those from Indo-Atlantic group. However, all the mapping populations mentioned were composed of parents belonging to one genetic group, the Southeast Asia and Pacific group. As a result, about 84% of DNA fragments generated in the Malayan Yellow Dwarf × Laguna Tall mapping population were non-polymorphic indicating that the two parents share identical alleles at a large number of loci.

Based on the experience and the information already generated, a large mapping population has now been created in Sri Lanka comprising 350 individuals arising from a cross between Sri Lanka Red Dwarf (Southeast Asia and Pacific group) and Sri Lanka Tall (Indo-Atlantic group) to obtain maximum segregation of traits and sufficient number of meioses to analyze. Another mapping population, particularly for segregating for tolerance to *Aceria* mite, is being constructed in Sri Lanka between tolerant Sri Lanka Yellow Dwarf and a highly susceptible Sri Lanka Tall palm (Perera 2006). Generation of new mapping populations including phytoplasma-resistant breeding material is being focused in Jamaica (unpublished).

12.8.6 Synteny Studies

Efforts are being undertaken to investigate the possible synteny of the oil palm and coconut genomes as both palm species are diploid and have the same chromosome number, $2n = 32$ (unpublished). Synteny would possibly speed up research by increasing the marker density on the respective linkage maps through the exchange of DNA markers.

12.8.7 In Vitro Culture

Coconut tissue culture has a long history dating back to the 1970s. Since then the problem of cloning coconut has been addressed in a number of research centers worldwide. Initial attempts were on reversion of floral meristem into vegetative growth but later on, most efforts were concentrated on somatic embryogenesis. During the past decade, the in vitro plant regeneration protocol has been improved, and a limited number of clonal plantlets obtained mainly from zygotic tissues has been produced in a few laboratories (Chan et al. 1998; Verdeil et al. 1999; Fernando et al. 2003). However, success is very limited due to numerous constraints. The regeneration efficiency is far from adequate though a small number of vegetatively propagated coconut palms have been established in the field. Due to very poor response of coconut tissues to in vitro conditions it is classified as one of the most recalcitrant species to regenerate in vitro (George and Sherrington 1984).

12.9 Seed Production

Many farmers still practice selection of their own mother palms for obtaining seed nuts for planting, thus selection of palms is based on farmer's long term observations on the performance of each palm. However, in many countries, improved seeds are produced by government and/or private sector organizations and seedlings raised are sold to farmers. When improved seeds need to be produced on a large scale they are produced in isolated seed gardens. In the case of hybrid seeds, the mother trees are planted in isolated seed gardens and daily emasculation of inflorescences is carried out by removing the upper part of each spikelet that carries male flowers. The remaining female flowers receive pollen by natural means, from male parents inter-planted at low ratio within the seed garden, or artificially from pollination labourers visiting each receptive palm, either by using pole-mounted pollen blowers or by using ladders and hand-pollinating each female flower with small brushes. When improved seeds are produced from mass selected high yielding palms or from paired crosses between selected trees, these are planted in the seed garden and allowed to naturally inter-pollinate. The second generation seeds obtained this way are then distributed to farmers for planting. However, production of improved seed often does not meet the demand from farmers, and hence the balance seed nut requirement is obtained from superior mother palms selected in high yielding blocks from high yielding estates. Seed from those trees results from open pollination.

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Chapter 13
Olive

Luciana Baldoni and Angjelina Belaj

13.1 Introduction

13.1.1 Overall Importance of the Crop and Production Areas

The olive (*Olea europaea* L.), grown on over 8 million hectares, is the second most important oil fruit tree crop worldwide after oil palm and its cultivation is traditionally concentrated in the Mediterranean area. The total olive oil production for the 2006–2007 season was 2,859,500 tons (International Olive Oil Council (IOOC) data). Southern European countries account for about 74.9% of the world production, with Spain being the main producer (38.7%), followed by Italy (21%) and Greece (12.9%). Other important olive oil producers are Turkey, Tunisia and Syria (17.1%) as well as Jordan, Morocco and Algeria.

Seventy percent of the olive oil produced globally is consumed in the Mediterranean area, but its demand is rapidly increasing in other countries. For example, the consumption of olive oil in the USA grew from 88,000 tons in 1990 to 251,000 tons in 2007, in Japan from 4,000 to 31,000, and in Australia from 13,500 to 40,000 tons (IOOC data).

13.1.2 Major Problems of Olive Cultivation

The income from olive production is quite low due to the low mean production per unit area of about 2 t/ha of olives, the low content of oil rarely exceeding 24% of fresh weight (depending on variety, agro-climatic conditions and extraction method) and the high costs of cultivation. Fruit production starts 3–5 years after planting, and olive orchards can survive almost indefinitely due to the longevity of the species. However, alternate bearing and the costs of cultivating old trees generally force the reduction of the lifetime of olive orchards to no more than 50–60 years. In order to minimize the costs of production,

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new models of cultivation have been developed based on the complete mechanization of harvesting and pruning, the most time-consuming steps in oil production.

The olive is a long-living evergreen tree that grows up to 15 m at maturity. Its life span is typically longer than 500 years, and trees older than 2,000 years are still under cultivation in numerous regions (Fig. 13.1). Flowers are generally hermaphroditic and wind pollinated, and most cultivars are self-incompatible.

The olive fruit is a drupe (Fig. 13.2) and, in contrast to other oil crops, the oil accumulates in the mesocarp, and only 3–4% of the oil is derived

Fig. 13.1 Ancient olive still under cultivation in Apulia (Italy). Olive trees may survive for thousands of years and many of them can be found along the entire Mediterranean area of cultivation. Plants like this represent the most ancient cultivated living plants of the world

Fig. 13.2 Green olive drupes; at ripening the colour will turn to reddish or black

from the seed. The best olive oils are extracted from olives harvested at the green-turning-to-black stage of ripeness; they have to be immediately milled to minimize oxidation and enzymatic reactions.

13.1.3 Types of Olive Oil and Characteristics

Virgin olive oil is mechanically extracted by pressing or centrifuging crushed fruits without any chemical treatment. Main component of olive oil triglycerides is the mono-unsaturated oleic acid (18:1), which represents 57–80% of total fatty acids, followed by linoleic (18:2) (7–19%), linolenic (18:3) (0.6–0.8%), palmitic (16:0) and stearic (18:0) acids (Salas et al. 2000).

Due to the mechanical processing, virgin olive oil represents a fresh-squeezed fruit juice containing, other than triglycerides, a vast range of microconstituents (more than 230 compounds) including phenolics, tocopherols, aliphatic and triterpenic alcohols, sterols (and their precursor squalene), hydrocarbons and volatile compounds, representing up to 20% of the fresh olives and about 2% of virgin olive oil (Obied et al. 2008). The phenolic compounds of olive and virgin olive oil show a peculiar composition that can not be found in any other vegetable oil. They include different types of hydrophilic phenols, such as secoiridoids, lignans, flavonoids, phenolic alcohols, and phenolic acids. Secoiridoids are the most important class of phenolics and they arise from simple structures, like tyrosol and hydroxytyrosol, to quantitatively more important,

conjugated forms, like oleuropein and ligstroside (Servili et al. 2004). The protective activity of olive oil on the prevention of a variety of tumors and important chronic and degenerative diseases may be credited to these minor constituents unique to virgin olive oil. There is extensive background demonstrating the effectiveness of olive oil and some of its compounds on human health.

Oleuropein, exclusively present in olive and in a few other related taxa, is a non-toxic secoiridoid known to exhibit several biological properties, many of which may result from their antioxidant and free radical scavenger activity. A number of observations elevate oleuropein from a non-toxic antioxidant into a potent anti-tumor agent with direct effects against tumor cells (Hamdi and Castellon 2005; Giamarellos-Bourboulis et al. 2006). Beauchamp et al. (2005) have recently identified *oleocanthal* (deacetoxy ligstroside aglycon), a compound present in newly pressed extra-virgin olive oils that was shown to be a 3–4 times more potent inhibitor of Cox-1 and Cox-2 than ibuprofen, a potent modulator of inflammation and analgesia (Galli 2006). *Squalene* is a triterpene containing six isoprene units and representing a key intermediate in the biosynthetic pathway to steroids in plants and animals. It has been demonstrated to protect against skin cancer (Newmark 1999). *Minor compounds* are also responsible for the organoleptic qualities, taste and flavor of extra virgin olive oils, which may distinguish oils originating from different regions.

As defined by the European Union Council Reg. (EC) 1513/2001, there are additional categories of olive oils: *Refined oil* is obtained from low quality virgin olive oil after chemical and physical refining treatments, which lead to removal of contaminants or degraded compounds. *Olive oil* consists of a blend of refined and virgin olive oil. Finally, *olive-pomace oil* is obtained by treating olive pomace with solvents.

Within the category of virgin olive oil the extra virgin is far more valuable than most other vegetable oils, due to the low percentage of free fatty acidity ($<0.8\%$) and the superior taste, but its production is costly and time consuming. For this reason adulteration is very common, mainly represented by blending premium extra virgin olive oil with seed oils or cheap olive oil. In order to reduce frauds, valorize excellence and defend the origin of best products, a number of regulations and standards have been defined by the International Olive Oil Council and by the Codex Alimentarius Commission. Moreover, the European Union has established the option of a protected designation of origin (EU Commission Reg. (EC) 1898/2006) for olive oils of important regional and traditional origins.

The quality of extra virgin olive oils strongly depends on the cultivar of origin, which affects the content of specific polyphenols and aromatic compounds controlling taste and flavour of the oil. Agro-climatic factors, harvesting time and oil extraction technologies may have a negative effect on the olive oil quality, altering the fatty acid and phenolic composition.

DNA fingerprinting is a powerful aid for the identification of olive oil provenance, and fingerprinting methods have been applied to trace the varietal origin of batches of olive oil (Muzzalupo et al. 2007; Busconi et al. 2003; Breton et al. 2004; Pasqualone et al. 2004; Testolin and Lain 2005).

13.1.4 Ancient and Recent History of Olive Cultivation

Olives have been extensively cultivated along the Mediterranean basin since 3000 BC and have had an enormous impact on the economy, history, culture, and environment of the area. Ancient Greeks and Romans considered olive oil a sacred substance and used it as food, medicine, soap, fuel for lamps and as base for perfumes. There are a great number of archaeological remains showing the cultivation, extraction, commerce, and consumption of olive oil from the main civilizations along the Mediterranean region.

The first evidence for olive cultivation is seen during the Minoan age, when olives were cultivated on the island of Crete (3000–1500 BC). They were then cultivated by the Egyptians (2000 BC) and grown in an almost specialized form. In 1000 BC olive started to be cultivated in Palestine and, between the ninth and eighth centuries BC, olive growing was seen in Greece and in North African coasts surrounding the Mediterranean Sea, where it was introduced by the Phoenicians. Thanks to the Phoenician and Greek shipping routes, olive trees reached the coasts of Sicily and Spain, where they were widely diffused in the fifth century BC. Between the sixth and fourth century BC their cultivation was established in many regions of Italy and Spain by the Romans. By the first century AD, olives were a cash crop for the Romans, who imported oil from the most remote colonies of the empire, mainly Spain and North Africa. Olive cultivation declined during the Medieval age but increased again after the 15th century, up to the present extension of cultivated area in the Mediterranean (Mastrangelo 1982).

After several unsuccessful attempts to introduce olive cultivation in Central and North America during the 18th century, olive production has recently started in new countries such as Argentina, Chile, Mexico, USA (California), New Zealand, Australia, and South Africa, that now represent 1.4% of world olive production.

13.2 Origin and Domestication

The poor historical documentation on cultivar pedigrees and the fragmented information available on olive paleobotany have failed to provide definitive conclusions on the origin of the cultivated olive, but numerous hypotheses are currently under evaluation. Palynological, anthropological, and archeological evidence (Watts et al. 1996; Carrion and Dupré 1996) have demonstrated the presence of sporadic forms of olive during the last glaciation (18000 BC) in the western and eastern Mediterranean regions.

Oleasters have been directly exploited for oil extraction since 4500–4300 BC as evidenced by archaeological and paleobotanical findings from Spain (Terral et al. 2004; Rodríguez-Ariza and Montes Moya 2005). Some studies on olive domestication have asserted that cultivars moved westward with human migrations

(Besnard et al. 2001a; Belaj et al. 2002). Other recent evidence has pointed out that the domestication process started simultaneously at both ends of the Mediterranean (Lumaret et al. 2004; Breton et al. 2006). Analyses of archaeological charcoal and olive stones have effectively dated domestication to the end of the Bronze Age in the north-western Mediterranean area (Terral 2000; Terral et al. 2004; Rodríguez-Ariza and Montes Moya 2005).

The first cultivars were probably selected from trees bearing large fruits and/or high oil content and were vegetatively propagated, either via cutting or grafting onto indigenous oleasters. However, considering the long life of olive plants, there has been relatively little selection and probably only a few generations separate the presently cultivated forms from their progenitors (Liphschitz et al. 1991).

Taking into account recent results obtained by molecular analysis of sets of wild olives and cultivars from different Mediterranean regions, it has been assumed that olive trees have undergone different selection/domestication processes in different regions. The contribution of local wild plants to the development of varieties has been demonstrated only in restricted areas where wild olives, very ancient cultivated trees, and local varieties shared a large portion of variability. In other areas of the western Mediterranean, in contrast, the clear distinction between oleasters and cultivars has confirmed that local cultivars did not develop from local oleasters but were introduced from abroad and propagated by grafting onto local oleasters (Baldoni et al. 2006).

13.3 Varietal Groups

13.3.1 Cultivated Olive Germplasm

Olive germplasm has not suffered significant genetic erosion, maintaining almost intact its entire variability, given that turnover with new genotypes has not occurred, and that the species has great longevity and a good capacity to survive without cultivation. Thus, more then 1,200 varieties are still under cultivation, 79 national and international collections hold about 4,200 genotypes and more than 5,300 cultivar names are recognized (Bartolini et al. 1998). Over two thirds of cultivars are present in the southern European countries (538 in Italy, 183 in Spain, 88 in France, 52 in Greece and 45 in Turkey) (Bartolini et al. 1998), and many other local varieties and ecotypes contribute to the richness of the olive germplasm. Olive represents, therefore, an unusual case among horticultural crops and its germplasm could constitute a particularly rich source of variability to be used directly or for future breeding.

Olive cultivars can be considered as varieties of unknown origin, most of them originating from empirical selections made by growers from naturally cross-bred genotypes over many centuries and propagated by cutting or grafting. Evidence for multilocal selection of most cultivars has been repeatedly demonstrated (Besnard et al. 2001b; Rotondi et al. 2003).

The genetic diversity of cultivated populations shows a complex patchy pattern (Baldoni et al. 2006; Owen et al. 2005). Few cultivars are dispersed over widespread areas, whereas the majority of varieties are highly localized. Cultivars may either have a non-autochthonous origin or have been derived by selection from local oleasters (Lumaret et al. 2004; Breton et al. 2006).

Up to now, identification of each variety has been delayed by confusion about the names given to each genotype, the low intervarietal genetic distances, the intra-cultivar variability and the putative presence of asymptomatic viruses that may affect the plant phenotype.

13.3.2 Cultivar Classification

The geographical distribution of olive cultivars and their economical importance are considered the main criteria for their classification. According to this system, Barranco and Rallo (2000) divided olive cultivars into four categories: main, secondary, dispersed and local. Main cultivars are those which account for either a large portion of the acreage or predominate in one or more olive districts. Secondary cultivars are not predominant in any district but form the basic cultivars of some orchards. Dispersed and local cultivars are isolated trees in various or single districts, respectively. The agronomical performance of main cultivars has been widely evaluated, while for most of the others information is unavailable.

13.3.3 Identification of Olive Cultivars

Until recently, the variability of *O. europaea* germplasm has been reported with respect to morphological descriptors. Understanding the amount and distribution of genetic variability among cultivars by means of molecular markers has been the main goal for most of research on olive trees.

Various types of molecular markers have been widely applied over the last decade, particularly in investigations aiming at studying the variability of olive cultivars and at developing tools to determine their origin and detecting frauds on olive oil varietal composition (Hess et al. 2000; Rallo et al. 2000; Sefc et al. 2000; Guerin et al. 2002; Owen et al. 2005; Montemurro et al. 2005; Vargas and Kadereit 2001; Carriero et al. 2002; Cipriani et al. 2002; Bandelj et al. 2002; Sarri et al. 2006).

Isozymes were the first molecular markers used (Trujillo et al. 1995). Since then, cultivar identification has been based on DNA markers such as RAPDs using different protocols (Mekuria et al. 1999; Belaj et al. 2002; Gemas et al. 2000; Sanz-Cortés et al. 2001; Gonzalo-Claros et al. 2000). AFLP data are available on a wide number of cultivars (Angiolillo et al. 1999; Baldoni et al. 2000; Sanz-Cortés et al. 2003; Sensi et al. 2003; Owen et al. 2005), ISSRs (Pasqualone et al. 2001; Vargas and Kadereit 2001) have also been used and SCAR markers have been developed from RAPDs (Hernandez et al. 2001).

To date, very few SNP markers have been identified in olive. Based on the sequence of candidate genes and their frequency along coding sequences, it has been estimated that there is one SNP in every 190 base pairs (Reale et al. 2006; Consolandi et al. 2007).

For cultivar characterization simple sequence repeats (SSRs) have become the most popular kind of marker (Sefc et al. 2000; Rallo et al. 2000; Carriero et al. 2002; Cipriani et al. 2002; De la Rosa et al. 2002; Díaz et al. 2006a; Sabino Gil et al. 2006), and it has been demonstrated that only three SSR markers are able to distinguish more than a hundred olive genotypes (Sarri et al. 2006). In a recent paper a set of the most effective SSR markers has been selected and proposed for varietal characterization (Baldoni et al. 2009).

DNA markers used to identify olive cultivars have also been applied for DNA tracking of olive oils to test their varietal composition (Breton et al. 2004; Pasqualone et al. 2004; Pafundo et al. 2005; Testolin and Lain 2005; Doveri et al. 2006; Muzzalupo et al. 2007; Consolandi et al. 2008).

13.4 Genetic Resources

13.4.1 Taxonomy and Distribution of Olea europaea

The genus *Olea* belongs to the Oleaceae family, sub-family Oleideae. The genus includes two sub-genera: *Olea* and *Paniculatae*, the former is divided into sections *Olea* and *Ligustroides*. According to recent revisions of *Olea europaea* taxonomy, the species includes six sub-species based on morphology and geographical distribution (Green and Wickens 1989; Green 2002):

- subsp. *europaea*, represented by two botanical varieties: cultivated olive (var. *europaea*) and wild olive (var. *sylvestris*), both present throughout the whole Mediterranean basin;
- subsp. *cuspidata*, distributed along south Asia, on the Arabian peninsula, and throughout east and south Africa;
- subsp. *laperrinei*, restricted to the Sahara region;
- subsp. *maroccana*, present in Morocco;
- subsp. *cerasiformis*, typical of Madeira island;
- subsp. *guanchica*, restricted to the Canary Islands.

Wild and cultivated olives are diploid ($2n = 2x = 46$), predominantly allogamous and their genome size is about 1,800 Mb (Loureiro et al. 2007; Besnard et al. 2008).

13.4.2 Natural Diversity of Olive

The geographic distribution of variability within the *Olea* genus and the genetic relationships among the wild (oleasters) and cultivated olives have been studied using various molecular methods (Angiolillo et al. 1999;

Besnard et al. 2002a,b; Lumaret et al. 2004; Baldoni et al. 2006; Breton et al. 2006; Belaj et al. 2007; Rubio de Casas et al. 2006).

The wild relatives of cultivated species exhibit traits such as biotic stress resistances, adaptation to extreme environmental conditions, plant vigour and architecture, which could be utilized in olive breeding. Model plant genetic systems and the molecular genetic resources that are currently available are greatly enhancing our ability to identify adaptive or stress-responsive genetic determinants. But natural diversity is still an underexploited sustainable resource for olive that could enrich the genetic basis of cultivated plants with novel alleles to improve both productivity and adaptation.

The potential value of wild olive trees and related species as a source of agronomically interesting traits has never been evaluated, thus severely restricting the ability of breeders to develop new genotypes by introgression of superior alleles into cultivated varieties. Oleasters growing under the drought-salt-heat complex conditions and ultra-millennially aged olive trees surviving in adverse environments have only occasionally been submitted to phenotypic evaluation, and there is a lack of serious prospecting surveys to characterize wild olive populations (Mulas et al. 2004; Belaj et al. 2007).

Up to now, most work has concentrated on evaluating the distribution of variability between cultivated and wild olives and on establishing the genetic relationships among the different *O. europaea* subspecies that are distributed beyond the Mediterranean area.

13.4.3 Wild Olives

Wild olive (*Olea europaea* subsp. *europaea* var. *sylvestris*), also known as oleaster, has colonized diverse environments along the Mediterranean basin, characterised by semi-arid climatic conditions with different altitudes, vegetative communities and soils, including those with extreme levels of drought, low temperatures and salinity (Baldoni et al. 2006). Wild plants occur in the same areas as domesticated olive, in the *maquis* and in uncultivated sites, and show some morphological differences from cultivars, such as a bushy plant shape and small fruit size (Terral and Arnold-Simard 1996).

The contribution of oleasters to the evolution of cultivated olive is still questionable, and a widely debated problem relates to the distinction between real oleasters and feral plants derived from the natural dissemination of cultivars. In fact, both forms occur in the same ecological sites and their appearance is very similar. Different criteria have been proposed to clearly distinguish the two forms based on geo-ecological parameters (Lumaret et al. 2004) or on molecular markers (Baldoni et al. 2006; Breton et al. 2006). It is believed that oleasters have originated in the eastern Mediterranean, and that wild olives present in the western Mediterranean basin could be feral (Besnard et al. 2002b).

In previous studies performed through the analysis of chloroplast, mitochondrial, and nuclear DNA polymorphisms, it has been shown that eastern and western Mediterranean oleaster populations are strongly differentiated from one another (Besnard et al. 2001b, 2002b; Lumaret et al. 2004).

It has been hypothesized that humans could have brought eastern-specific chlorotypes to the west, probably bringing plant material (or olive fruits) from the eastern to western Mediterranean (Besnard et al. 2002b). The linkage disequilibrium between widely-spread chlorotypes and nuclear markers characteristic to eastern oleasters can be explained by the common origin of these polymorphisms in a wild population from the east. These results support the hypothesis that western oleasters could be feral forms as a result of an eastern introduction and a gene flow from olive groves towards wild populations.

Studies carried out by the use of allozyme markers (Lumaret et al. 2004) have pointed out the genetic evidence that genuine oleasters still survive locally in the west Mediterranean, as shown by west-specific alleles found in wild olives collected in forests potentially containing genuinely wild forms according to environmental, historical, and demographic criteria. Western populations are more closely related to the wild populations of the Canary Islands. Populations of wild olive seem to be restricted to a few isolated areas of the native Mediterranean forests, where pollen and stones may be wind/bird-distributed.

Other studies, performed on oleaster populations of restricted areas on both sides of the Mediterranean pointed out significant differences between east and west areas (Rubio de Casas et al. 2006). The highest genetic diversity was found at the extreme western side of Mediterranean and in the Balearic islands. Additionally, long-lasting isolation of the northern populations of the Iberian Peninsula appears to be responsible for a significant divergence. Gene flow estimates demonstrated that genetic material seems to be exchanged frequently among populations in accordance with the predominant outcrossing between wild and cultivated individuals. Birds eating olives (Rey and Alcántara 2000) may enable long distance dispersal and make exchange of migrants common even between distant regions. Pollen circulation can also occur over long distances, enhancing lineage admixture.

The cline of genetic diversity revealed by chloroplast and SSR markers was explained by oleaster re-colonization of the Mediterranean basin from refugees after the last glacial event, located in both eastern and western regions (Breton et al. 2006). Based on different population analysis methods, it has been shown that oleasters are equally present in the eastern and the western Mediterranean, and that they are native and not derived from cultivars. It is also likely that gene flow has occurred in oleasters mediated by cultivars spread by human migration or through trade and animals.

The evaluation of olive differentiation at a microscale regional level has shown more complex results (Baldoni et al. 2006). Levels of interpopulation genetic variance between wild olive populations present in distant islands and highly differentiated from mainland oleasters, which only partially represent

real wild olives, were very low, even if the populations were clearly distinguishable from those of other areas. Other wild olives represent feral plants, spread in the same uncultivated areas where real oleasters still survive. The low level of differentiation among wild plants of distant isolated regions is difficult to explain for natural populations. Factors that may be considered include a common ancestral genetic pool, a lack of differential selective pressure and a reduced number of divergent generations between the different populations, likely representing refugial relics of the same population.

13.4.4 Related Subspecies

Analyses performed on rDNA, cpDNA, and mtDNA polymorphisms have characterized three distinct main clades: *O. europaea – O. laperrinei – O. maroccana – O. cerasiformis (= europaea* phylum) of the Mediterranean region, Sahara and northwest Africa, *O. cuspidata – O. chrysophylla (= cuspidata* phylum) of Asia, and *O. africana (= africana* phylum) for east and south Africa (Besnard and Bervillé 2002; Besnard et al. 2002a,b).

AFLP analyses showed that subsp. *laperrinei* and *maroccana*, from northwest Africa, showed a high similarity with the Mediterranean cultivated and wild olive and a clear distinction of the Australian taxa from those of east Africa and Asia, which clustered together (Angiolillo et al. 1999).

The phylogenetic relationships between the *Olea europaea* subspecies and other related taxa were assessed by nucleotide variation in non-coding cpDNA regions and by cpDNA RFLPs, distinguishing four groups: the taxa of northwest Africa and Mediterranean region (including the cultivated olive), the *O. europaea* forms from southeast Africa, those from Asia, and finally *Olea capensis* and *O. lancea,* both belonging to a distinct subgenus *Ligustroides* (Baldoni et al. 2002; Lumaret et al. 2000).

Two tandemly repeated sequences isolated by Katsiotis et al. (1998) localized on the chromosomes by in situ hybridization are present in oleasters and in subsp. *chrysophylla* and *africana*, but are absent in other genera of the Oleaceae family.

13.5 Major Breeding Achievements

Despite the pressure to improve productivity and agronomic performance of olive cultivars and in spite of the economic importance of the crop, there have been few efforts to produce new olive cultivars.

Exploration of phenotypic variability in agronomic characters has led to the identification of valuable clones within numerous olive cultivars of various Mediterranean countries (Suárez et al. 1990; Lavee et al. 1995; Bartolini et al. 2002; Grati-Kammoun et al. 2002). However, in spite of the significant efforts made towards clonal selection, very few clones have shown outstanding performance (Loussert and Berrichi 1995; Tous et al. 1998).

Very few studies have addressed the selection of clonal rootstocks. Preliminary work addressed the influence of rootstocks on scion performance. Clonal rootstocks have been selected with high rooting ability and the capacity to control scion vigour (Baldoni and Fontanazza 1990). Other selected rootstocks can control scion vigour and resistance to frost injury (Pannelli et al. 2002). The use of cvs. Souri, Muhasan and Barnea as rootstocks under dry conditions did not show any significant effect on tree vigour, shape and fruit production after ten years from planting (Lavee and Schachtel 1999).

Similarly to clonal selection, the use of induced mutagenesis has not been encouraging, and so far has succeeded in producing of only a compact mutant of the cv. Ascolana Tenera (Roselli and Donini 1982).

The evaluation of minor local cultivars has recently been exploited to identify individuals highly adapted to extreme environmental conditions (Pannelli et al. 2003; Rotondi et al. 2003).

The long generation time of olives has severely hindered both classical breeding and genetic studies (León et al. 2004a; Santos-Antunes et al. 2005). But now the development of new protocols to force seedling growth has made it possible to greatly reduce the length of the juvenile phase, even if the evaluation of the agronomic performance of mature plants still requires at least five years of experimentation (Santos-Antunes et al. 2005). Furthermore, the genetic control of major traits under selection is still unknown (De la Rosa et al. 2003). Tree vigour, leaf size, and fruit shape seem to be controlled by major genes showing dominance (Bellini et al. 2002a), while the inheritance of other characters such as fruit size, flowering intensity, fruit set, ripening time, and yield remains uncertain (Bellini et al. 2002a; Parlati et al. 1994).

In spite of these drawbacks, various classical breeding programs, performed by intervarietal crossing have been reported from different countries such as Turkey (Arsel and Cirik 1994), Morocco, Spain (Rallo 1995; León et al. 2004a, 2007), Tunisia (Trigui 1996), Israel (Lavee et al. 1999, 2003), Greece (Pritsa et al. 2003), Italy (Fontanazza et al. 1998; Bellini et al. 2002b) and Iran. In Australia, the University of Adelaide has recently established a breeding program to select for quality oil production in feral olive populations derived from natural dissemination of cultivars previously introduced in Australia and well adapted to that environment (Sedgley 2004). In any case, at present, the procedures of selection are still in progress and very few new genotypes have arisen from these breeding programmes.

Maalot, a new cultivar resistant to *Spilocaea oleagina*, has been selected from a selfed F_1 progeny of a semi resistant seedling probably of cv. Chemlali (Lavee et al. 1999). From seedling populations obtained by unknown parents, two other cultivars were selected, 'Barnea' with vigorous and upright growth and 'Kadesh' as a table olive (Lavee 1978; Lavee et al. 1986). The new cultivar 'Askal', a hybrid from the cross 'Barnea' × 'Manzanillo', was selected for its adaptation and good performance in high-density olive orchards (Lavee et al. 2003).

Three new olive cultivars ('Arno', 'Tevere' and 'Basento') were released from the progeny of the cross 'Picholine × Manzanillo' (Bellini et al. 2002b) and their

performance is still under evaluation. The new cultivar 'Fs 17' was selected among seedlings obtained from free pollination of the Italian olive cultivar 'Frantoio' (Fontanazza et al. 1998).

Very recently, the new cultivar 'Chiquitita', derived from a cross between 'Picual' and 'Arbequina', was obtained in a Spanish cross-breeding program. This new cultivar is characterized by early bearing, high oil content and high yield efficiency, while its low vigour, compact canopy and pendulous branches make it very suitable for high density orchards (Rallo et al. 2008).

In the same olive breeding program, preliminary results obtained from a comparative trial of the first 15 selections (León et al. 2007) indicated that some early bearing genotypes could be released as new cultivars. Additionally, some selections have shown a low vigour and could be kept for high-density hedgerow orchards.

13.6 Current Goals of Breeding

The primary goals in olive breeding are directed towards overcoming current limiting factors for production. These include: shortening the unproductive period, increasing fruit number and size, increasing oil content and quality (fatty acid composition, phenol content, etc.), reduction of alternate bearing, dwarfing or modifying tree architecture to facilitate mechanical pruning and harvesting, improving resistance to pests, in particular olive fruit fly, *Bactrocera oleae,* and diseases such as leaf peacock spot, caused by *Spilocaea oleagina*, Verticillium wilt, *Verticillium dahliae* and olive knot, *Pseudomonas savastanoi*. Other important objectives are to improve cold tolerance to allow cultivation in colder areas and to promote self-fertility in order to reduce reliance on pollinators.

Tree architecture and vigour are particularly important because the height of the trees prevents mechanical harvesting and pruning, thereby increasing the costs of cultivation.

Although olive is considered a species adapted to semi-arid climates, its productivity is strongly reduced under dry conditions, and thus there is great interest in developing new drought-tolerant cultivars as well as those that can thrive on saline and heavy soils.

Rootstock selection is focused on the ability to control scion vigour, to improve the resistance to pathogens, mainly *Verticillium*, and to abiotic stresses, namely water stress.

13.7 Breeding Methods and Techniques

13.7.1 Classical Breeding

Main steps for olive breeding are: (i) establishing the inventory of the existent varieties and determining their agronomic value, and (ii) breeding by hybridisation and selection. The first point has received particular attention and is

under way in various Mediterranean countries. The second step has been initiated in Spain and in a few other countries.

13.7.2 Clonal Selection

Clonal selection aims at uncovering valuable genotypes within a variety. Most selection programs in olive have so far relied on clonal selection and are based on the assumption that natural mutations generating any positive alteration in traits of agronomic interest may occur in long-living plants, in which they may be maintained by vegetative propagation (Rallo 1995; Belaj et al. 2004). Prospecting surveys to identify outstanding trees within a variety, either for agronomical or technological characters, are the first steps for clonal selection. Vegetatively propagated progenies of these individuals are then tested in comparative trials. Individuals performing better than standard samples of the corresponding cultivar for specific characteristics are selected and their vegetatively propagated clones represent the new clonal selections. The selected individuals most commonly retain the original cultivar name and acquire an identifying clone code. Most of the reported works on clonal selection in olive have followed this methodology, but most of them have stopped at the first step. This method of selection has demonstrated a low efficiency, and only a few selected clones have gained commercial relevance and have been propagated by the nursery industry (Loussert and Berrichi 1995; Tous et al. 1998).

13.7.3 Sanitary Selection

Systemic pathogens, such as viruses and phytoplasms, are sources of variation in vegetatively propagated plants. The application of molecular diagnostic techniques (such as RT-PCR) has allowed to detect the presence of different viruses (Faggioli et al. 2005) which may be symptomless but affecting plant morphology. Little information on the incidence of these diseases on agronomic characteristics is available and has given contradictory results (Clara et al. 1997; Martelli 1998; Bartolini et al. 1998). For that reason in the last years the sanitary selection, i.e. original plant material free from systemic pathogens, has become a technique of olive selection (Bottalico et al. 2004).

13.7.4 Breeding by Intervarietal Crossing

The adequate choice of parents is of great importance for the achievement of the objectives of a breeding program. Thus, a detailed knowledge of cultivars' identity and of their agronomic performance as well as the amount and distribution of their genetic variability is crucial to broaden the genetic base of new cultivars (Belaj et al. 2004; León et al. 2004a).

In olive breeding, the length of the juvenile period has traditionally been one of the main drawbacks. Under normal conditions olive seedlings begin to set fruits 15–20 years after germination (Santos-Antunes et al. 2005). This may

explain the various attempts by olive breeders to study the juvenile period and to develop protocols to shorten it (Lavee et al. 1996; Santos-Antunes et al. 2005; De la Rosa et al. 2006).

The protocols under use to perform intervarietal crosses in the olive consist of adding pollen of the paternal variety to bagged branches of the maternal parent, chosen among self-sterile cultivars (Lavee 1990; Fontanazza and Baldoni 1990; De la Rosa et al. 2004). Fruits are collected at ripening and, after removing the endocarp, seeds are germinated under controlled temperature and humidity. Plantlets are grown in a greenhouse under continuous light until they reach a minimum height, then they are moved to the field where they undergo the procedures of agronomic evaluation and selection of outstanding genotypes. After the pre-selection phase, plants are vegetatively propagated and compared in experimental trials for the final selection (Lavee et al. 1999; León et al. 2004b; Santos-Antunes et al. 2005; León et al. 2007).

An alternative way to overcome the problems of the long juvenile period is the selection of early bearing genotypes (Pritsa et al. 2003; De la Rosa et al. 2006). The relationships between seedling phenotypes and their agronomic behaviour at the mature phase are also very important. The initial results of a comparative trial of some genotypes under selection indicate that seedlings with a short juvenile period also show a short unproductive period when vegetatively propagated (León et al. 2007). It has also been demonstrated that there is a strong correlation between the resistance to *Spilocaea oleagina* of seedlings and that of adult plants (De la Rosa et al. 2006; Lavee et al. 1999; León et al. 2007).

The first evaluations of olive progenies have shown wide ranges of variation for all evaluated characteristics (Lavee et al. 1999; León et al. 2004a,b). Significant correlations have been observed among many traits. The most relevant correlations were found between oil content and oleic acid concentration, which was negatively correlated with palmitic, palmitoleic and linoleic acid percentages (León et al. 2004b).

Up to now, crossing programs have been performed only between cultivars, but the use of wild olives in future crosses should introduce useful variability, as wild genotypes may contain characteristics rare or absent in cultivated olive germplasm. So far, only one case of an interspecific cross has been reported using *Olea chrysophylla* Lam (Lavee 1990).

At the moment, olive breeding programs using intervarietal or interspecific crosses are mainly carried out in Spain, Israel and Australia.

13.7.5 Marker Assisted Breeding

The very preliminary works performed on olive genomics are far from producing effective results toward the selection of new cultivars by the use of molecular tools. For that reason and considering the lack of knowledge on the real useful variability already present in the cultivated and wild olive germplasm, attention has been focused in the last ten years on the evaluation of such germplasm.

As far as the use of molecular markers in olive breeding programs, a SCAR marker was proved to be linked to leaf peacock spot tolerance, as reported by Mekuria et al. (2001). Recently SSR markers have proved to be useful for paternity testing in olive progenies (De la Rosa et al. 2004; Díaz et al. 2006a; Mookerjee et al. 2005) as well as for the study of parental cross compatibility (Díaz et al. 2006b). These studies have evidenced the high frequency of contamination with undesirable pollen in seedlings (Santos-Antunes et al. 2005; León et al. 2004b). SSR markers have also proved to be useful for unequivocally identifying selections from a breeding program (Díaz et al. 2007).

One of the major contributions of molecular markers in breeding is the construction of genetic maps and the detection of QTLs. The first mapping population in olive consisted of a progeny derived from the cross between two highly heterozygous cultivars, Leccino and Dolce Agogia. Dolce Agogia is resistant to the most important olive pathogens such as *Spilocaea oleagina* and *Verticillium dahliae* (Bartolini et al. 1998; Gonzales-Lamothe et al. 2002), whereas Leccino is susceptible or medium tolerant to these biotic stresses. The linkage map was based on dominant (RAPDs and AFLPs) along with a small number of codominant markers (RFLPs and SSRs, De la Rosa et al. 2003). The Leccino map covered 2,765 cM and included 249 markers, falling into 22 major and 17 minor linkage groups (the latter each involving less than four markers). The Dolce Agogia map was of similar length (2,445 cM) and included 236 markers arranged in 27 major and three minor linkage groups. Besides, a candidate gene for stearoyl-ACP desaturase, which is a key enzyme for the conversion of 18:0 stearic acid to 18:1 oleic acid, the main component of olive oil was mapped on a linkage group of cv. Leccino (De la Rosa et al. 2003). At present, with the aim to construct a reference linkage map, this first map is being completed by a wider use of codominant markers such as SSRs and SNPs.

A second linkage map was constructed by Wu et al. (2004) based on RAPDs, SCARs and SSRs, exploiting the progeny of a cross between the cultivars Frantoio and Kalamata. The greater use of codominant markers allowed the integration of the two parental maps to generate 15 linkage groups covering 101 loci and 879 cM with a mean inter-marker distance of 10.2 cM.

At present, no further olive genetic mapping data is available, no QTLs have been detected, and neither is there any detailed analysis known on genome organization.

13.8 Integration of New Biotechnologies in Breeding Programmes

The very long generation time of olive has delayed the recovery of superior olive cultivars through conventional breeding. Genetic transformation represents a powerful alternative technique for accelerating the development of superior cultivars. For the introduction of genes controlling specific traits via transgenetics, many studies have been performed in the 1980s through 2000 in order to develop the biotechnological tools necessary to transform and regenerate olive

plants. But the restrictions from the European legislation and the public concern about the use of genetically modified plants, especially for traditional products such as olive oil, have dramatically decreased investigation on such topic during the recent years.

Here, the goals and main achievements obtained on the different aspects of olive genetic transformation will be summarized. Most importantly, olive transformation research has to address the identification and evaluation of genes and specific promoters for useful traits and the development of efficient protocols for regeneration from cell and tissue cultures of elite cultivars. Many potentially useful genes have been isolated from different species which could be introduced into olive separately or in combination. However, genetic transformation also requires the development of genotype-independent procedures based on the transformation of meristematic cells with high regeneration potential and/or the use of regeneration-promoting genes.

13.8.1 Organogenesis and Regeneration

Organogenesis represents a first step to somatic embryogenesis, as adventitious buds developing from explants of micropropagated plantlets may generate embryogenic cultures.

Shoot organogenesis and complete plants from mature phase explants of important cultivars have been achieved by Mencuccini and Rugini (1993), and thereafter applied routinely with minor modifications on the media and regeneration conditions. New rooting has been induced by inoculating the basal part of in vitro microcuttings with *Agrobacterium rhizogenes*. The application of putrescine increased rooting rate and basal callus formation (Rugini 1992).

Significant progress has also been achieved on the improvement of regeneration systems, in particular by somatic embryogenesis. Somatic embryos have been induced from immature zygotic embryos of various cultivars (Leva et al. 1995), from cotyledons of mature zygotic embryos (Pritsa and Voyiatzis 1999) and from seeds using radicle segments as explants. Somatic embryogenesis from tissues of elite olive cultivars has been difficult to achieve and has been reported only for two cultivars, 'Canino' and 'Moraiolo' (Rugini and Caricato 1995). Secondary somatic embryos originate from the epidermis or from the first sub-epidermal layer of embryos, mainly from their basal part. A limited number of cells of the primary explant seems to be involved in the formation of embryo's primordia (Benelli et al. 2001).

13.8.2 Genetic Manipulation

Olive explants have been transformed by the use of *Agrobacterium rhizogenes* through microprojectile-mediated DNA delivery on sporophytic explants or zygotic embryos (Mencuccini and Rugini 1993). Even if transgenic callus has been produced from leaf petioles of in vitro growing shoots, somatic embryos represent the most suitable tissue for transformation, because they may continuously develop secondary embryos (Rugini et al. 2000).

Genes used for transformation of olive were mainly *rol* genes of *A. rhizogenes* T-DNA cloned in *A. tumefaciens* LBA4404. Transformation with the entire T-DNA of *A. rhizogenes* may increase rooting efficiency, but only chimeric plants have been obtained (Rugini 1992). The *rol*ABC genes allow morphological plant characteristics to be modified, but somatic embryos and plants have been obtained only from cv. Canino (Rugini et al. 1999), whereas in all other cases no regeneration has been obtained. Transformation with the *osmotin* gene increases defence against fungal pathogens, and olive transgenes have been regenerated and evaluated in field trials (Rugini et al. 1999). Olive plants expressing the *osmotin* gene under the 35S promoter have shown reduced growth. Experiments are in progress to transform 3 genes from tobacco (o*smotin* + *chitinase* + *PR1*) in one construct.

Neomycin phosphotransferase II (*npt II*), which encodes resistance to kanamycin, has been most widely used as a selective marker. To increase public acceptance of transgenic olives, the procedures of selection should be improved, possibly by replacing traditional selection markers. The development of methods avoiding the use of antibiotic-dependent selection or allowing elimination of marker genes from transformed plants is a research priority in coming years.

Other methods of genetic modification include induced mutations. By irradiating rooted cuttings of cv. Ascolana Tenera with gamma rays, dwarf plants have been obtained (Roselli and Donini 1982). Irradiation of 'Frantoio' and 'Leccino' plantlets has produced mixoploid mutants showing dwarf habit, self-sterility and late blooming (Rugini et al. 1996).

13.8.3 Other In Vitro Technologies

Haploid recovery. Unlike the great interest in obtaining homozygous plants to isolate mutants, identify recessive alleles and facilitating whole genome sequencing, little work has been devoted to generating haploid plants (to be used for self-fertilization) from in vitro cultured anthers, and no regeneration has been obtained (Perri et al. 1994).

Polyploids. Tetraploid plants have been produced by irradiation of 'Leccino' and 'Frantoio' plantlets. Triploid plants have been obtained by pollinating mixoploid or tetraploid plants with regular haploid pollen.

Protoplast culture. Viable protoplasts have been isolated from hypocotyls, cotyledons, and leaves of micropropagated shoots, but it has been impossible to obtain regeneration (Canas et al. 1987).

Somaclonal variation. Somaclonal variation has been observed in mature olive plants regenerated through somatic embryogenesis of the 'Frangivento' cultivar obtained from embryogenic tissue induced on immature cotyledon portions. Plants have shown two types of variation: BOS (bushy olive somaclone), characterized by reduced plant height, leaf, inflorescence and fruit size, and COS (columnar olive somaclone), characterized by increased plant height, canopy volume and fruit size. The causes of this variation remain unknown (Leva and Petruccelli 2007).

Cryopreservation. With respect to medium- and long-term conservation, promising results have been obtained by slow growth storage and cryopreservation. In 'Arbequina' 30% survival of shoot tips has been obtained following their desiccation to 30% moisture content and direct immersion into liquid nitrogen (Martinez et al. 1999). Utilizing vitrification and one-step freezing in liquid nitrogen for shoot tips excised from in vitro 'Frantoio' shoot cultures, Lambardi et al. (2000) have achieved 15% survival, following re-warming to 40°C and plating on re-growth medium. A higher percentage (38%) of cryopreserved embryogenic cultures was obtained when using embryogenic cultures such as proembryonal masses and somatic embryos, and recovered embryogenic tissues showed enhanced proliferative and morphogenic activity. The encapsulation-dehydration procedure proved ineffective for cryopreservation (Martinez et al. 1999) but has potential applications in olive propagation (Micheli et al. 1998). Generally, cryogenic methods can be applied for long-term conservation of olive germplasm and the establishment of in vitro repositories, which could safeguard olive biodiversity.

13.9 Concluding Remarks

Studies on olive genetic resources have been intensively carried out in recent years, while a serious gap is envisaged for what concerns breeding activities and genomic research. Efforts are currently put on by many research groups to rapidly cover these areas.

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Chapter 14
Safflower

Hans-Henning Mündel and Jerald W. Bergman

14.1 Introduction

Significant contributions to understanding and manipulating domesticated safflower, *Carthamus tinctorius* L., for improvement of the crop, have been made by scientists in numerous countries. A sampling of such contributions will be presented in this chapter. However, two countries stand out in terms of the amount of research carried out on safflower, namely India and the USA. India produces more safflower and has more safflower researchers than any other country. The USA has contributed considerably to the academic training involving safflower over the past half century, to scientists from around the world. Contributions from India and the USA will be highlighted in this chapter.

14.2 Origin and Domestication

Cultivated safflower may have its origins from the two related species, wild safflower, *Carthamus oxyacanthus* M.Bieb. from Afghanistan and adjoining countries (e.g. Pakistan) and from the saffron thistle, *Carthamus lanatus* from Ethiopia (Chavan 1961).

Using wide-ranging sources, Weiss (1971) determined that safflower, *Carthamus tinctorius* L., has been recorded as being grown for centuries in a wide area covering southern and western China, much of India and westward across present-day Pakistan, Afghanistan, Iran, Iraq, northern Saudi Arabia, Kazakhstan, Turkey and numerous other 'Middle Eastern' countries; as well down the Nile Valley of Egypt and Sudan, and Ethiopia. Egyptian mummies were anointed with a ceremonial ointment using a safflower dye, prior to binding, 4,000 years ago. Safflower seed packets and garlands of florets were found with such mummies (Weiss 1971). While the main use of safflower was

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for foods, for spice and colouring and dying of clothing, it has been known as an edible oil crop in the ancient Mesopotamian region over 2,000 years ago (Weiss 1971). In China safflower, introduced around the 2nd century BC (Chavan 1961), has been used almost exclusively for medicinal purposes by use of its florets applied topically or in a tonic tea in both ancient and modern medicine (Li and Mündel 1996). The use of carthamin tablets for food colouring, rouge and medicine have been described in Hebrew writings from the 2nd century AD, while both European and Japanese pharmacopoeias of the past listed safflower among their medicines (Chavan 1961; Weiss 1983). Just prior to the 10th century, the Persian physician-pharmacist, Mesua (originally called Yahya ibn Masawayh), provided drawings of safflower as recognizable as being *C. tinctorius*, for around Baghdad as well as from India (Chavan 1961).

Before the discovery of aniline dyes, benzene derivatives, in the early 19th century, natural products such as indigo (for blue) and safflower – for yellow, orange and red pigments from the flower petals – were important sources of vegetable dyes for colouring fabrics (Knowles 1982).

Since the 20th, century and probably as early as the 19th century, safflower has been grown mostly for its edible oil in India, where it is cultivated in many states (Chavan 1961). Commercial oil production from safflower began in the United States of America (USA) in Nebraska in the 1940s and expanded to California by 1950 where production dominates as well as in the Northern Great Plains of eastern Montana and North Dakota (Knowles 1980). Sizable production also occurs in Mexico and in much smaller areas in numerous other countries (Li and Mündel 1996). Almost half of the worldwide production of safflower, or close to 1.5 million ha is grown in India, in many states mostly in central and southern regions; the remainder is grown typically in drier regions of many countries (Sastry 1997).

14.3 Species Groupings Related to Breeding of Cultivated Safflower

A botanical classification for safflower, in both Latin and English, is given by the USDA (Anon. 2007a) as follows: Kingdom Plantae – Plants; Subkingdom Tracheobionta – Vascular plants; Superdivision – Spermatophyta – Seed plants; Division Magnoliophyta – Flowering plants; Class Magnoliopsida – Dicotyledons; Subclass Asteridae; Order Asterales; Family Asteraceae – Aster family; Genus *Carthamus* L. – distaff thistle.

As the topic of this chapter is safflower breeding, only the species of most likely relevance to the actual breeding will be discussed in detail. Khidir (1969) described the probably most basic ploidy condition of *Carthamus* as the one of $2n = 24$ chromosomes, which is predominantly outcrossing with occasional selfing. Beside the cultivated *C. tinctorius*, with $2n = 24$, species that produce fertile natural as well as artificial F_1 and F_2 hybrids with the cultivated species

are: *C. oxyacanthus* M.Bieb., known as 'wild safflower', *C. palaestinus* Eig and *C. persicus* Desf. ex Willd. (Syn. = *C. flavescens* Spreng) (Ashri 1974). Thus, only these three wild species of *Carthamus*, which are closely related to the cultivated safflower, contribute variability to the gene pool from beyond the species *C. tinctorius* itself. While cultivated safflower, *C. tinctorius*, is self-compatible and self-pollination as well as out-crossing occur naturally, *C. oxyacanthus* is a mixture of self-incompatible and self-compatible types; *C. palaestinus* is a self-compatible species; and *C. persicus* is entirely self-incompatible (Knowles 1982).

Ashri and Knowles (1960) classified the genus *Carthamus* into four sections, based on chromosome numbers. More recently however, López-González (1989) developed a classification system, based on anatomical, chorological (biogeographic, related to distribution) and biosystematic information. In this system, the two genera *Carthamus* and *Carduncellus* are replaced by four new genera: *Phonus, Lamottea*, *Carthamus* and *Carduncellus*. Species of the three genera *Phonus, Lamottea* and *Carduncellus* are classified as perennial and have 24 chromosomes in their genomes, whereas the newly circumscribed genus *Carthamus* contains only annual species, and has members of 20, 22, 24, 44 and 64 chromosomes, including several putative allopolyploid species. The geographical distribution for *Carthamus* is west and central Asia as well as in the Mediterranean region; of the four genera, only this new genus **Carthamus** is further subdivided into sections, with the species indicated. Section *Carthamus* has 24 chromosomes and includes the following species: *C. curdicus* Hanelt, *C. gypsicola* Ilj., *C. oxyacanthus* Bieb., *C. palaestinus* Eig, *C. persicus* Willd. and *C. tinctorius* L. Section **Odonthagnathius** (DC.) Hanelt (incl. Sect. *Lepidopappus* Hanelt) has 20 or 22 chromosomes and includes the following species: *C. boissieri* Halácsy, *C. dentatus* Vahl, *C. divaricatus* Beguinot & Vacc. (with 22 chromosomes), *C. glaucus* Bieb., *C. leucocaulos* Sm. and *C. tenuis* (Boiss. & Bl.) Bornm. Section **Atractylis** Reichenb., with a presumed *x* number of 11, contains numerous polyploids, including the following species: *C. lanatus* L., *C. creticus* (*C. baeticus* [Boiss. & Reuter] Nyman) and *C. turkestanicus* M. Popov. Subsequent molecular studies have clarified the problem of the generic limits of *Carthamus*, confirming *Carthamus* as a natural group, using molecular phylogenies based on DNA sequences (Vilatersana et al. 2005). Analyses of genome size showed that the species of section *Carthamus* form a distinct cluster, with the most invasive *Carthamus* spp. exhibiting an increased genome size but a decreased chromosome number, with respect to the other taxa of the genus (Garnatje et al. 2006).

While numerous studies on crossing among *Carthamus* species and associated cytogenetics have been carried out in the past, relatively few actual crosses have been recorded towards improvement of the domestic safflower using the wild or weedy relatives. A sample of these is described below. The three wild species mentioned above, also having 12 pairs of chromosomes, all in the new genus *Carthamus* subsection **Carthamus**, namely *C. oxyacanthus, C. creticus* and *C. palaestinus*, have basic fatty acid compositions similar to

those for the commercial *C. tinctorius* safflower types. However, some collections could be used to raise levels of palmitic acid (Knowles 1972). While the lack of seed dormancy can be a shortcoming in commercial safflower, where continuous rainfall after maturity result in seed germinating in the head, crosses with *C. palaestinus* have been successful in transferring dormancy to the domesticated species (Kotecha and Zimmerman 1978). Crosses of common safflower to *C. persicus* indicated that cold tolerance in the early growing stage could be transferred to the domesticated species (Zimmerman and Buck 1977).

14.4 Genetic Resources

Over the decades, various researchers conducted safflower collecting expeditions, with perhaps the most comprehensive being those of Paulden F. Knowles in 1957 (Knowles 1959) and 1964–1965 (Knowles 1965). These expeditions resulted in the deposition of major accession items in the USDA World Collection of Safflower, which has become a major source for safflower breeders and germplasm researchers around the world. Access to this material is available via the Regional Plant Introduction Station in Pullman, Washington, USA. In 1958, Knowles collected samples from 14 countries from India westward through the Middle East, North Africa, and southern Europe and in 1964–1965, from nine countries from India in the east, westward to include Egypt, Sudan and Spain, which includes both cultivated, wild and weedy safflower species.

Some of the major national safflower collections, evaluations and documentations, include those of China, India and the USA.

The Safflower Research Group of the Beijing Botanical Garden of the Chinese Academy of Sciences, with the support of IBPGR since 1989, recorded a total of 2,051 accessions from 49 countries, and 465 specimens from within China. Extensive evaluations, based on complete grow-outs of the germplasm at Beijing, have been reported in English (Li et al. 1993). Similar evaluations at Urumqi, in Xinjiang in western China, have been reported in Chinese (Wang Zhaomu and Fan Lin 1991; Wang Zhaomu 1993).

Safflower research in India is coordinated from Solapur, in Maharashtra State, where the Germplasm Management Unit (GMU) is the major repository for world safflower germplasm in India, with 6,115 accessions assembled from 38 countries (Mehtre et al. 1995). The GMU in cooperation with the Project Coordination Unit (Safflower) at the Mahatma Phule Agricultural University and the Directorate of Oilseeds Research (DOR) in Hyderabad, has coordinated the systematic safflower germplasm handling and documentation since the early 1980s when characterization of indigenous and exotic collections and elimination of duplicate entries reduced the original 9,000 accessions to 1,196 (Rao et al. 1991).

Zhang and Johnson (1999) compiled a directory of safflower germplasm collections, listing 15 countries as having *Carthamus* collections. Only those 11 countries with more than 10 accessions and permitting exchange of germplasm, even if somehow restricted, are included in Table 14.1. Where additional information could be obtained (either from researchers in those countries or the on-line IPGRI Directory of Germplasm Collections [Anon. 2007b]), such numbers, with a date, are included. Numbers of accessions refer to *C. tinctorius* unless otherwise stated. Only such species that are expected to hybridize with *C. tinctorius* and produce fertile offspring are included here.

As summarized by Li and Mündel (1996), safflower seed is an 'orthodox' seed in terms of its storage behaviour, viability of seed is maintained best by storing well-dried seed at low humidity and at low temperatures. In dry environments, safflower seed equilibrates at around 6–7% moisture. Based on the International Board for Plant Genetic Resources (IBPGR) guidelines established, 'medium-term storage' can be accomplished by storage at 4°C and 30% relative humidity; 'long-term' storage can be effected at –20°C. To the extent possible, with the financial resources provided, centres storing the collections outlined below use those or similar sets of conditions. Unfortunately, however, a number of collections are stored at ambient temperature and humidity. This results in a great potential loss of viability and accumulation of mutations as viability is reduced.

It should be noted that *C. oxyacanthus*, which has been introduced to North America, is now considered a noxious weed in the USA, appearing on the USDA Federal Noxious Weed List of banned plants on June 7, 1999 (Anon. 1999). Indeed it is known as a serious weed in its native habitat of northern India, Pakistan, west to Iraq (Ashri and Knowles 1960) and in Australia where it had also been introduced (Weiss 1983). Thus, safflower breeders are cautioned.

In the USA, Johnson et al. (1999) studied one thousand safflower accessions to: (1) provide oil and meal evaluation information for a major portion of the United States Department of Agriculture (USDA) safflower (*Carthamus tinctorius* L.) collection, (2) compare ranges, variances and means between 203 core and 797 non-core accessions, and (3) determine if region of origin could be differentiated based on accessions' oil and meal characteristics. While the core was not fully representative of the non-core accessions, it captured a large fraction of the diversity in oil and meal factors present.

In India, Dwivedi et al. (2005) developed a core subset of safflower based on 12 morphological descriptors and geographic information on 5,522 safflower accessions; stratifying the accessions by country of origin, and data on 12 descriptors. About 10% of the accessions were randomly selected from each of the 25 clusters to constitute a core subset of 570 accessions. Mean comparisons of the descriptors indicated that the genetic variation available for these traits in the entire collection was preserved in the core subset. This core subset thus selected provides an opportunity to evaluate agronomic and seed quality traits and resistance to abiotic and biotic stresses and to identify diverse germplasm with beneficial traits for enhancing the genetic potential of safflower.

Table 14.1 Safflower germplasm collections of countries permitting exchange

Country (sources)	Accessions (date)	Institute	Storage conditions	Availability
Australia (from 27 countries)	486 (2003)	Australian Temperate Field Crops Collection Horsham, Victoria	Medium- & long-term	Small amounts
Canada (duplicates in India)	34	Plant Gene Resources of Canada, AAFC, Saskatoon	Long-term	Yes
China (from over 50 countries)	2784	Inst. of Oil Crops Res. YAAS, Kunming, Yunan- duplicates of most at CAS, Beijing and XAAS, Urumuqi, Xinjiang	Short-term	Partial
	178	Inst. of Crop Germplasm Resources, CAS, Beijing	Long-term	Mutual exchange
Ethiopia (indigenous)	197 (2007)	Inst. of Biodiversity Cons. and Research, Addis Ababa	Short-term	MTA needed
Germany	167 (2007)	IPK, Genebank, Gatersleben	Long-term	Yes
C. lanatus	31 (2007)	IPK, Genebank, Gatersleben	Long-term	Yes
	118 (1995)	Fed.Centre for Breeding Res. of Cultiv. Plants (BAZ), Quedlinburg	Not known	Not known
India	2393	Nat. Bureau of Plant Genetic Res., New Delhi (Also: Regional Station of NBPGR, Akola)	Long-term	Yes
Mexico (from 22 countries)	1504	Instituto Nacional de Investig. Agricolas, Iguala	Medium- term	Restricted
	120	Inst.Nac. de Investig. Forestales, Agricolas y Pecuarias, Mexico City	Not known	Not known
Romania	31 (2002)	Gene Bank of Suceava, Suceava	Medium-term	Restricted
Russia (from over 40 countries)	418 (2002)	NI Vavilov Inst. of Plant Industry, St. Petersburg-duplicates at Kuban Expt. Station of VIR, Krasnodar Region and Uzbek Res. Inst.of Plant Ind., Tashkent, Uzbekistan	Medium-term	Yes
Turkey (indigenous)	27	Aegean Agricultural Research Inst., Menemen, Izmir	Medium- & long-term	Yes, following gov't regulations
United States of America	2288	Western Regional Plant Introd. Station, USDA-ARS, Pullman, Washington - duplicates of most held at Fort Collins, Colorado	Medium- term	Yes
Carthamus lanatus 7				
Carthamus lanatus ssp. *turkestanicus* 4				
Carthamus oxyacanthus 90				
Carthamus palaestinus 3				

14.5 Major Breeding Achievements

Genetic improvement of safflower, though a self-pollinating species but showing variably low to considerable outcrossing, has relied to a great extent on exploiting the existing variability in cultivated varieties and land races, and to a limited extent also on crosses with closely related species (Ashri and Knowles 1960).

In India, where more safflower is grown than anywhere else in the world, safflower landraces have been cultivated over millennia. Thus it is not surprising that there are and have been more safflower breeders and other researchers in India than in any other country, trying *inter alia* to improve the productivity of local safflower. Screening for resistance to a number of the common safflower diseases has generally not directly resulted in resistant varieties in India, but has provided useful resistant or moderately resistant germplasm to use (Mündel and Huang 2003). The All India Coordinated Research Project on Oilseeds (AICORPO) was initiated in the late 1960s and from the early 1970s on included also safflower. This project included mainly public research but also a private research center, the Nimbkar Agricultural Research Institute (NARI), in Maharashtra State, in addition, the Maharashtra Hybrid Seed Company (MAHYCO) includes safflower as one of its crops. Aside from developing high yielding cultivars for the diverse regions growing safflower, these programs have developed a wide range of plant types, such as appressed, semi-compact, spreading, in both spiny and non-spiny versions, with a result of 18 high-yielding varieties and four hybrids of regional and multi-rgional importance (Sujatha 2006). The first-ever spineless variety, JSI-7, was developed in Indore and released in 1990, to facilitate the introduction of safflower in non-traditional areas of safflower cultivation, realizing that harvesting is done manually (Anjani et al. 2006). The same report mentions the release of the spineless NARI-6 in 2000 by NARI, providing dual income to farmers, as the florets can easily be collected from non-spiny safflower after the crop matures and sold for food and textile dye.

In the USA, safflower breeding has been carried out in numerous states, from the 1940s on in Utah, Nebraska, Arizona, then California with public breeding at the University of California at Davis, and several private companies being involved. From the 1960s on, a major safflower breeding program was commenced in eastern Montana, at Sidney. A series of varieties was released with resistance to alternaria leaf blight (caused by the fungus *Alternaria carthami* Chowdhury) (Bergman et al. 1985, 1987, 1989). These varieties were developed by mass-selection of resistant plants from crossing of existing varieties in a disease nursery initiated in the early 1960s. In anticipation of the need for high oleic safflower varieties, this program developed a series of such varieties named Montola and year of release, starting with Montola-2000 (Anon. 2007c). As infection with safflower rust (caused by *Puccinia carthami* Cda) at the seedling stage can cause serious yield reduction in

warm soils, five improved safflower lines (PCA, PCM-1, PCM-2, PCN, and PCOy) have been developed, each carrying a different dominant gene for rust resistance (Zimmer and Urie 1970). Also stem and root rot resistant varieties, resistant to *Phytophthora drechsleri* Tucker, have been developed over the decades, including Gila, US10, VFR-1, Dart, and most California cultivars (Mündel and Huang 2003). Anecdotal evidence from numerous countries indicates that the highly-variable variety Gila (Dave Rubis – personal communication), released from the Arizona program in 1958, has in following decades been grown in more countries and covering more area than any other single variety. From the 1970s on, some of the varieties of the private breeding company, SeedTech, headquartered in Woodland, CA, have dominated commercial production areas in the US. This included specially S-208 and S-541, with their high-oleic variety, S-317 being produced mainly in a rather confined area in California.

Contributions to disease resistant varieties and germplasm have also been made by safflower programs in countries with very modest safflower breeding programs. For example, in Australia the cultivar Sironaria was developed in a backcrossing program to Gila, selecting plants to incorporate resistance to races of *A. carthami* (Harrigan 1987); and in Canada, Saffire was developed by mass-selection from a bulk derived from selections from India, having good field resistance to head rot, caused by *Sclerotinia sclerotiorum* (Lib.) de Bary (Mündel et al. 1985).

Important examples of major screening of safflower germplasm against insect and diseases, as well as describing in detail thousands of lines are those from China and Israel, in which important germplasm lines have been identified for use in breeding programs world-wide (Ashri 1971; Li et al. 1993; Wang Zhaomu 1993).

14.6 Current Goals of Breeding

Genetic improvement in safflower has been commonly aimed at improvement of yield, oil and other agronomic characteristics, including resistance to diseases, insects and abiotic stresses.

Over the decades, one of the common breeding goals has been and continues to be the increase of oil content. One way this has been attempted is by the reduction in the ratio of seed hull of the achenes to the total seed. While a typical commercial safflower seed may be white (Fig. 14.1) as shown in Saffire, various genetic variations have been developed with resultant reductions in the see coat, and hence increases in oil content, ranging from 42 to 50% in California (Knowles 1982). Thus, partial hull (Fig. 14.2), reduced hull (Urie and Zimmer 1970) (Fig. 14.3), and striped hull (Fig. 14.4) can be used for commercial varieties.

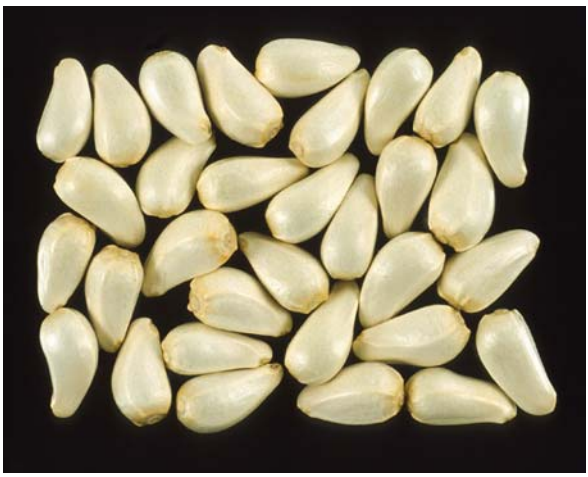

Fig. 14.1 White seeds of Saffire safflower

Fig. 14.2 Partial hull seeds

Typically the fatty acid composition of safflower has been over 70% linoleic acid, a polyunsaturated fatty acid, and with also a significant percentage oleic acid, a monounsaturated fatty acid. The genetics of the fatty acid composition has been determined and manipulated for different uses. Over the past decade high oleic acid safflower is favoured by nutritionists and for frying, hence by the commercial trade. Knowles (1972) summarized information on the genetics of fatty acid composition. Three alleles at one locus govern the levels of linoleic and oleic acid, with the intermediate levels being temperature sensitive: linoleic contents being high

Fig. 14.3 Reduced hull seeds

Fig. 14.4 Striped hull seeds

under cool temperatures and oleic contents being high under high temperatures. As the genetic control of fatty acid composition in safflower is expressed in the embryo (Knowles 1983), F_2 segregations can be determined from single seeds on an F_1 plant.

The All India Coordinated Research Project on Safflower, has identified the following goals as their thrust for the Xth Plan (2002–2007) of the Indian government (Damodaram 2006):

- Development of spineless varieties with high yield and high oil content possessing resistance to *Alternaria* wilt and aphids.
- Exploration of heterosis through the development of CMS, maintainer and restorer lines.
- Development of seed production technology for the hybrids already released through GMS background.
- Wilt and *Alternaria* disease management and aphid management through IPM technologies.
- Development of region-specific crop production technology for higher production.

14.7 Crossing Techniques and Breeding Methods

14.7.1 Crossing Techniques

Safflower is a predominantly self-pollinating crop. However, depending on the genetics and environmental conditions, outcrossing may be considerable (Li and Mündel 1996). A diversity of bees, as well as other insects, are attracted to safflower pollen and nectar, and thus contribute to outcrossing. Thus, to ensure planned crossing, flowers must be emasculated prior to pollen shedding.

While specific procedures in use by different breeders may differ somewhat, the following provides a generally acceptable procedure for crossing safflower. In this case a greenhouse or phytotron is assumed for crossing, thus insect contamination of the pollen parent is obviated. As indoors heads may be fewer than in the field, staggered planting over several weeks is advisable, to ensure pollen availability when stigmas of the designated females are receptive. Considerable variation may be observed in different safflower germplasm lines or varieties. The calyx is removed and at least the first (outermost) whorl of flowers, which is typically female-sterile (Knowles 1980) when the first individual flowers have elongated and are about to or have just appeared above the bracts (Fig. 14.5). Once the first flowers have elongated, which can occur inside the calyx in some material, typically anthers have dehisced and pollination has already been effected.

In preparation for crossing, the outer corolla tubes of each flower are gently broken by holding with slender tweezers and twisting slightly until a snap is felt just below the origin of the anther filaments. Then the upper part of the corolla tubes with the five attached fused anthers is slid upwards and outwards over the style and stigma, ensuring that the latter is not damaged nor breaking off the style. This can best be accomplished by taking the tip of the closed corolla lobe with the tweezers and gently pulling it up and off the rest of the flower head (Fig. 14.6). Immature buds in the centre of the head should be cut off with the tweezers.

Fig. 14.5 Developmental stage for preparing flowers for crossing

Fig. 14.6 Emasculation by sliding corolla tube upwards

The next day, if the styles have elongated considerably (Fig. 14.7), indicating that the stigma would be receptive, pollen can be added by using whole flowers from the pollen parent (Fig. 14.8), if adequate flowers are available. Pollination can commence an hour or two after daylight, natural or artificial, and continue

Fig. 14.7 Elongated styles

Fig. 14.8 Pollination

for several hours. If pollen is in short supply, one pollen-rich style of the pollen-parent may be used to pollinate a number of stigmas of the female emasculated parent. The pollinated capitula is covered with plastic bags, appropriately labeling the male and female with dates of emasculation and pollination on small tags. After a few days the successful crosses will set seed and to prevent high humidity build-up inside the head, at least the top of the plastic bag can be cut off, or the bag can be removed completely (Fig. 14.9). As soon as seeds mature they may be harvested. Delays may result in crossed seeds falling off.

Fig. 14.9 Head of crossed seeds

At the NARI in Phaltan, India, a mass-emasculation technique was developed (Deshmuk and Ranga Rao 1991). At flower initiation, 5–10 fully developed capitula from the top four or five branches of each plant are covered with polythene bags in the field. The remaining branches are pruned off. Temperature and moisture build-up inside the bags prevents dehiscence of anthers. When 50% flowering is attained, the bags are removed, flowers are pollinated with the desired pollen source and the bags are again closed. This may be repeated for three successive days. Once flowering is complete, tissue paper bags are used to replace the polythene bags to reduce moisture and hence disease accumulation in the head. This technique has been shown to permit up to 10 times as many crossed seeds to be produced in the same time as

emasculation of individual florets. However, this mass-emasculation technique is only effective at the moderate temperatures of December and early January at Phaltan. At higher temperatures, the pollen is sterilized in the bags.

14.7.2 Breeding Methods

14.7.2.1 Pure-Line Selection and Mass Selection

Pure-line selection is the oldest and most extensively practised method of crop improvement for safflower. Weiss (1983) mentions that the term 'pure lines' was then used rather loosely and that many of the varieties resulted from selections in early generations following a cross. Thus, such varieties will in fact be mixtures of similar types, appearing quite uniform under commercial production. In India, 16 out of the 28 varieties released for commercial production since N-630 was released in 1942, have been developed by carrying out selection in the local landraces (6) and selections in varieties or germplasm lines (10 for these two groups together) (Nimbkar, personal communication 2007).

Mass selection from fields naturally infested with a multitude of diseases has been used in Montana, USA, to develop cultivars with improved field resistance to several diseases, including leaf blight caused by *Alternaria carthami* and bacterial blight caused by *Pseudomonas syringae* van Hall. This was followed by crossing to commercial varieties and resulted in such varieties as Oker, Hartman and Girard (Bergman et al. 1985, 1987, 1989).

14.7.2.2 Pedigree Breeding

Plant breeders of safflower have generally used variations on the pedigree method for handling segregating generations (Knowles 1989), selecting for highly heritable characters (e.g. early maturity, disease resistance) beginning from single F_2 plants. To improve seed yield, oil content and other desirable traits in safflower, breeders have often followed the pedigree method. Starting with the release of A-1 in Annegiri in 1969, almost a dozen safflower varieties have been released by both government and private breeders in India using the pedigree method (Nimbkar, personal communication 2007).

14.7.2.3 Backcross Breeding

Backcrossing has been used to introduce specific characters, especially disease resistance, into otherwise good commercial cultivars. For example for transferring the *Fusarium oxysporum* (wilt) resistance genes to the wilt-susceptible variety Nira (Singh et al. 2003) in India. The wilt resistant varieties developed by using the backcross method are presently in multilocation evaluation for assessing their adaptability and yielding ability and to identify the most suitable one for commercial production.

14.7.2.4 Recurrent Selection

Recurrent selection programmes have also sometimes been used in safflower improvement, when the proper tools were available. For example, in a programme begun in 1970 in Arizona, USA, Rubis (1981) used structural male sterility associated with the thin-hulled gene (*th th*) to enforce outcrossing and produce lines highly resistant to root rot caused by *Phytophthora* spp. The thin-hull gene has shown crossability of 98–100%. Flooding, along with high temperatures at flowering time, following moisture stress imposes a very strong selection pressure for resistance to phytophthora root rot. Enforced fertilization by pollen among the surviving plants resulted by the use of this thin-hulled gene. Thus, a complete cycle of recurrent selection was achieved in a single year. The survival rate of safflower plants increased from 14% in the best plots in 1972 to 85% in 1980, while the survival rate of the check variety (Royal) was 0%.

Based on a genetic analysis of seed yield, oil content and their components of four Indian and seven US safflower lines in an 11 × 11 diallel cross, Ramachandran and Goud (1981) suggested a combination of breeding methods, such as biparental mating followed by reciprocal recurrent selection, for the simultaneous improvement of seed yield and oil content.

14.7.2.5 Hybrid Breeding

As safflower is largely self-pollinated, though outcrossing does occur naturally, significant heterosis is expected for such economically important factors as yield and oil content. Thus, efforts to produce commercially viable hybrids has been ongoing for many years.

Genic male sterility, identified in safflower by Heaton and Knowles (1982), has been considered for use in hybridization to produce high-yielding cultivars. However, manual removal of male-fertile plants in crossing blocks has generally made this procedure prohibitively expensive where labour costs are high.

In studying the effectiveness of early-generation selection for yield by using various yield components in hybrid safflower, Patil et al. (1994) used only fertile plants of 25 hybrids developed with parents selected on the basis of good general combining ability. The segregating progenies studied included five dominant genetic male sterile parents crossed with 10 male parents. While test weight and seeds per capitulum were ineffective in selecting for yield, capitula per plant and individual plant yield were useful early-generation selection criteria.

USA

Rubis (1967) identified a form of structural sterility, linked with the thin-hull mutant. However efforts to develop hybrids using this system failed because of a high percentage of selfed female plants, which adversely affected yield

(Sujatha 2006). Furthermore, seeds with the thin-hulled character are readily damaged on commercial harvesting.

Hill (1991), working for Cargill at the time, initiated a program to produce commercially viable safflower hybrids in 1972. This system for the development of cytoplasmic male sterile (CMS) lines relied on the use of the wild safflower, *C. oxyacanthus* as female and the domestic safflower, *C. tinctorius* as restorers of CMS and as recurrent males. Because of dominance of the restorers (R-lines), F_2 populations were required from each of the backcross females starting at BC_3 and selecting only the sterile plants. Hybrid testing began in 1983, with gradual improvements in oil content and absence of pappus on the seeds. Various partnerships had been developed between A.B. Hill and his Safftech Hybrid Safflower company and initially two other California safflower companies developing safflower varieties, SeedTec and Cal West Seeds (Hill 2005), then mainly with Montana-based Safflower Technologies International. Yield increases in the hybrids had been recorded from multi-location trials and birdseed safflower hybrid commercialized. High-oil, with 44% oil, B-lines are expected in test hybrids by 2008 or 2009. This system is not available to the public.

India

With significant heterosis of up to 177% for seed yield and 80% for oil being reported for safflower, led to the development of both dominant and recessive genetic as well as cytoplasmic male sterility systems in India (Sujatha 2006; Singh and Nimbkar 2007). Five hybrids, including dominant GMS, recessive GMS and CMS systems, have been released in India over the past 10 years (Nimbkar, personal communication 2007). Aside from improvements in yield, these hybrids variously have moderate to high field resistances to *Alternaria* wilt and leaf spot and three show tolerance to the black aphids as well.

Hybrid seed production, using the genetic male sterility systems, necessitates labour-intensive roguing before flowering of 50% male fertile (MF) plants appearing in the female parent (GMS line) (Sujatha 2006). The roguing of MF plants in the hybrids based on non-spiny genetic male sterile lines is economically viable. However, in case of spiny genetic male sterile lines due to presence of spines on the plants, roguing of MF plants is cumbersome and tedious and hence is commercially not feasible.

The development of cytoplasmic-genetic male sterility system is underway at the NARI, Phaltan, Maharashtra (Singh et al. 2001) and at the Directorate of Oilseeds Research (DOR), Rajendranagar, Hyderabad (Anjani 2005). The system developed in Hyderabad employed an interspecific cross between *C. tinctorius* and *C. oxyacanthus,* with the latter being the donor of the sterile cytoplasm. Single gene segregation for male sterility to male fertility, showed dominance and that *C. tinctorius* possesses a nuclear fertility restorer gene (*Rf*), with hybrid progeny carrying the cytoplasmic male-sterile (CMS) cytoplasm of *C. oxyacanthus*. Thus male sterility occurred with the homozygous recessive

condition (*rfrf*) in a sterile *C. oxyacanthus* cytoplasm background, but not in the normal cytoplasm of *C. tinctorius*. The utilization of a CMS system for hybrid development at these institutes is hindered due to the unavailability of suitable male sterility maintainer genotypes. However, another Maharasthra private seed company, MAHYCO, has not only developed the male sterility maintainer genotype for the sterile cytoplasm but has developed and released the first CMS-based safflower hybrid MRSA-521, expressing high wilt resistance, for commercial cultivation in India during 2006–2007. With the development of CMS-based hybrid MRSA-521, released by MAHYCO in 2006, it is expected that hybrid seed will be made available much more cheaply to the producers as compared to the seed of GMS-based hybrids in safflower. This should lead to the rapid expansion of hybrid safflower area in India. In India, the area under hybrid safflower is increasing gradually, however it still comprises less than 5% of the total safflower grown in the country.

Apart from the development of GMS and CMS systems, the NARI has developed thermosensitive genetic male sterility (TGMS) in safflower (Nimbkar, personal communication 2007). In this system, during winter, which is the normal safflower-growing season, TGMS lines show complete male sterility, with temperatures ranging from 10 to 32°C, behave as fertile genotypes when grown under summer conditions, with day temperatures during pre flowering and flowering ranging from 19 to 42°C. The hybrids developed from such lines show complete fertility in both winter and summer seasons. The TGMS hybrids have exhibited an average increase if yield of 15–20% over the best GMS hybrids. Multilocation evaluation of TGMS-based hybrids will be started in the winter 2007–2008.

14.8 Integration of New Biotechnologies in Breeding Programs

Safflower offers potential in the use of Agrobacterium-mediated gene transfer. *Agrobacterium tumefaciens*-mediated transformation and regeneration of transgenic safflower using the variety Centennial was accomplished by Ying et al. (1997). However, root regeneration was at a low percentage. A protocol for regeneration of plantlets with well-developed root systems was developed by Tejovathi and Anwar in 1993.

The genetic modification of crop plants for use as protein factories has been pursued for nearly a decade as a potential method of meeting the high volume demands made on the pharmaceutical industry. Safflower is a host species with growing appeal for genetic modification for biopharming for the production of pharmaceuticals, proteins, enzymes, and safflower oil modifications for specialty oil markets.

A Calgary, Alberta, Canada-based company, SemBioSys Genetics Inc., has found safflower to be a very attractive host for the production of high value proteins such as pharmaceuticals and industrial enzymes. SemBioSys

genetically transforms safflower tissue so that the proteins of interest will accumulate in the seed of the mature transgenic plant. The patented Stratosome™ Biologics System involves the genetic attachment of commercially viable target proteins to oleosin, the primary protein coating the oil-containing vesicles (oil bodies) of the seed (Fig. 14.10). This attachment allows the target protein to be purified along with the oil body fraction which floats to the surface of a ground seed/water slurry upon centrifugation (Fig. 14.11) (van Rooijen

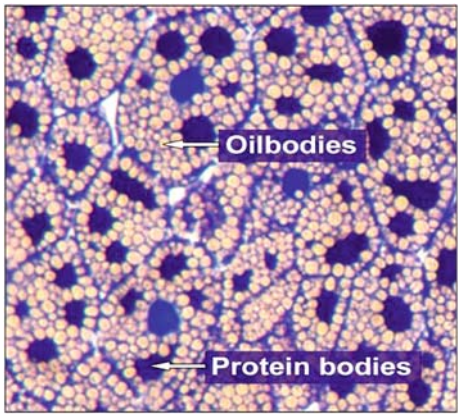

Fig. 14.10 Oil bodies (oleosomes) and protein bodies in cells of oil-bearing tissue

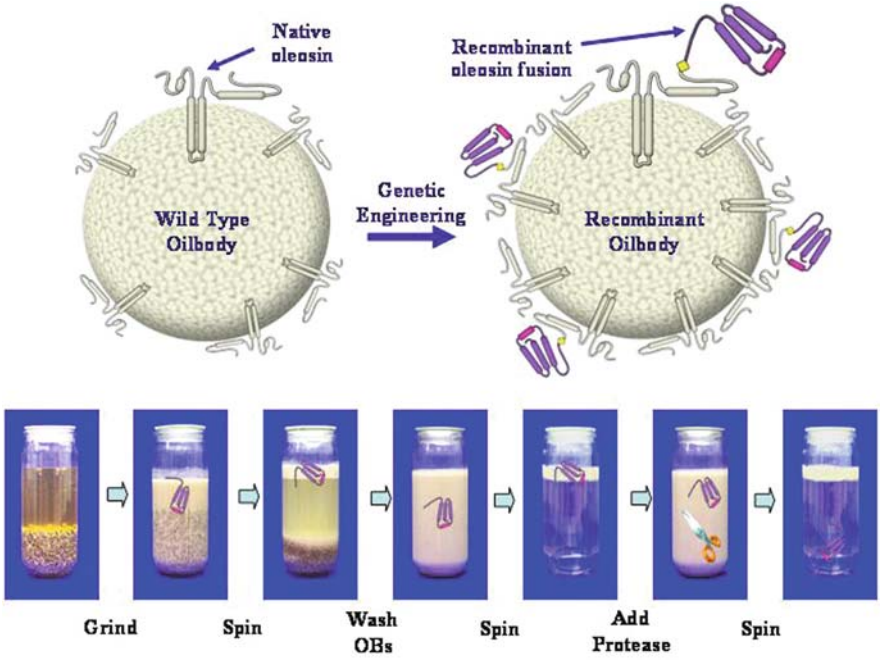

Fig. 14.11 Schematic representation of purification of recombinant proteins produced in genetically engineered oil body membranes

et al. 1992). This initial purification step gives the system a favourable process advantage over other transgenic plant systems. In addition to the purification advantage of the Stratosome™ Biologics System, the attachment of proteins to the oil bodies of safflower has been shown to stabilize intracellular accumulation of foreign proteins as well as providing a useful attachment matrix and delivery benefits for downstream applications.

The uncontained, outdoor production of so-called 'Molecular Farming' crops such as SemBioSys' GM safflower offers the potential for economical, huge-scale production of pharmaceuticals, indeed 'molecular pharming,' and industrials but, understandably, must be regulated for proper material confinement. The Canadian Food Inspection Agency (CFIA) (in Canada) and the United States Department of Agriculture (USDA) (in the USA) have placed a host of restrictions on the numerous crops that are used for Molecular Farming purposes but many of the features possessed by safflower make it a lower risk production platform. Several inherent agronomic qualities such as a low tendency to weediness, low seed dormancy and the large degree of self-pollination translate into a system that is much easier to confine so that target products don't mingle with food or feed. The role that safflower plays in North American agriculture also lends benefits to its use as a pharmaceutical factory. Safflower is not a major food or feed crop in North America, acreages are relatively low and there are no weedy relatives with which it can cross to produce fertile hybrids. As the tiny molecular farming industry grows in North America, safflower has been receiving increasing attention as a host crop with great appeal.

SemBioSys Genetics, Inc. has formed strategic partnerships with other companies to utilize genetic modified safflower varieties for products that include GM varieties that accumulate omega-3 and omega-6 fatty acids, human insulin, carp growth hormone, and apolipoprotein-A1 for the treatment of cardiovascular disease. Commercial production of these GM varieties is expected to be accomplished in the next several years and will stimulate further advances in safflower genetic engineering and biotechnology. A comprehensive review article on the 'Advances in Safflower Biotechnology' by Sujatha (2007) covers the topic in more detail for interested readers.

14.9 Seed Production

Maintaining genetic purity of safflower varieties starts with breeder seed to supply a pure seed source for certified classes of seed that meet seed certification standards. The seed originators supply a statement of the variety's origin, breeding procedure used in development, and detailed description of the plant and seed characteristics to seed certifying agencies. Any variants in the variety must be stated so field inspectors can recognize the variation from crop mixtures. The number of generations through which a variety may be multiplied

should be specified by the originator but usually will not be allowed to exceed three generations beyond breeder seed. The foundation class of seed must be the progeny of breeder or foundation seed. The registered class of seed must be the progeny of breeder or foundation seed and may be omitted by the seed originator. The certified seed must be the progeny of breeder, foundation, or registered seed.

Safflower may not be considered for certification if planted where safflower has been grown the previous two crop years. Seed certifying agencies recommend that safflower be planted on land immediately following a separable crop. Additionally, safflower is not recommended to follow other oilseed crops.

The minimum isolation requirement for pure seed production of safflower is at least 400 m from any other variety or non-certified field of safflower. The recommended minimum isolation requirement for pure hybrid seed production is 4.83 km. Off-type plants or identifiable variety mixtures must be removed prior to bloom or before pollination occurs. Specific field standards to pass field certification depend on the certified class of seed. Field standards restrict the number of plants of other varieties (none to 1 per 1,000 plants), inseparable other crops (none to 1 per 3,000 plants), noxious weeds (none). Safflower seed standards for certification specify a seed purity of 98% with a minimum germination of 80% and limit the percent inert matter (2%), other crop seeds (0.1–0.10%), weed seeds (0.01–0.10%) noxious weed seeds (0%), sclerotia (1 seed per 0.45 kg), and seed moisture of 8% or less.

Producers are responsible for cleanliness and maintenance of all equipment and storage facilities used in the planting, harvesting, and storage of the safflower. Approved seed conditioners are usually required for cleaning all certified classes of seed. Seed cleaning facilities must condition seed without introducing other crop or variety mixtures in cleaning, treating, handling and bagging each seed lot of safflower.

Pure seed production growers should rely heavily on herbicide, insecticide, and fungicide control of safflower pests as an integral and necessary part of pure seed production. The following herbicides are labeled for weed control in safflower (USA): paraquat, Eptam (EPTC), trifluralin, sonalan, metolachlor, clethoderm, and sethoxydem. Azoxystrobin is labeled for foliar application to control *Alternaria* leaf spot. Insecticide and fungicide seed treatments and foliar applied treatments are available and recommended to control insects and diseases when potential problems may occur in pure seed production fields.

The Montana Seed Growers Association (2006), the North Dakota State Seed Department (1986), and the California Crop Improvement Association (2003) seed certification standards were used as references for this section. Relatively minor variations are expected in these and other jurisdictions.

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Chapter 15
Poppy

Jenö Bernáth and Éva Németh

15.1 Introduction

Poppy (*Papaver somniferum* L.) has been utilised and cultivated since prehistoric times (Tétényi 1997). The narcotic and nutritive values of its products were recognised in ancient Egypt, Greece and Rome. Hippocrates (460–377 BC) was among the first to emphasise the medical advantages of poppy and its preparations. He also recognised the nutritive property of poppy seeds. Poppy spread from its Central Asian gene centre through the Roman Empire, where cultivation for food and medicinal utilisation started probably at the same time in all provinces. After the Roman period poppy cultivation continued both in Europe and Asia. However, opium became the main product in Asia, while poppy seed and oil were utilised in Europe.

The opium production in Southeast Asia (Golden Triangle), West Asia (Golden Crescent) and other territories is still going on using traditional methods. The production is regulated by local consumption, market possibilities and political considerations. A political reason for opium production was obvious during the 'opium-war' between Great Britain and China (1838–1842). At present the policy of international organisations (WHO, FAO, UNODC) is oriented towards gaining control of opium production in order to reduce its illicit trade and consumption.

In Europe, a large amount of poppy oil had been produced for food use at the end of the 18th and in the first half of the 19th century in France (Provance, Alsace) and Germany. Later, the importance of poppy oil declined, and industrial application remained the main branch of poppy utilization. In the 19th century the manufacture of morphine started in small pharmaceutical companies using opium imported from Turkey and Persia. About 45% of the world's morphine production is still based on this traditional source (Bryant 1988), and 800–100 tonnes of licit Indian opium are processed annually.

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In the first half of the 20th century a remarkable advance was achieved for extracting morphine and related compounds from poppy straw. The invention of the Hungarian pharmacist Kabay in 1928 opened a new perspective for the industrial utilization of poppy (Bernáth 1998). Based on his method the alkaloids, especially morphine had been extracted from capsules. Before, straw was known as a waste, which had to be separated from the seed as the final step of commercial poppy cultivation. Using the new method a high seed quality crop and a valuable raw material for pharmaceutical industry were available at the same time. The invention resulted a continuous increase of the poppy cultivation area in Europe and most recently in Australia. Considering the licit production area and quantity of harvested poppy straw and seed, the main European producers are Hungary, Spain, France, Romania, Slovakia, the Czech Republic and Poland (INCB 2000). Germany, Austria and the Netherlands also produce smaller amounts of poppy seed, almost exclusively for local utilisation. In Turkey the annual poppy production is about 70,000 ha (INCB 2000). In the late 1960s, Australia appeared as a new poppy producer; the cultivation area located in Tasmania increased up to 21,000 ha till 2001.

In spite of the great pharmaceutical and nutritional value of poppy, a large amount of the crop is cultivated for illicit purposes. According to data of Bryant (1988) around 40,000 tonnes of opium is produced world-wide, and only 5% of it is used as a raw material of industrial production or source of seed. This contradictory situation was recognised even at the beginning of the 20th century, and international efforts were initiated against drug abuse and illicit traffic; as a result of the international harmonisation a special board was brought into existence for checking and advising global tendencies (Bernáth 1998). In European countries, parallel to administrative regulations, a new strategy was adopted by governments interested in large scale cultivation of poppy. Utilising the huge biodiversity of the species, selection had been intensified into three main directions (Bernáth and Németh 1999):

- Producing cultivars with high alkaloid content (1.5–2.5% morphine or thebaine), which are reserved for pharmaceutical and large-scale seed production, only. This genetic pool can be cultivated in strictly controlled agricultural areas.
- Selection of cultivars with low morphine content (less than 0.1%). These cultivars are suggested for seed/oil production in any agricultural region.
- Ornamental cultivars showing special morphological characteristics of flower and capsule. This type of cultivar can be grown without restriction because of low morphine content and small cultivation area.

Consequently, the appearance of new types of cultivars required a revision of cultivar registration and DUS testing, for which new evaluation procedures had to be developed (Köck et al. 2001).

15.2 Origin and Domestication

Although poppy is one of the few species utilised or even cultivated in prehistoric time, its origin is not yet fully clear. Until the 1930s *Papaver setigerum* D.C. was considered as the ancestor of poppy, which was supported by the finding of fossils. It became clear that poppy was known by the cavemen living in the territory of Spain, France, Germany and Hungary 4–5 thousand years BC. This proves that some form of poppy was widely know and utilised in ancient time (Tétényi 1997). However, based on cytogenetic investigations (Hrishi 1960), large differences between *P. setigerum* and the cultivated poppy were found questioning the *P. segiterum* origin. It now appears acceptable that the gene-centre of cultivated poppy is Central Asia, especially in the territories of Iran and Afghanistan.

On the basis of written historical records the gene center of poppy had to be located in western Asia (Simmonds 1976). This is supported by early historical records, which show ritual and therapeutic-like applications of the plant in this area. The name of the plant appeared in the classic literature such as in Odyssey and Iliad of Homer. Kritikos and Papadaki (1967) summarizing the historical evidence concluded that the Greeks portrayed their divinities Hypnos, Nyx and Thanatos with poppies. The first written record dates back to the eighth century BC. In the Corinth region a city was named Mekone (Poppy-town); the name of the town may reflect the fact that there was an extensive cultivation of poppy, or it was the place of first discovery of the plant. In early records the plant was mentioned as a tool for attaining an easy and painless death. Hippocrates (460–377 BC) was one of the first who emphasised the medical advantages of poppy and its preparations. He mentioned that the plant was frequently used in medicinal preparations in unripe, ripe and baked forms, and he also recognised the nutritive property of the seeds. Herakleides (340 BC) reported the plant to be used as a tool for euthanasia in some Greek islands. People, especially women took poppy to shorten the time until natural death.

Dioskourides (first century AD) distinguished between several kinds of poppy. The 'cultivated' or 'garden' poppy was used in baking bread. Two types were known in this category, plants with elongated capsules and white seeds, and plants forming involuted and elongated capsules with black seeds. From the present botanical point of view, those types represent the species *Papaver somniferum*. Another group named 'flowering' poppy showed high hypnotic properties and may refer to the species *Papaver hybridum*. The 'wild' poppy group may be equivalent to the species *Papaver orientale*. Later, Pliny mentioned an 'intermediate' type between the 'wild' and 'cultivated' poppy, which may be *Papaver rhoeas*.

There is evidence that poppy was cultivated by Sumerians, Babylonians and Assyrians at about 3–6 thousand years BC. On the clay tables of Sumerians the production method of poppy juice was detailed, which was collected very early in the morning; it was called 'gil' and was used for curing. Poppy was also

known in early ancient Egypt, to where it was introduced from abroad, particularly from Greece and Babylon.

The name and preparations of poppy appear both in the Bible and in the Talmud. Probably the plant head 'rosch' refers to the capsule of *Papaver setigerum*.

The exact time of the introduction of poppy into India is under discussion. Probably it had been introduced at the time of the invasion of Alexander the Great (fourth century BC). The Persians carried poppy for the needs of their army. Later, no evidence of poppy application in India is existing till the seventh century AD. However, it is hard to believe that opium was unknown by the Indian physicians till that time.

The phylogenetic origin of opium poppy was approached from different points of view. Some botanists (De Candolle 1883; Fedde 1909; Soó 1968; Hammer and Fritsch 1977) assumed that opium poppy originated from *P. setigerum*. Another hypothesis was that *P. glaucum* is the ancestor of the opium poppy (Rothmaler 1949), and by others, a parallel evolution of *P. somniferum* and *P. aculeatum* was supposed (Reckin 1971). A triploid hybrid origin of *P. somniferum* ($2n = 22$) was assumed as well, which was supported by morphological evidence derived from interspecific crosses (Kadereit 1986). The crosses of *P. glaucum* ($2n = 14$) with *P. gracile* ($2n = 14$) produced progeny with capsules similar to *P. somniferum* subsp. *setigerum*, but leaves and petals similar to subsp. *somniferum*. Nevertheless, the triploid origin of *P. somniferum* was also supported by cytogenetic studies. Thus, an F_1 hybrid of subsp. *setigerum* ($2n = 44$) with subsp. *somniferum* ($2n = 22$) has $2n = 22$ and when selfed, the F_2 descendants were mostly $2n = 28$, regaining the original basic number of the genus *Papaver* ($x = 7$).

At present, the classification of Vesselovskaya (1975) seems to be widely accepted. Her system is made up of three levels: the subspecies based on origin (geography), morphology (height and branching of axis, shape of capsule) and physiology (life cycle). However, none of these classifications dealt with differences in alkaloid biosynthesis. An infraspecific classification of opium poppy was established by Tétényi (1963) based on the diversity of alkaloid synthesis and accumulation. Chemoconvars 'Morphinan' and 'Isoquinoline' as well as their chemoprovars were distinguished. This classification was developed taking into consideration the two demethylation pathways and the response to photoperiod (Tétényi 1989).

15.3 Genetic Resources and Varietal Groups

In the first half of the 20th century, cultivation was mainly based on traditional populations, and selected poppy varieties were scarcely known. In Germany, Heeger (1956) mentions five registered cultivars, while he emphasizes the dominance of landraces without selection in other countries. In Hungary, the

science-based breeding had started around 1930 (Köck et al. 2001). Today, the varietal background of the production shows characteristic differences among cultivation areas of the world:

- In areas of highly developed industrial production (West Europe, Australia), patented strains are used without cultivar registration by the varietal authority. Because of industrial interest, detailed information on them is almost fully missing. These materials are homogenous, developed by different breeding methods and selected for special production characteristics.
- In other countries (Central Europe) the selected cultivars are registered by the national variety offices, similar to the varieties of other agricultural crops. The registration authorities investigate candidates according to the valid DUS guideline for *Papaver somniferum*. The official European Union cultivar-trials for poppy are performed in Hungary. Seed and basic information of these cultivars are available. However, in case of industrial varieties, the propagation material is practically distributed only by the processing factory to their agricultural producers. For the breeders' interest the best varieties may also be patented.

The registered varieties present in the European variety list (CPVO) are shown in Table 15.1 based on their primary utilization. *Industrial cultivars* of high alkaloid content are used for extraction and processing of pharmaceuticals. Therefore, until recently, the prime goal of breeding was the increase of alkaloid levels. During the last decades, the morphine content was increased from 2–2.5‰ ('Fertődi zárttokú' 1952) up to 18–20‰ ('Minoán' 2005). The *culinary varieties* are primarily used by households and in food processing,

Table 15.1 Poppy cultivars (*Papaver somniferum* L.) registered in European Union (Anonymus 2007c)

Industrial (capsule)	Double use (capsule and seed)	Culinary (seed)
'A- 1' – Hungary	'Bergam' – Slovakia	'Albin' – Slovakia
'Alfa' – Hungary	'Edel-Weiss' – Austria	'Agat' – Poland
'Botond' – Hungary	'Gerlach' – Slovakia	'Albakomp' – Hungary
'Buddha' – Hungary	'Kék Duna' – Hungary	'Ametiszt' – Hungary
'Csiki kék' – Hungary	'Major' – Slovakia	'Aristo' – Austria
'Extaz' – Romania	'Malsar' – Slovakia	'Florian' – Austria
'Evelin' – Hungary	'Marathon' – Slovakia	'Kozmosz' – Hungary
'Kék Gemona' – Hungary	'Marianne' – Netherland	'Josef' – Austria
'Lazur' – Poland	'Sokol' – Czech Rep.	'Michalko' – Poland
'Medea' – Hungary	'Opal' – Slovakia	'Mieszko' – Poland
'Minoan' – Hungary	'Parmo' – Denmark	'Przemko' – Poland
'Monaco' – Hungary	'Rubin' – Poland	'Zeno' – Austria
'Nigra' – Hungary	'Rosemarie' – Netherland	'Zeno 2002' – Austria
'Riesenmohn' – Germany		'Zeta' – Austria
'Tebona' – Hungary		

where either the seed or the oil extracted from the seed is utilized. To date, no varieties have been developed for an increased seed oil content or for a particular fatty acid composition.

The basic element in regulation of poppy cultivation and avoiding drug abuse seems to be the use of proper cultivars. The production of morphine rich cultivars is permitted exclusively under strict control (UN Agreement, signed in 1988). On the other side, a minimum value or traces of morphine in the capsules create the basis for uncontrolled cultivation. However, no uniform limits between high and low alkaloid or morphine content cultivars have been established yet. In Germany 0.01% maximal morphine value is allowed in dry capsules; in Hungary this limit will be 0.2% from 2010 on (Németh and Bernáth 2007), whereas in the majority of countries no exact distinction level is existing. For this reason, both industrial and culinary variety groups include a wide range of alkaloid contents. For the industrial use we can find cultivars of medium level (0.2–0.8%), e.g. 'Edel-weiss', but also ones of maximum alkaloid level (above 1.5%), e.g. 'Botond'. The case is similar for the culinary varieties: only 'Przemko', 'Mieszko', 'Mihalko' and 'Ametiszt' have practically morphine-free capsules (alkaloid level below 0.1%), whereas others exhibit different accumulation potentials of up to 0.4–0.5% (Table 15.1). Traditional cultivars usually exhibit a larger variability both in morphology and alkaloids, whereas varieties released during the last 10–20 years are phenotypically more uniform and stable. The most recent Hungarian varieties show a clear separation between either industrial or dietary utilisation with respect to their morphine content (Fig. 15.1).

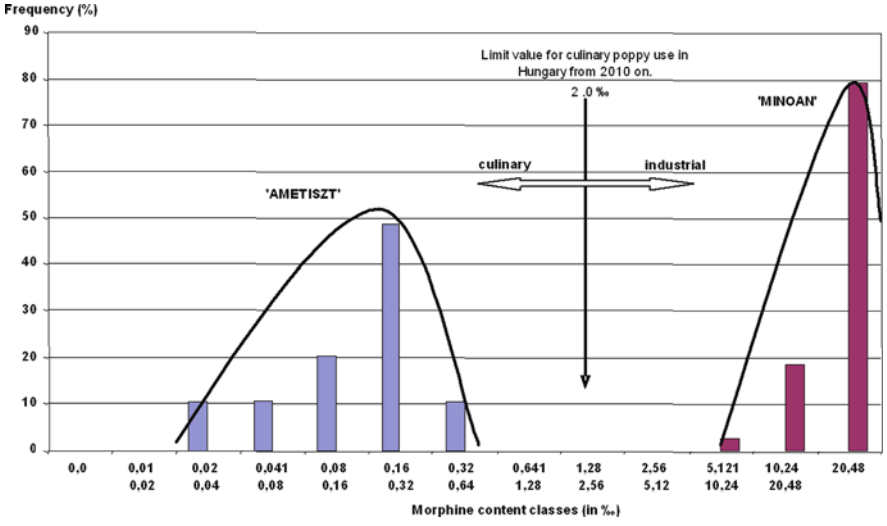

Fig. 15.1 Separation of culinary and industrial cultivars based on the morphine content of capsules (Németh and Bernáth 2007)

In several European and Asian regions, the majority of cultivated materials are landraces even today, i.e. populations without any systematic breeding. They are well adapted to the local conditions, but usually are far from fulfilling the requirements of intensive agriculture. In Romania, a great diversity was found in the investigated morphological characteristics of local poppy populations (Handrea 1996). In Turkey, the seed market offers populations characterised by flowers of mixed colour and shape (Anonymous 2007a). Indian landraces consist of 20–25 basic types, which have given rise to a wide diversity by intermixing and hybridisation during long years of cultivation (Singh et al. 1997). Representative cultivars for this region are 'Telia-1', 'Bhakua', 'Aphuri', 'Kantia pink', 'Galania', 'Mandraj' or 'Kasuha'. Differences include plant height, flower colour, capsule shape, morphine content and vegetation length. An intensive development of genetic resources is carried out in producer countries at present (Balci et al. 2007; Singh et al. 2003), but local populations and strains may assure a wide and prosperous genetic basis for developing new cultivars.

15.4 Current Goals of Breeding

The following are the major goals of current poppy breeding which are independent of the type of utilisation:

Increase of yield had always been a primary aim of breeding. Poppy yield includes the seeds, capsules and alkaloids in Europe and Australia, latex and opium in India. The production of active materials is based on dry matter yield and the accumulation level of active materials such as alkaloids or seed oil (Bernáth 1986). However, dry matter production is a complex composed of morphological (e.g. number and size of capsules) or physiological features (e.g. length of vegetation period, floral biological properties, nutrient uptake and utilisation, stem stability). Thus, improving these characteristics by breeding indirectly rises the yield level and profitability of the crop (Singh et al. 1997; Németh 2002).

Morphological homogeneity is considered a basic requirement for variety registration. Previously, colour of flowers and seeds were the main features (Mórász 1979), whereas today homogeneity of several other characteristics needs to be assured according to the UPOV guidelines and examination methods (Anonymous 2007b).

The significance of *resistance breeding* has increased during the last 20 years connected with intensified poppy cultivation. Today, efforts for the establishment of genotypes resistant against diseases such as downy mildew (*Peronospora arborescens*) (Singh et al. 2003), damping off (*Pythium dissotocum*), poppy mosaic virus (PoMo-1) (Sattar et al. 1995) and others are carried out mainly in India. In Europe, successful selection against poppy stem rot has been practised (Hörömpöli 1998). Although none of the poppy accessions tested till now proved to be completely resistant against all diseases, there are wide differences

in the disease reaction of genotypes, which provides a considerable genetical pool for resistance breeding.

Recently, *resistance against abiotic factors* seems to gain more attention. Although spring sown poppy can be grown under a wide range of ecological conditions, frost tolerant genotypes might have an economical significance especially in Central Europe. Poppy sown in autumn and overwintering at 4–6 leaf stage usually has a superior potential compared to a spring sown crop. It is based on some advantageous features: higher yields, earlier ripening, avoiding the mass appearence of weevils (*Ceutorrhynchus* spp.) even at reduced plant protection. However, an overwintering production presumes frost tolerance. Therefore breeding of autumn sown 'winter' type varieties appears important in poppy breeding for Central Europe (Dobos and Vetter 1997). The respective populations have been destined primarily for culinary purposes, but recently development of high alkaloid winter poppy types for the pharmaceutical industry has been started as well (Németh and Bernáth 2007).

Further goals of poppy breeding for special characteristics are dependent of the utilisation area:

The *alkaloid content* of capsules has been the key feature of poppy for pharmaceutical purposes in Europe and Australia; since the mid of the last century, mainly the level of morphine has been in focus. Increase of accumulation to at least 1% and later 2% or even higher were achieved by selection. Since the last 1–2 decades, a further growth of alkaloid accumulation can only be assured through advanced breeding methods such as deliberate hybridization or genetic transformation (Levy and Milo 1998). Through development and broadening of the profile of pharmaceutical industries in the 1980s, new demands appeared for cultivars accumulating other alkaloids such as narcotine, noscapine, codeine or thebaine (Balci et al. 2007; Bernáth and Németh 1999). Hybridization or mutation induction were applied in breeding for these new demands (Bernáth and Németh 2005; Millgate et al. 2004). In India, poppy breeding has always been focused on the increase of latex yield and its morphine content. Recently, the development of cultivars morphine-rich in straw has been started as well (Singh et al. 1997).

The *lack of alkaloids* is of high significance for safe seed consumption by the confectionary industry, bakeries and households (Anonymous 2006). Poppy cultivars accumulating minimal levels of alkaloids might be produced, processed, transported and sold without severe restrictions and control all over the world. The need for such cultivars for avoiding illicit use of capsules had been recognized almost 50 years ago by Kopp et al. (1961). Development of cultivars low in total alkaloids seems to be of high importance in countries such as Austria, Slovakia, Hungary or Poland, where poppy seeds are a widespread and popular dietary component. Monitoring of the fields could be accomplished easily if the 'alkaloidless' (alkaloid content below 0.1%) character is linked to a simply observable marker trait such as flower colour, shape of leaves or capsules etc. (Liersch et al. 1996).

Seed colour used to be an additional breeding goal for the dietary utilisation; poppy seeds have a higher appreciation at the market if they are dark blue. The colour of the seeds is variable depending on pigments and the crystalline layer below the outer epidermis (Petri and Mihalik 1998). Beside the blue coloured cultivars, some varieties had been selected especially for white seed colour, advised for substitution of walnut in bakery products, e.g. 'Albin' (Anonymous 2005) or 'KP-Albakomp' (Németh 2002).

Increasing the *oil content* as the main seed component is less frequently mentioned as a breeding goal. Recently, the possibilities of selection for high oil yield and for strains rich in linoleic, palmitic or oleic acid, or containing palmitic, oleic and linoleic acids in about equal amounts had been discussed (Bajpai et al. 1999).

15.5 Breeding Methods and Techniques

15.5.1 Biological and Genetic Characteristics

Poppy is primarly an autogamous species, fertilisation occurs mostly before opening of the flowers (Heltmann and Silva 1978). However, allogamy is also occurring depending on variety, growing site, weather conditions, colour of the flowers and waxyness of stigma (Bhandari 1990; Patra et al. 1992). According to Morice and Louarn (1971), allogamy may reach 15–40% in case of European varieties, while Khanna and Shukla (1983) describe 0–70% out-crossing in India.

The inheritance of major characteristics of poppy (Table 15.2) has been summarized by Németh (2002). Plant height and branching of stem are dominantly inherited traits, in some crosses over-dominance was found (e.g. Tétényi et al. 1961).

The development of 'double petal' flowers as result of a modification of anthers was described either as a recessive character (Levy and Milo 1998) or as an effect of the multiallelic locus *Pl* (Belyaeva 1988). Monogenic inheritance with multiple allelism was also found to determine the colour of petals (Bhandari 1989); genotypes with irregular flowers or coloured petals have ornamental decoration value and may be useful as marker traits for phytochemical characteristics.

The leaves of poppy show a wide variation from the lacerate, pinnatisect to doubly serrated. Sharma et al. (1991) suggest a digenic inheritance, where the homozygous recessive individuals (lfr_1 lfr_1 lfr_2 lfr_2) exhibit the normal (lacerate) leaf shape and gene-dose effects produce transition forms. Downy mildew resistance appears to be a recessive trait most likely determined by several genes (Kandalkar et al. 1995). Multilocus regulation is also supposed for the colour of seeds which may be white, yellowish, greyish, brown, pink and blue at different darkness. Based on crossing experiments, Leake and Pershad (1920) cited in Verma et al. (1999) assumed three genes playing a role in the regulation

Table 15.2 Overview on the inheritance of major characters in poppy (Németh 2002)

Character	Inheritance	References
Plant height	Dominance, overdominance	Levy and Milo (l998)
	Heterosis	Sharma et al. (1997); Kálmán-Pál et al. (1987); Singh et al. (1999)
	Negative heterosis	Singh et al. (1995)
	Dominance/additive gene action	Shukla et al. (1999)
	Maternal effect	Khanna and Gupta (1989)
Lacerate leaf	Digenic, recessive	Sharma et al. (1991)
Double petal	Monogenic, recessive	Levy and Milo (1998)
	Polyallelism	Belyaeva (1988)
Divided petal	Monogenic, dominant	Belyaeva (1988)
Unusual stamina	Monogenic, partial dominance	Belyaeva (1988)
Petal colour	Monogenic, polyallelism	Bhandari (1989)
Capsule length	Heterosis	Singh et al. (1999)
Capsule size (big capsule)	Monogenic, dominant	Patra et al. (1992)
Number of capsules	Heterosis	Sharma et al. (1988); Sharma et al. (1997)
Seed yield	Polygenic	Levy and Milo (1998); Kálmán-Pál et al. (1987);
	Heterosis	Sharma et al. (1997); Singh et al. (1999)
Total alkaloid content	Monogenic, recessive	Straka et al. (1993); Nyman and Hall (1976)
	Polygenic	Nothnagel et al. (1996)
Morphine content	Intermediate	Morice and Louarn (1971)
	Heterosis	Kálmán-Pál et al. (1987)
	Negative heterosis	Lal and Sharma (1995)
	Overdominance	Kaicker (1985)
Narcotine content	Heterosis	Kálmán-Pál et al. (1987)
	Negative heterosis	Lai and Sharma (1995)
Codeine content	Heterosis	Kálmán-Pál et al. (1987);
	Negative heterosis	Lal and Sharma (1995)
	Polygenic	Tóthné-Lökös et al. (1997)
Thebaine content	Negative heterosis	Lal and Sharma (1995)
Papaverine content	Additive x dominant gene action	Shukla et al. (1999)
Latex yield	Non-additive effects	Shukla et al. (1997)
	Heterosis	Lal and Sharma (1995)
Peronospora resistance.	Recessive, polygenic	Kandalkar et al. (1995)

of seed colour; however, the action of these genes on pigment content of the parenchymatous layer only or also on the construction of the crystal cell layer, which contributes to the colour of seeds has not yet been established.

Heterosis and non-additive gene action may play important roles in poppy breeding. Stronger growth of hybrids compared with the parents was described by several authors (Dános 1965; Kálmán-Pál et al. 1987; Sharma et al. 1997). The diameter and mass of the capsules showed a considerable heterosis (22–53%) in many studies. As for the seed yield, the effect of heterosis may reach an even higher level (up to 167%), while the yield of opium may increase in hybrids by 44–50% (Levy and Milo 1998; Kálmán-Pál et al. 1987; Singh et al. 1999).

A basic difference exists between genotypes accumulating alkaloids and the ones unable to produce alkaloids in an extractable amount. According to Nyman and Hall (1974) the extremely low level of alkaloids is due to one or two recessive gene loci. Recent molecular-biochemical research revealed that the first step of alkaloid accumulation is the transformation of tyrosine into (S)-norcoclaurine. Overexpressing or silencing of tyrosine decarboxylase enzyme (TyDC) activity may basically determine the alkaloid level of the plant (Psenák 1998). It appears, that this enzyme is also active against dopa (dihydroxyphenylalanine), and the TyDC/DODC gene families consist of at least 16 genes which are organ and tissue specific (Facchini et al. 1998). Another less studied aspect of low alkaloid content is the anatomical constitution of the plant. Capsules of Swedish cultivar 'Soma' or Indian cultivar 'Sujata' are almost free of alkaloids due to its underdeveloped lactiferous vessels (Straka et al. 1993; Sharma et al. 1999), which might be inherited monogenically through a recessive allele. With respect to the spectrum and level of different alkaloids accumulated, there is still a lack of knowledge about the enzymes participating in these processes. According to the majority of studies dominance variance proved to be outstanding for morphine content. Recent molecular genetic results demonstrate that the biosynthesis of morphinane alkaloids is a complex process involving enzymatic and substrate feed back reactions beside transcriptional processes (Allen et al. 2004).

Studies on the heritability of different characters have also been summarized in Németh (2002). While relatively high heritability ($h^2 = 0.7$–0.8) was found for important agronomic traits (size and number of capsules, seed yield), the heritability is lower ($h^2 = 0.1$–0.2) in case of alkaloid accumulation. Nevertheless, significant genetic advance was achieved in selection for alkaloid concentrations (Bernáth and Németh 1999).

Genetic correlation between characters, particularly between alkaloids and other traits, may be important in selection. The yields of latex, seed and oil are positively correlated with each other (Sethi et al. 1990; Singh et al. 1995). Heltmann and Silva (1978) describe a positive correlation of morphine content with the number of capsules, stigmatic rays and height of plants, whereas other studies deny any connection between capsule characteristics and morphine (Ghiorghita et al. 1990). More likely, these connections depend on the genetic material used and are also modified by environmental conditions (Kálmán-Pál et al. 1989). For seed yield, Shukla et al. (2003) found the strongest correlation with capsule mass, but also positive correlations with plant height, capsule size and stem diameter.

15.5.2 Breeding Methods

In the presence of sufficient genetic variability of a starting population, selection from still existing landraces with wide distribution and high degree of adaptability may be efficient (Handrea 1996). In poppy, selection is mostly pactised as individual plant selection with progeny testing, which is assured by its good self-pollination ability and seed production. However, isolation of flowers is necessary because of the possibility of allogamous fertilisation.

The efficacy of selection for alkaloid content may be improved by environmental selection pressure. Under low temperature and poor light conditions narcotine and codeine can not be detected or are expressed at a low level. Selection under such conditions could help in identifying strains with high genetic potential for accumulation of these alkaloids (Bernáth et al. 1988). Selection under provocative conditions is also used in resistance breeding (Hörömpöli 1998).

Hybridisation had been used in poppy breeding since the 1940s. Both intra- and interspecific crosses of poppy may result in valuable new breeding material. Emasculation of flowers is carried out, when pollen grains are not mature and self-pollination has not yet occurred; after removing the stamina, isolation of stigma is necessary, and at the same time or during the following days pollination can be done. The effect of a male parent may appear already in the tissues of the mother plant. Bernáth and Németh (2003) proved metaxenia for the alkaloid content of the capsules. When an alkaloid free cultivar was pollinated by another one rich in morphine, the morphine content of capsules of the maternal parent increased by 0.9–7.5 mg/g.

The pedigree method of selection is most widely applied. According to Levy and Milo (1998), pedigree breeding was efficient in combining and fixing plant characteristics such as capsule yield, increased opium and seed yield as well as lodging resistance. Selection and stabilisation of desired genotypes is usually achieved in 3–4 generations. When crossing genetically distant cultivars, evaluation of the early progenies may be difficult due to the appearance of heterosis (Dobos and Vetter 1997). Significant reciprocal effects were also found in particular crosses. The maternal effect was most obvious in crosses between low and high alkaloid cultivars or cultivars of different alkaloid types (Bernáth and Németh 2005).

Backcrossing is practised after interspecific hybridization for elimination of wild-type properties and after mutation induction treatments. Shukla et al. (1999) proposed the development of high papaverine genotypes through backcrossing.

Hybrid breeding can be applied in poppy because both self- and cross-pollination is easily possible, and the seed propagation rate is high. Kaicker (1985) described, that heterosis effects can be utilized even for two generations because of mainly non-additive genetic effects for economically important characteristics (e.g. opium and seed yield). In contrast, Singh et al. (1999) found considerable inbreeding depression for six characters (e.g. seed yield) already in the F_2 generation. Selection of appropriate parents for hybrid development is carried out after testing for combining ability in diallel trials and improving genotypes by recurrent

or reciprocal recurrent selection (Heltmann and Silva 1978). Hybrid seed production has to be carried out by hand-pollination which limits seed production (Mórász 1979), as no source of male sterility is available till now. Although hybrids are mentioned occasionally (Sharma et al. 1997), no hybrid cultivars are produced at a commercial scale because of the hudge cost of seed production. Alternatively, development of synthetic varieties is practised more frequently and is considered the most suitable method in poppy breeding (Khanna and Shukla 1989). Synthetic varieties may keep up with F_1 hybrids in production capacity; besides, productivity does not decrease radically in the following generations and seed production cost is much lower.

Polyploid forms of poppy exhibited an increased morphine level and higher capsule numbers (Andreev 1963 cited in Levy and Milo 1998; Chauhan and Patra 1993). In Hungary, high expectations were held for polyploids (Kiskériné et al. 1977 cited in Németh 2002), but no practical results were achieved due to the decrease of seed production in tetraploid poppy (from full sterility to 7.0% fertility compared to diploids).

Characteristics such as male sterility, lack of opium production, increase in morphine accumulation, multiplication of capsule number, dwarf growth and early flowering were induced through either chemical mutagenesis or irradiation (Khanna and Singh 1975 cited in Levy and Milo 1998; Nigam et al. 1990). Shifting of biosynthetic pathways into a desired direction seems to be the most useful application of mutagenesis. As an example, non-narcotic (alkaloid-free and opiumless) poppy with high seed and oil yield was developed through mutagenic treatments using gamma rays (100–800 Gy) and ethyl methane sulphonate (EMS 0.4%) (Sharma et al. 1999). Mutants were also found which accumulate thebaine and oripavine but do not complete the biosynthesis into codeine and morphine (Millgate et al. 2004); others accumulate high levels of noscapine beside morphinanes (Ziegler and Kutchan 2005).

15.6 Integration of New Biotechnologies in Breeding Programmes

Isoenzyme markers may be applicable for cultivar identification, as demonstrated by Margl et al. (2001) using GOT, 6-PGDH, ACP, DIA, GDH, LAP, MDH and SAD enzyme systems which revealed 1–9 alleles in a total of 12 poppy genotypes. A genetic linkage map containing 87 markers in 16 linkage groups was constructed from a segregating population (Straka and Nothnagel 2002) and is intended for mapping morphine content loci.

Various studies have been carried out in order to identify and isolate the enzymes being involved in alkaloid formation of poppy. Over 30 enzymes participating in biosynthesis of benzylisoquinoline alkaloids have been isolated from cell cultures and are now characterised and partly purified. For many of the enzymes identified, the involvement of multiple cell types is suggested in alkaloid biosynthesis. As an example, while berberine bridge enzyme is localized to parenchyma cells of the root cortex, O-methyltransferases are

found in the pericycle, and – typically – codeinon reductase is only found in organs were laticifers are present (Weid et al. 2004).

Molecular cloning of pathway genes has been successful since the mid 1990s and respective cDNAs are known (e.g. Ounaroon et al. 2005; Ziegler et al. 2006). One of the first genes identified from poppy was that encoding tyrosine/3,4-dihydroxyphenylalanine decarboxylase (Facchini et al. 1998). Recently Ziegler and Kutchan (2005) constructed a specific cDNA library for identification of sequences responsible for morphinane biosynthesis. From over thousand unique sequences created and used for gene expression analysis, 27 sequences showed highly different expression when comparing poppy strains (*P. somniferum* and *P. bracteatum*) either containing morphine or noscapine.

Agrobacterium tumefaciens-mediated genetic transformation can be achieved in hypocotyl derived suspension cultures of poppy using antibiotic or herbicide resistance markers for effective selection of transgenic cells and plant regeneration through somatic embryogenesis (Nessler 1998). Chitty et al. (2003) reported a high rate of somatic embryogenesis in the creation of transgenic plants; measuring outcrossing rates they concluded that a distance of above 2.5 m between transgenic and neighbouring plants was sufficient for avoiding outcrosses. Several practical examples in genetic transformation of poppy have been achieved in recent years. Frick et al. (2004) transformed the berberine bridge enzyme cDNA into seedling explants of an industrial elite line. The selfed progenies of the regenerated plants showed an altered alkaloid profile which was heritable. Larkin et al. (2007) enhanced the activity of codeinone reductase enzyme thus increasing morphinan alkaloids (by up to 28% in the best transformants) in transgenic whole plants as a consequence of over-expression of codeinone reductase. More recently, genetic manipulation of the gene regulating (S)—N—methycoclaurine -3′hydroxylase resulted in significant changes of alkaloid level in latex. Overexpression of the gene induced a 450% increase of the total alkaloid level, while silencing of it by antisence cDNA caused a reduction of up to 84% (Frick et al. 2007).

15.7 Major Breeding Achievements

Breeding of poppy has resulted in advanced cultivars in Austria, Australia, Czech Republic, Germany, Hungary, Poland and Slovakia. Beside the variety list of the European Union (Table 15.1), various other cultivars and strains exist representing local selections or industrially utilized strains, especially in India.

15.7.1 Industrial Cultivars for Alkaloid Production

Widely grown cultivars already in the middle of the last century were e.g. ‘Mahndorfer’, ‘Strubes Blauer’, ‘Eckendorfer Blausamiger’ in Germany,

'Kompolti M', 'BC-2', 'Kék Duna' in Hungary and 'Reading' in England. During the last decades, new industrial cultivars had been developed with continuously increasing alkaloid contents of up to 2–3%, capsule yields of over 1,000 kg/ha and adaptation to intensive cultivation. In Hungary, cultivars accumulating other alkaloids beside morphine, e.g. 'Kék Gemona' (narcotine), 'Monaco' (codeine) or 'Tebona' (thebaine) are produced on large scale.

In India, high yielding cultivars are 'Rajhans Chetak', 'Kirtiman', 'JA-16' or the synthetic variety 'BROP-1' (Singh et al. 1997); the best genotypes assure an opium yield of 250–320 mg/plant with 15% morphine content. The cultivar 'Pusa Selection 1-3' provides more than 53 kg/ha opium (Kaicker 1985). Other strains ('MOP-539', 'G-38', 'BF-5-13') have been developed for downy mildew resistance.

In Australia, a population ('Top-1 poppy') representing a morphine less and thebaine rich mutant has been developed, which provides precursors for highly effective analgesiscs and for treatment of opioid addiction (Millgate et al. 2004).

15.7.2 Culinary Cultivars for Poppy Seed and Oil

Breeding of poppy started from populations cultivated for edible seeds and oil production. Oil content of poppy seed is in the range between 40 and 55%, and palmitic acid (C16:0, 10–12%), oleic acid (C18:1, 12–22%) and linoleic acid (C18:2, 60–75%) mainly make up the fatty acid composition. However, seed yield, oil content or fatty acid composition have never played a primary role in poppy breeding. During the last decades, breeding for culinary purposes focused on decreasing morphine content of capsules in order to avoid drug abuse and contamination of seeds.

The Swedish low-alkaloid cultivar 'Soma' is the result of a spontaneous mutation. It had been the base for developing of low-alkaloid varieties such as the Polish variety 'Przemko' with a morphine content of 0.05% and further lines even lower in morphine and with marker traits such as laciniated and coloured flowers (Liersch et al. 1996). Subsequently, cultivars 'Michalko' and 'Mieszko' have been developed for free seed utilisation and cultivation, blue seed colour, yield potential of 1–1.2 t/ha and oil content of 48–49%. From crosses between gene bank accessions followed by pedigree selection, the Hungarian culinary cultivar 'Ametiszt' had been developed which accumulates only 0.03–0.08% alkaloids in the capsules; besides, its purple-pink petals represent a good morphological marker of its non-narcotic characteristic (Németh et al. 2002). In India, the variety 'Sujata' was produced as a result of mutagenic treatment. The plant does not exude latex on lancing and seed oil content is in the range of 50.7–53.5% (Sharma et al. 1999).

In Central Europe, winter-sown poppy cultivars may reach superior yields compared to spring-sowing; therefore, improvement of frost tolerance is a main

breeding objective. The first winter-sown Hungarian cultivar 'Kozmosz' is too high in alkaloid level (0.4–0.7%) at present to be used in free production. The Austrian winter poppy cultivar 'Zeno Wintermohn' which had been developed from a land race, out-yields spring-sown cultivars by more than 30% reaching 1,450 kg/ha of seed (Dobos 1996); improved strains have been developed from that population recently.

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Chapter 16
Hull-Less Oil Seed Pumpkin

Tamás Lelley, Brent Loy, and Michael Murkovic

16.1 Introduction

Pumpkin is one of the oldest domesticated crops of the Neolithic revolution (Smith 1997; Piperno and Stothert 2003), and pumpkin seeds are consumed as a snack in many cultures throughout the world. Since the bitter flesh of wild *Cucurbita* species is usually inedible, very likely seeds were the first parts of pumpkin eaten by humans (Whitaker and Bemis 1964).

In the volume 'Vegetables I' of the 'Handbook of Plant Breeding' two separate chapters are dealing with 'Pumpkin and Winter Squash' (Ferriol and Picó 2008) and 'Summer Squash' (Paris 2008), respectively. Most of the relevant information on breeding cucurbits as vegetables is handled in those two chapters. Hull-less oil seed pumpkin (*Cucurbita pepo* subsp. *pepo*), however, is a specialty oil crop of Central Europe with a number of specific problems not covered by those chapters, which justifies an in-depth treatment of the subject in the present contribution.

16.1.1 Use of Hull-Less Pumpkin Seed As a Food Crop

The first definite proof for cucurbit cultivation in Styria, Austria was found in the legacy of a farmer dating back to 1697, in which 54 l of pumpkin seeds were recorded (Riegler 2004, loc.cit. Teppner 2004). Several references to pumpkin cultivation in the region and the use of pumpkin seed oil in the early 18th century can be found in Riegler (2004, loc. cit. Teppner 2004) and Kundegraber (1988, loc. cit. Teppner 2000, 2004). The seeds used for oil production were manually de-hulled (Hlubek 1860, loc. cit. Teppner 2004). Based on indirect evidence as summarized by Teppner (2000, 2004), the approximate time of emerging and spreading of a naked seeded, also called thin-coated or hull-less

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mutant of *Cucurbita pepo* subsp. *pepo* in the southwestern part of the then Austro-Hungarian Monarchy appears to be dated between 1870 and 1880. Due to the popularity of pumpkin seeds and especially the seed oil, the advantage of the hull-less seed type, allowing a much more efficient oil extraction, was quickly recognized, leading to its rapid dispersal in the whole region. Essentially, this mutation turned pumpkin into an oil crop. Pumpkin was grown in Austria in 2006 on 18,151 ha with an average seed yield of 0.61 t/ha.

The first hull-less oil-pumpkin variety, '869 Feldkürbis', appeared in a seed catalog of 1915 (Teppner 2004). The first variety derived from a cross between a hull-less oil pumpkin and a Vegetable Marrow genotype, combining the bush growth habit of the latter with the hull-less seed type of the former, was registered in Austria from 1955 to 1974 under the name of 'Tschermaks Ölkürbis'. Tschermak also wrote the first extensive paper on the hull-less pumpkin (Tschermak-Seysenegg 1934). The scientific name, *C. pepo* subsp. *pepo*, var. *styriaca* I. Greb., was conferred to the Styrian oil-pumpkin by Grebenščikov (1950). At present five open pollinated and three hybrid cultivars of oil pumpkin are registered in the Austrian variety list (AGES 2008).

In the USA Curtis (1948) first recognized the potential value of hull-less seeded pumpkin for producing a high quality vegetable oil and for use as a snack seed, and initiated the first breeding program at the University of Connecticut. However, Curtis left Connecticut before he could bring this project to fruition. In breeding work at the University of New Hampshire, two hull-less seeded, small-fruited squash cultivars were released, 'Sweetnut' in 1960 and 'Eat-all' in 1965, followed by the bush pumpkin, 'Tricky Jack' in 1969. However, the first variety of a naked seeded oil-pumpkin to receive more national acclaim was 'Lady Godiva' released by the USDA in 1972. Phenotypically, 'Lady Godiva' shows close similarity to Styrian oil pumpkin.

16.1.2 Origin of the Hull-Less Seeded Phenotype in Pumpkin

Grebenščikov (1954) suggested a common origin in Styria for all hull-less pumpkin genotypes around the world. The absence of any trace of lignin in the seed coats of F_2 progenies derived from crosses of hull-less genotypes from Argentina, South Africa and China with completely hull-less strains of Styrian oil-pumpkin substantiates the postulation of a common genetic background for the hull-less phenotype, and that all of them were probably directly descended from the same Austrian ancestor (Zraidi 2005). Further evidence was provided by Stift et al. (2004) by means of a genetic distance analysis, carried out using SSR markers and comparing Styrian cultigens with hull-less genotypes from China, South Africa, and Argentina. The hull-less phenotype likely evolved from the rare occurrence of a single recessive mutation event as proposed by Grebenščikov (1954), and in Austria there would have been a strong incentive for selection of such a phenotype given the interest in oil seed pumpkin. This

would certainly have been followed by subsequent breeding efforts, perhaps through cross breeding, to select for modifying genes which further reduced lignification of the seed coat. Corroborating the speculation of initial appearance of a single gene mutant in *C. pepo* is the intriguing report by Zhou Xianglin (1987) of a hull-less genotype of *Cucurbita moschata* Duchesne, discovered by the author in a landrace collection in the Shanxi province in China. The hull-less seeds are, however, white, due to a complete lack of protochlorophyll in the chlorenchyma layer, whereas Styrian oil pumpkin seeds are green. Successful crossing of this genotype with zucchini and further crossing the F_1 with Styrian oil pumpkin yielded a progeny showing a clear 1:1 segregation suggesting that these two genes may be alleles (Pachner and Lelley unpublished results), representing a classical example of Vavilov's parallel variations (Vavilov 1920).

16.2 Nutritionally Relevant Components of Pumpkin Seeds

The main nutritionally relevant components of pumpkin seeds are protein and oil. Albumins and globulins, in the range of 59% of the crude protein, are the most prominent protein fractions. The oil content was evaluated by Murkovic in different breeding lines. Breeding lines and varieties commonly used for commercial production of seeds have an oil content of 46.9/100 g (standard deviation 2.4/100 g, $n = 184$, Murkovic, unpublished results). The main fatty acids of pumpkin seeds are palmitic acid (C 16:0), stearic acid (C 18:0), oleic acid (C 18:1), and linoleic acid (C 18:2). These four fatty acids comprise more than 98% of all occurring fatty acids. Linoleic acid is the most prominent, followed by oleic acid and palmitic acid. The values are shown in detail in Fig. 16.1. All other fatty acids are present in minor concentrations. The Chinese variety of *Cucurbita moschata*, 'Zhou', shows a similar fatty acid distribution (C16:0 16.6%, C18:0 7.6%, C18:1 17.3%, C18:2 56.7%). Linoleic acid belongs to the group of essential fatty acids which is modified metabolically to eicosanoids. A low level of eicosanoids arising from an undersupply with linoleic acids is partly responsible for the well known deficiency symptoms of linoleic acid (Innis 1996).

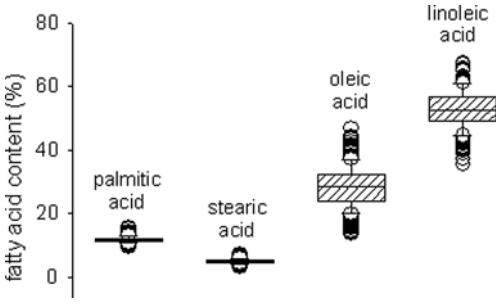

Fig. 16.1 Distribution of fatty acid content in pumpkin seeds ($n = 400$; updated from Murkovic et al. 1996a)

Depending on climatic conditions, the fatty acid composition is subject to variation. When the temperature is lower during the last weeks of seed filling, there will be a shift from oleic to linoleic acid. For example, in the year 1994 the average temperature from July to October was 15.8°C, resulting in a relative concentration of linoleic acid of 48%, whereas in the following years the temperature was below 15.2°C and linoleic acid rose to 57% (Murkovic et al. 1999).

Tocopherols present in the seeds are dominated by γ-tocopherol (41–620 mg/kg), the concentration of which is 5–10 times higher than the concentration of α-tocopherol (0–91 mg/kg) (Fig. 16.2). Additionally, a significant amount of α-and γ-tocotrienols is present. In contrast to the fatty acids, the content and distribution of tocopherols is not influenced by the climate during the ripening period (Murkovic et al. 1996b). In addition to the vitamin E activity and radical quenching properties, tocotrienols are known for their potential to reduce the incidence of breast cancer (Nesaretnam et al. 2007).

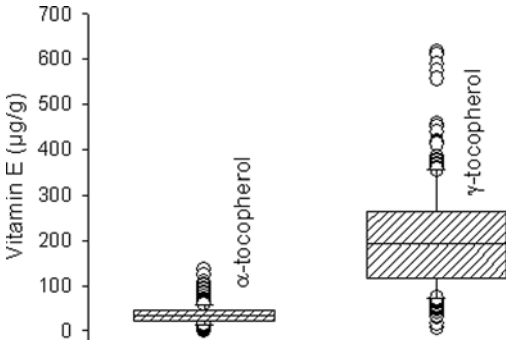

Fig. 16.2 Content of α- and γ-tocopherol in pumpkin seeds ($n = 400$; updated from Murkovic et al. 1996b)

Pumpkin seeds also contain phytosterols that are dominated by Δ7-sterols (Table 16.1). The amount of Δ5-sterols is significantly lower than in other seed oils. Due to this unique phytosterol composition of the seed oil, the analysis of phytosterols can be used to detect adulteration of pumpkin seed oil with other

Table 16.1 Summary of sterol and secoisolariciresinol (SECO) content in pumpkin seeds and seed oil. Values are means of three replications with standard deviation (from Murkovic et al. 2004)

	24-ethylcholest-7-enol, 24-ethylcholest-7,22-dienol and co-eluting sterols (µg/g FW)	Total amount of sterols[1] (µg/g FW)	SECO (µg/g FW)
Seeds	860 ± 30	1710 ± 80	3.8
Oil	2060 ± 10	4030 ± 110	n.d.

n.d.: below limit of quantification.

[1] includes e.g. Δ7-mono- and diunsaturated sterols with 29 C-atoms, dimethylsterols, campesterol and stigmasterol.

plant oils. Mandl et al. (1999) found 1,160 μg/ml total phytosterols in pumpkin seed oil out of which 58 μg/ml was β-sitosterol. In addition to the 5-α-reductase inhibiting properties, phytosterols are known for their cholesterol lowering effects (Thompson and Grundy 2005). However, the concentrations of phytosterols present in functional foods that are used to lower the serum cholesterol level are much higher than in pumpkin seeds.

Carotenoids found in the pumpkin flesh and the seeds are physiologically important, especially lutein, which may reduce risk of the development of age related macula degeneration (AMD, Stringham and Hammond 2005). Matus et al. (1993) found some carotenoids in the defatted seed meal, undoubtedly originating from inner seed coat (chlorenchyma), the primary source of carotenoids in seeds of *C. pepo* (Loy, unpublished results). The main components of the press-residue were lutein (3,3′-dihydroxy-α-carotene = (3R,3′R,6′R)-β,ε-carotene-3,3′-diol; 52.5%) and β-carotene (β,ε-carotene; 10.1%). In addition to the above mentioned pigments, small quantities of violaxanthin, luteoxanthin, auroxanthin epimers, lutein epoxide, flavoxanthin, chrysanthemaxanthin, 9(9′)-*Z*-lutein, 13(13′)-*Z*-lutein, 15-*Z*-lutein, (central-*Z*)-lutein, α-cryptoxanthin, β-cryptoxanthin, and α-carotene (β,ε-carotene) were identified. These carotenoids are not only found in the seeds, but also in the flesh of the fruits (e.g. Murkovic et al. 2002; Azevedo-Meleiro and Rodriguez-Amaya 2007).

Another group of physiologically active substances are lignans, especially secoisolariciresinol is a compound of interest. The concentration in the seeds was determined by the group of Sontag (Murkovic et al. 2004, see Table 16.1) being 3.8 μg/g, whereas Adlerkreutz and Mazur (1997) found a significantly higher amount of 200 μg/g. This group also reported the presence of other estrogens like genistein and daidzein in pumpkin seeds, but the concentrations of these isoflavones are several orders of magnitude lower compared to soybean products. Lariciresinol was identified by Sicilia et al. (2003) at trace levels. Experimental evidence in animals has shown clear anti-carcinogenic effects of pure lignans in many types of cancer. Many epidemiological results are controversial, partly because the determinants of plasma metabolites are very different in different countries. The source of the lignans seems to play a role, because other factors in the food obviously participate in the protective effects.

The seeds contain different protochlorophylls in the innermost layer of the chlorenchyma (Teppner 2004). Chlorophylls are not formed inside the fruits, since the complete synthesis requires a light induced reaction (Mukaida el al. 1993). During the process of oil pressing, these pigments are extracted into the oil providing its typical, dark green color. Since chlorophylls are known as photosensitizers in lipid oxidation, this makes it necessary that the oil is stored in the dark. The oil of Chinese *Cucurbita moschata* variety 'Zhou', mentioned above, may be more resistant to photooxidation, since the seeds do not contain these pro-oxidant chlorophyll precursors.

16.2.1 Antioxidant Activity of Pumpkin Seed Oil

Fruhwirth and co-workers developed a method that uses a fluorescence marker, 1-palmitoyl-2-(2-(4-(6-phenyl-E-1,3,5-hexatrienyl)phenyl-carbonyl) – *sn*-glycero-3-phosphocholine (DPH-DC)). As the fluorophore is sensitive to oxidation, a reduction of the fluorescence indicates the oxidation process. The oxidation experiments were carried out under physiological conditions at 37°C. Using this test, the pumpkin seed oil as well as extracts from seeds with non-polar solvent showed a very high antioxidative capacity compared to other edible oils. The protection against oxidation was attributed partly to the presence of tocopherols and partly to polar substances that also react with the Folin-Ciocalteu reagent. This reagent is commonly used to determine polyphenols (Fruhwirth et al. 2003).

In Austria pumpkin seeds are used for curing bladder and prostate associated symptoms in traditional medicine. In modern phytotherapy the enriched polar extracts of pumpkin seeds are used for curing benign prostate hyperplasia (BPH). The hypothesis of the pharmaceutical action is based on the inhibition of 5-α-reductase. The enzyme 5-α-reductase (testosterone 5-α-reductase, EC 1.3.99.5) converts testosterone to dihydrotestosterone (DHT), which is the active male sex hormone. The enzyme is a nuclear membrane bound NADPH-dependent δ-3-ketosteroid 5-α-oxido-reductase (5-α-reductase). The testes and adrenal glands secrete testosterone into the blood stream. Then a considerable amount of this substance, diffusing into 5-α-reductase containing cells, is irreversibly converted to DHT. Both testosterone and DHT produce androgen-mediated effects; however, DHT is significantly more potent. DHT promotes the development of prostate cells and BPH, and possibly serves as a promoter for prostate cancer. Therapy with 5-α-reductase inhibitors such as finasteride causes decreased serum prostate DHT levels, regression of BPH, and a reduction of serum prostate specific antigen (PSA). PSA is a prostate specific marker, an elevated level of which may indicate prostate cancer (Brawley et al. 1994; Bartsch et al. 2002).

Together with other phytopharmaceuticals (*Pygeum africanum* – african plum; *Populus tremula* – aspen; *Serenoa repens* – dwarf palm; *Echinacea purpurea* – purple cone flower; *Secale cereale* – rye; *Hipoxis rooperi* – South African star grass; *Urtica dioica* – stinging nettle; *Aletrius farinose* – Unicorn root), also *Cucurbita pepo* was studied for its ability to relieve symptoms related to BPH and alter 5-α-reductase activity. What is normally investigated in these studies are parameters like modified Boyarsky symptom score, International Prostate Symptom Score (IPSS), quality-of-life (QOL) index, maximum urinary flow (Q_{max}) and postvoid residual urine volume (PVR). Most of the studies, in which the efficiency of phytotherapeutic agents for BPH was tested, have been carried out without controls. The difficulty of such studies is their lack of considering a high placebo effect of 40–60% observed in several controlled studies (Lowe and Ku 1996). Additionally,

the active principle is normally not known, with the result that the dose of the active ingredient is also unknown, since a standardization of the extract is not possible (Dreikorn et al. 2002). A placebo-controlled study (Bach 2000) showed that within 12 months a significant reduction of IPSS was obtained by administering a polar pumpkin seed extract compared to the placebo, whereas other parameters investigated (Q_{max}, QOL, prostate volume, PVR) did not change.

16.2.2 Treatment of Symptomatic BPH with β-Sitosterol

A 6-month randomized double blinded trial with an 18-month follow-up has shown that β-sitosterol improved the parameters related to BPH (modified Boyarsky symptom score, IPSS, QOL index, Q_{max} and PVR). A dose 20 mg of β-sitosterol, along with minor amounts of β-sitosterol-β-D-glucoside and other phytosterols, was applied 3 times daily. The active ingredients in this mixture are unknown (Berges et al. 2000). If β-sitosterol was the active ingredient in this study, and this is compared with pumpkin seeds, this is equivalent to 50 ml of oil (results from Mandl et al. 1999), or 15 g of oil, if the total amount of phytosterols is considered (Murkovic et al. 2004). Mandl et al. (1999), however, found significantly lower values of β-sitosterol in the pumpkin seed oil. Thus, for an uptake of 20 mg of phytosterols a much higher amount of oil has to be consumed.

The evidence for the efficacy of phytotherapeutic agents in the treatment of symptomatic BPH is inconclusive. Although a few studies were published which showed positive effects of pumpkin seed extract on prostate symptoms, a recommendation of these products at this point still lacks sufficient scientific support. However, since many of the medicines used in clinical practice are derived from the plant kingdom, it is conceivable that these agents do have beneficial effects in the treatment of BPH. The widespread usage and demand for these agents certainly warrant further well-designed, long-term, placebo-controlled studies (Lowe and Ku 1996). Taking all health promoting substances into account (linoleic acid, vitamin E, lutein, lignans, phytosterols) pumpkin seeds certainly contribute to a healthier diet.

16.3 Genetics of the Hull-Less Seed Character

The seeds of *C. pepo* normally are covered by a thick, leathery, whitish to ocher coat consisting of five cell layers (hull), of which at least three are strongly lignified (Fig. 16.3a). The testa of the pumpkin seed is of maternal origin, thus seeds inside the fruit should theoretically show the same seed coat genotype. Styrian oil pumpkin seeds are characterized by a dark green color, unique for

Fig. 16.3 Hulled (**a**) and hull-less (**b**) seeds of *C. pepo*

this genotype, due to a complete lack of lignification of any of the testa layers (Fig. 16.3b). The outer testa layers collapse, resulting in a transparent layer through which the high protochlorophyll content of the well-developed fifth layer, chlorenchyma, comprising up to 12 cell-layers (Stuart and Loy 1983) becomes visible. Protochlorophyll is the immediate precursor of chlorophyll.

Several studies on the seed coat character and its inheritance were carried out in the early 1950s (Heinisch and Ruthenberg 1950; Schöniger 1950, 1952, 1955; Grebenščikov 1954; Prym von Becherer 1955). There is general agreement on the existence of a major dominant gene responsible for the wild type seed coat. In a cross involving a hulled and a completely hull-less genotype, the seed type in 3/4 of the F_2-plants is clearly hulled, 1/4 being hull-less. Nevertheless, these hull-less seeds may show variation with respect to the amount of lignin deposited in the testa, ranging from its complete absence up to a clear lignification, represented by a thin layer covering the whole seed surface. This condition will be referred to as 'residual lignification'. To account for this variation, different genetic interpretations were put forward, such as the presence of a major recessive gene (Schöniger 1950; Grebenščikov 1950), a minor gene and/or modifiers (Schöniger 1952, 1955; Grebenščikov 1954; Stuart 1983), or a multigenic model (Mudra and Neumann 1952; Teppner 2000, 2004).

Histological investigations demonstrated that the seed-coat development up to 10 days post-anthesis is similar in both wild and hull-less seed types. It results in five clearly distinguishable tissue layers: epidermis, hypodermis, sclerenchyma, aerenchyma and chlorenchyma (Stuart and Loy 1983). The effect of the mutation on reduced lignification of the sclerenchymatous and hypodermal layers and reduced polysaccharide deposition in the epidermis becomes clearly visible by 20 days post-anthesis. While in the hulled wild type the second, third, and fourth tissue layers become strongly lignified, in the hull-less types due to the lack of lignin deposition these tissue layers collapse forming a transparent hyaline (Schöniger 1950; Stuart and Loy 1983).

Typically, Styrian oil pumpkin lacks any residual lignification. Crossing Styrian oil pumpkin with zucchini results in a clear 3:1 segregation, 3/4 being

completely hulled and 1/4 showing variation with respect to residual lignification. This variability extends from a complete lack of lignin to a complete coverage of the seed surface by a thin lignified layer. Recently, an attempt was made to clarify the genetic background of this residual lignification (Zraidi 2005). Three oil pumpkin varieties were crossed with one crookneck and two zucchini genotypes, respectively. Analysis of the three F_2-populations proved that the seed coat phenotype is primarily controlled by a single major dominant gene *H*, as was first suggested by Schöniger (1950) and confirmed by Grebenščikov (1954). The homozygous dominant *HH* and the heterozygous genotypes *Hh* produce completely hulled (3/4) seed type (Type 1), while seeds of the *hh* genotypes are hull-less (1/4), but with a varying expression of residual lignification. This varying expression of the hull-less seed type was classified following Schöniger (1950, 1955) as seed coat Type 2 (Fig. 16.4a), Type 3 (Fig. 16.4b) and Type 4 for completely hull-less seeds (Fig. 16.3b).

Fig. 16.4 Hull-less seeds of Type 2 (**a**) and Type 3 (**b**)

Schöniger (1955) attributed this variation to the presence of a second, incompletely dominant minor gene *N,* the effect of which is not discernible in the presence of the major gene *H*. In homozygous dominant condition it is responsible for the production of a thin coat, covering the whole surface of the seed (Fig. 16.4a, Type 2). In heterozygous condition, this thin coat is partial and develops only in parts of the seed surface usually spreading from the margin towards the middle (Fig. 16.4b, Type 3). The recessive alleles produce completely hull-less seeds (Type 4, Fig. 16.3b). Similar results were reported by Stuart (1983), but to account for a low occurrence of completely hull-less segregants (Type 4) and approximately equal proportions of Type 2 and Type 3 phenotypes, a three gene model was invoked, one major gene plus two modifiers. The three gene model could explain the F_2 segregation results, but not the testcross results. However, it was noted that 'Tricky Jack'and 293A, the hull-less parents used in the inheritance study, exhibited seasonal differences in expression of the hull-less trait, varying from Type 2 to Type 4 (Stuart 1983).

Schöniger's hypothesis of two genes was first criticized by Grebenščikov (1954). Not being able in his experiment to prove the presence of a second gene for the phenotypic variation of the hull-less seeds, he accepted the suggestion of the presence of a major dominant gene (*H*) segregating in three to one hulled versus non-hulled types. For the varying expression of a thin coat

observed on the non-hulled seeds, however, he proposed modifiers of which the inheritance 'can not be explained on the base of Mendelian segregation'. The genetics of the seed coat character was also investigated by Teppner (2000) using a *C. pepo* genotype originating from Georgia. This genotype he later classified as *C. pepo* var. *georgica* with a seed coat which is thinner than in the wild type but thick enough to hide the chlorenchyma and to possess a lignified margin. This type was named semi-thin by Teppner. He crossed this genotype with hull-less Styrian oil-pumpkin and found that the semi-thin character of the Georgian genotype was dominant. The F_2-generation segregated in thick coated, semi-thick, semi-thin and thin coated types. In his final conclusion Teppner suggests that to account for all the variation in seed coat phenotypes 6–12 genes could be responsible (Teppner 2000).

The results of Zraidi (2005) clearly confirm the existence of a major completely dominant gene conferring the hulled wild type character to *C. pepo*. If this gene is named *H* (Schöniger 1950), the hull-less type has the genotype *hh*. In the new gene-list of Paris and Brown (2005), both genes *h* and *n* are described conferring the hull-less character to seeds of *C. pepo* (*hh*) (Schöniger 1950) and of *C. moschata* (*nn*) in which a hull-less seeded genotype was discovered by Zhou (1987). Crossing this hull-less genotype, No. 6518, with a hulled one he found a clear 3:1 segregation of hulled versus hull-less types. This line is now named 'Zhou'. As described earlier, results of the crossing experiment of Pachner and Lelley (unpublished results) strongly suggest that this is the same gene as in *C. pepo*. Hence, for priority reasons, this gene should be named *h* instead of *n* as proposed by Zhou (1987), especially because for the residual lignification of the hull-less seeds Schöniger (1950) postulated a minor incompletely dominant gene, which she named *N* or *n*, and the existence of that gene is not yet disproved.

At the histological level, as described in Zraidi (2005) and Zraidi et al. (2003) the thick coat covering the seeds of the hulled types was due to the strong lignification of the three testa cell layers: hypodermis, sclerenchyma and aerenchyma. If on the seed surface of the hull-less segregants a continuous thin coat was observed (Type 2, Fig. 16.4a), this was always accompanied by a continuous but weakly lignified sclerenchyma cell layer in the histological picture (Fig. 16.5b) as was already indicated by Schöniger (1950). According to Zraidi (2005), it is possible that the dominant major gene *H* in the hulled type is responsible for the lignification of all but the chlorenchyma and epidermal cells, while the incompletely dominant gene *N* is responsible exclusively for the lignification of the sclerenchyma layer in hull-less Type 2 and Type 3 seeds. Consequently, completely hull-less seeds (Type 4) result from the homozygous recessive state of both genes (*hhnn*). It is worth mentioning that the histological appearance of hull-less *C. moschata* is similar to that of hull-less *C. pepo*, Type 4. The seeds are, however, white because of the absence of protochlorophyll in the chlorenchyma layer of *C. moschata*. The hull-less F_2 progeny of Zhou × Waltham Butternut shows a residual lignification comparable to Type 2 in *C. pepo* (Pachner and Lelley unpublished results).

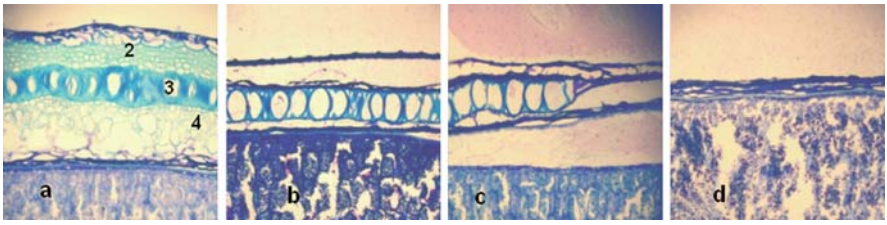

Fig. 16.5 Hulled seed coat (**a**) with all three testa layers, i.e. hypodermis (2), sclerenchyma (3) and aerenchyma (4) strongly lignified; Type 2 hull-less seed coat (**b**) with a continuously lignified single sclerenchyma cell-layer; Type 3 seed coat (**c**) with discontinuous sclerenchyma cell-layer; Type 4 hull-less seed coat (**d**) with all cell layers collapsed

In his study, Zraidi (2005) followed the segregation of only the non-hulled types in three different F_2 populations. In the F_3, segregation patterns of the selfed generations of Type 2 and Type 3 seed phenotypes did not give a clue about the genetic determination of residual lignification. They produced all three hull-less seed types in irregular ratios. Residual lignification was found even in the selfed progeny of completely hull-less genotypes. One major reason for a possible misclassification of F_2 seeds could be the substantial within fruit variation of residual lignification (Fig. 16.6). Within-fruit variation of seed type was reported by Grebenščikov (1950) and Teppner (2000), and within fruit variation could be observed even in Type 1 segregants in Zraidis material (Fig. 16.7, Pachner and Lelley unpublished data). The difficulty in classifying hull-less seeds notwithstanding, the existence of modifying genes is also demonstrated by the prevalence of numerous breeding lines in the US that show a preponderance of seeds representing one or the other of the three hull-less seed types, Type 2, Type 3, or Type 4 (Loy, unpublished observations).

In histological preparations of the progenies of some of the hulled (Type 1) segregants Zraidi (2005) found a second lignified sclerenchyma cell layer (Fig. 16.8a) assembled on the top of the primary one, which is typical for the seeds of the hulled parental genotypes (Fig. 16.5a). This second sclerenchyma

Fig. 16.6 Range of variation of residual lignification of Type 3 hull-less seeds in a single fruit

Fig. 16.7 Within-fruit variation of seed lignification in Type 1 segregants

cell layer exhibited variation frequently observed in the testa of Type 3 hull-less segregants (Fig. 16.8b,c). He did not exclude the possibility, that the same *N* allele is accountable for the existence of this second sclerenchyma cell layer in the hulled as for the single continuous sclerenchyma layer in Type 2 hull-less segregants (Fig. 16.5b).

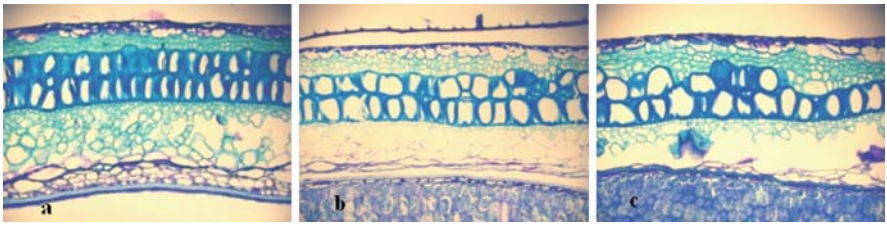

Fig. 16.8 Seed coat histology of hulled segregants (Type 1) of a cross hulled × hull-less pumpkin (Zraidi 2005) with two sclerenchyma cell layers (**a**) and varying expression of the second lignified sclerenchyma layer (**b** and **c**)

Zraidis observations lead to the conclusion that Schöniger's (1950) assumption of a second incompletely dominant gene (*N*) might indeed be mainly responsible for the residual lignification of genotypes homozygous recessive (*hh*) for the major lignification gene. Nevertheless, seasonal differences in the expression of the hull-less phenotype attributed to differential gene expression by Stuart (1983) and within-fruit variation, which may become visible in segregating progenies in all four seed type categories, so far has not allowed unequivocal clarification of the genetic determination of residual lignification.

16.4 Current Goals of Oil Seed Pumpkin Breeding

Although the major use of hull-less seeded pumpkins has been for oil production in central Europe, there is an expanding market for the seed as food, especially as snack food and in confectionary products. In addition, it is

conceivable that a sandwich spread could be developed and marketed much in the way peanut butter is utilized in North America (Curtis 1948). Although much of the acreage of oil seed pumpkin in Europe and China is associated with small farms and hand cleaning of seeds, for products of pumpkin seeds to continue to be competitive with foods from other crops such as peanut, soybean and sunflower, seed yields must be elevated and varieties have to be bred that are resistant or tolerant to some of the more common diseases.

16.4.1 Increasing Seed Yield

Currently, the most popular varieties of oil seed pumpkins being cultivated in Europe are rather large in size weighing 3–7 kg (Winkler 2000). Smaller-fruited varieties have been introduced (Berenji 2000; Winkler 2000), but varieties with larger fruit are apparently preferred, presumably because many farmers still remove seed from the fruit by hand. Large fruits tend to have larger seed and a more open cavity with looser placental tissue, so that it is easier to remove the seed by hand. In pumpkin, changes in total plant biomass (biological yield) produced per hectare is largely a function of cultural conditions such as fertility, weed control, climate, length of the growing season and plant density, rather than a function of genetic variation for greater photosynthetic efficiency. Thus, for genetic improvement in seed yield, strategies must be employed to increase the proportion of photosynthate partitioned into reproductive versus vegetative tissues, and in particular, assimilates being partitioned into seed. Most large-fruited oil seed pumpkin varieties are inefficient in this context, mostly because the ratio of pericarp tissue to seeds is extremely high.

16.4.2 Components of Yield

The two overriding factors contributing to crop yield are production of total plant biomass (biological yield) and the harvest index (HI) or proportion of biological yield that is converted into reproductive biomass. Because changes in biological yield are largely brought about by changes in cultural techniques, genetic increases in yield among the major crop species have largely been accomplished by increasing the harvest index (Donald and Hamblin 1976). High HI values for fruit load in pumpkin can be achieved by breeding more compact (bush) cultivars that produce a heavy fruit load on a restricted vine (Broderick and Loy 1990). In the case of seed production, not only is prolific fruit production per unit area important (high HI), but also the proportion of total assimilate in the fruit that is partitioned into the seed. Two indices have been developed to measure the efficiency of seed production within the fruit (Loy 1991), the 'seed index' (SI), defined as seed dry biomass divided by total fruit biomass, and seed dry biomass per kg fruit fresh weight (FW), a seed yield

efficiency measure that Nerson (2002) termed the 'seed yield index' (SYI). To calculate these indices it is necessary to determine fruit fresh weight, % dry weight (DW) of mesocarp tissue, and seed FW and DW per fruit. In addition, it is usually important to select for seed size, and so seed size (mg) and in some cases, individual parameters of seed size (seed width, length and thickness) are also important variables to consider. In breeding results obtained at the University of New Hampshire from 1995 to 1999, seed yield per fruit was positively correlated with fruit size ($r = 0.68$), but seed yield per fruit did not continue to increase in fruit larger than about 3 kg (Fig. 16.9A). In a similar fashion, seed size was positively correlated with fruit size ($r = 0.58$), but seed size did not progressively increase in the best selections in fruit larger than about 2.5 kg (Fig. 16.9B). On the other hand, the highest seed yield indices (SYI) were obtained in fruit weighing between 0.5 and 1.5 kg (Fig. 16.9C). Both the seed index and seed yield index are highly correlated with seed yield (Chretien and Loy 2000), but the seed yield index is an easier parameter to measure and gives as high or higher correlations to seed yield than the seed index. In breeding work at the University of New Hampshire, seed yield indices of small-fruited (0.7–1.5 kg) lines have been increased from 17 to 20 g seed/kg in initial breeding lines (Loy 1991) to 50–65 in more advanced lines developed from additional breeding cycles (Chretien and Loy 2000; Cui and Loy 2002). Winkler (2000) has recently reported increasing seed yield indices from 15 in older cultigens to 30 in newer breeding lines of oil seed pumpkin.

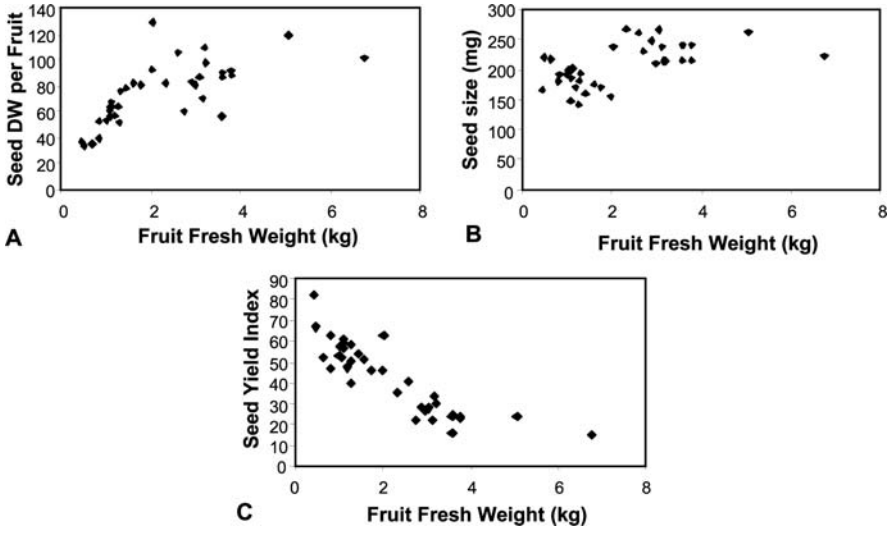

Fig. 16.9 Relationship of fruit fresh weight to seed DW per fruit (**a**), seed size (**b**), and seed yield index (**c**) in seed pumpkin selections; seed yield index = g seed/kg fruit FW

16.4.3 Seed Size and Seed Number

Large seed size should not be a necessity for oil seed pumpkins harvested mechanically as long as a high proportion of seeds per fruit is recovered during harvesting, whereas larger seeds in the range of 170–220 mg are more desirable for the snack seed industry. High seed yield indices and therefore high seed yields are associated with fruit weights in the 0.5–1.5 kg range, so this raised the question of whether consistently large seed size could be achieved in smaller fruit. In a study reported by Carle and Loy (1994), a large-seeded (size range of 202–259 mg) hull-less accession (PI 285611) from Poland with a mean FW of 5.2 kg was crossed to a small-fruited (0.6 kg) breeding line (NH29-13-5-4) with relatively small seed (116–167 mg). In the F_2 population generated from this cross, seed weight was positively correlated with fruit weight, but the correlation was not strong ($r = 0.48$). Fruit weight was also positively correlated with seed width ($r = 0.40$) and seed length ($r = 0.54$), but showed little association with seed thickness ($r = 0.14$). The correlations might have been higher with more small-fruited segregants in the population, because only one fruit in the entire population of 450 plants even approached the small size of NH29-13-5-4. The results, nonetheless, suggested that relatively large seed size could be attained in fruit much smaller than PI 285611, especially if selecting for thick seeds. One selection (NH396) from the F_2 population above had a fruit size of 1.4 kg and relatively large seed size. Inbred lines developed from NH396 have mean seed sizes in the 180–220 mg range and fruit size between 0.7 and 1.5 kg, and have been used to produce productive F_1 hybrid varieties (Cui and Loy 2002). Using thick or plump seeds as a major selection criterion has been further fortuitous because poor tip fill is less of a problem in such seeds. For the snack seed industry good tip fill is an important attribute because seeds with poor tip fill do not puff well during the roasting process.

There are physical restrictions to how many moderately sized seeds can be contained in a fruit with a fresh weight of 0.7–1.5 kg. Maximum FW seed size as determined by maximum seed coat expansion occurs about 20 days after pollination (DAP, Vining 1999). Likewise, maximum fruit FW in a small-fruited cultigen is attained by 20 DAP (Berg 2004). Maximum seed numbers per fruit are determined by the number of ovules in the ovary, but because of seed abortion, developed seeds per fruit are usually considerably less than the maximum number of ovules. Maximum seed numbers vary according to genotype, but seed counts in individual fruit as high as 691 have been reported in small-fruited cultigens (Carle and Loy 1996). In high yielding hull-less cultigens having fruit weights between 1.0 and 1.5 kg and seed weights of 170–200 mg, the seed cavity is almost completely filled and average seed numbers under field conditions typically vary from about 275 to 425 (Cui and Loy 2002). It may be possible to make small genetic gains in seed numbers in large-fruited cultigens, and thereby increase seed yields. In the early 1950s, Neumann (1952) and Mudra and Neumann (1952) reported increasing seed yields by 10% or greater

by selecting genotypes with four or five carpels, rather than the common three. In general though, in examining seed number distribution in numerous hull-less cultigens with a range of fruit size and moderately large to large seed, it has been observed that high seed numbers per fruit are not strongly associated with fruit size (Loy, unpublished data).

16.4.4 Bush Growth Habit

Genetically dwarfed pumpkins, referred to as bush, are characterized by shorter internodes, thicker stems, longer and thicker petioles, earlier flowering, and a higher ratio of pistillate to staminate flowers than typical vine genotypes (Loy 2004). The potential advantages of the bush habit of growth are faster leaf canopy development at high density planting because of their uniform growth pattern, more uniform fruit maturation because only one or two fruits are set at high density planting, higher harvest indices (fruit to total biomass), and easier cultivation for weed control. At least some of these attributes were recognized by early breeders of oil seed pumpkin. As mentioned before, in the 1930s Tschermak-Seysenegg incorporated the bush or short internode habit of growth into oil seed pumpkin by crossing an oil seed pumpkin with a bush marrow squash resulting in 'Tschermak's Ölkürbis' (Teppner 2000). Another European bush variety of note, 'Giessener', produces elongate fruit weighing about 3.0–3.5 kg on a bush plant. Fruits contain an abundance of moderately large seeds, but the seed yield index varies from low (14; Berenji 1986) to moderately high (34; Loy, unpublished data). To increase uniformity in maturation and greater productivity, two small-fruited bush varieties, 'Sepp' and 'Markant', were developed and released in Austria in the mid-1990s. Also in the 1990s, the bush variety 'Olinka' was developed in former Yugoslavia (Berenji 2000) and has also been registered in Hungary and Slovenia.

In a breeding program at the University of New Hampshire, USA, to develop hull-less seeded pumpkins for the snack food trade, the hull-less seeded variety 'Tricky Jack' was used as a source of the bush gene. In 1986 two small-fruited, bush breeding lines of hull-less pumpkins developed from this program, NH14-40-6 and NH55-7-20, were evaluated for components of seed yield using a gradient density planting scheme with densities from 7,200 to 36,000 plants/ha (Loy 1991). Fruit size was moderately decreased at plant densities above 12,000 plants/ha, with mean fruit size being reduced from 1.2 kg to 0.8–0.9 kg at the highest plant density. On the other hand, fruit number per plant for the two breeding lines was reduced by more than 50%, from 3.5 to 3.6 at the lowest plant density to 1.5–1.7 at the highest plant density. Seed yields were highest, 768 kg/ha and 942 kg/ha, respectively, for NH14-40-6 and NH55-7-20 at the highest plant density, but seed yield indices were very low (17.5–18.2). Planting densities between 18,000 and 24,000 plants/ha have proven to be near optimum

for producing high yields in more productive varieties developed during the 1990s from this breeding program (Cui 2005; Cui and Loy 2002).

The greatest utility of the bush habit of growth has been for hybrid seed production. Ethephon, a growth regulator, releases ethylene upon decomposition, converting monoecious plants to female flowering (Lower and Miller 1969; Robinson et al. 1970). Bush plants can be treated with ethephon early in development, causing plants to produce only female flowers for an extended period. Therefore, a bush line treated with ethephon and serving as a female parent, can be planted adjacent to a male pollinator line to produce hybrid seed without hand pollination. Vine plants cannot be efficiently converted to femaleness by this method. However, by using a vine breeding line as the male parent, F_1 hybrids are produced that have a phenotype intermediate between the bush and vine growth habit. In *Cucurbita pepo*, bush-vine hybrids generally display a bush habit early and a more vining growth habit later in the season. For pumpkins, this growth habit is generally considered more favorable than the homozygous bush growth habit. Although homozygous bush cultivars lend themselves to high density planting and easier weed control, one disadvantage is that bush cultivars often lack sufficient vegetative growth to support their fruit load, and this can adversely impact mesocarp dry matter and seed yields (Loy 2004). Peak mesocarp dry matter occurs about 30–35 days after pollination (Culpepper and Moon 1945), but near maximum seed fill requires about 55 days after fruit set (Vining and Loy 1998). In bush cultivars, peak vegetative growth occurs shortly after flowering, and this is soon followed by progressive leaf senescence (Broderick and Loy 1990; Cui 2005). If sufficient vegetative growth and photosynthetic leaf area are not attained by the time of fruiting, then photosynthates may be potentially limiting for later stages of seed fill. However, if enough assimilates are translocated from leaves and stored as starch in mesocarp tissue by 35 DAP, then remobilization of mesocarp reserves to the developing seed may be sufficient to attain good seed fill (Loy 2000).

To justify hybrid seed production, hybrid varieties must perform appreciably better than OP varieties in terms of uniformity, seed size and overall seed yields. Berenji (1986) produced six inbred breeding lines by selfing six existing open pollinated varieties, one of which was bush 'Giessener'. All of the breeding lines but one had fruit size greater than 2.0 kg. The inbreds were intercrossed to produce 15 F_1 hybrids which were evaluated for seed size, fruit weight, seed weight per fruit, yield per plant, and percentage of oil. High parent heterosis was obtained in 13 out of 15 hybrids for seed weight per fruit and fruit weight. High parent heterosis was also evident in 7 out of 15 hybrids for seed yield indices; however, the highest seed yield index was only 28.4, and all but three of the hybrids had seed yield indices below 22 g/kg fruit FW. In spite of the low seed yield indices, all hybrids showed high parent heterosis for seed yield per plant, and in one instance, the seed yield was more than doubled in the hybrid. Correlation analysis indicated that specific combining ability was more important than general combining ability in affecting yield components.

Cui and Loy (2002) reported on a study of heterosis in two experimental hybrids (NH1050 and NH1051), using parents that had been selected for high seed yield indices. Fruit biomass per plot was 32% higher in hybrid NH1051 than in the highest yielding parent. Mean fruit FWs of 1.49 kg for NH1050 and 1.33 kg for NH1051 were 28 and 32% higher, respectively, than in the largest fruited inbred parents. Seed yields per plot in both hybrids were about 25% higher than the highest yielding parent, but yield differences were statistically significant only for NH1051, with plot yields extrapolating to 2,088 kg/ha. Mean seed numbers per fruit were 428 for NH1050 and 396 for NH1051, and both hybrids exhibited highly significant high parent heterosis for seed number (24 and 35% increases) but not seed size, agreeing with previous observations by Carle and Loy (1996). Coefficients of variation for mean seed size among fruit were lower in NH1051 (3.7%) than in either parent (6.6 and 8.2%), reflecting greater seed uniformity of the hybrid. Although no high parent heterosis was exhibited for seed yield indices, the seed yield indices were high for all cultigens, ranging from a low of 43.4 to a high of 60.7 g seed/kg fruit FW. Two bush $\times$ vine F_1 hybrid seed pumpkins have been released by University of New Hampshire for introduction into the commercial market, 'Snackjack' and 'Snackface'. The former variety produces small (0.8–1.4 kg) fruit and was developed as an attractive ornamental pumpkin as well as for hull-less seed production. The latter variety, a sister hybrid to NH1051, was developed for commercial seed production for the snack food industry. It produces fruit in the 1.0–1.5 kg range, seed size between 170 and 210 mg, and yields between 1,500 and 2,300 kg/ha (Cui 2005;) at a spacing of 18,000 plants/ha (0.3 $\times$ 1.8 m). Seed color in 'Snackface', produced by the inner seed coat, is medium rather than the dark green preferred for oil seed pumpkins.

16.4.5 Disease Resistance

Probably the two most prevalent diseases in regions growing *C. pepo* pumpkins are powdery mildew (PM) and several viruses. Cucumber mosaic virus (CMV), squash mosaic virus (SqMV), papaya ringspot virus (PRSV), zucchini yellow mosaic virus (ZYMV) and watermelon mosaic virus (WMV) are usually the most prevalent viruses in pumpkin. In many temperate regions PM does not overwinter, and hence, infestations usually occur after fruit set. Because seed fill is not completed until late in fruit development, heavy infestations of powdery mildew can adversely impact photosynthate production and seed fill.

16.4.5.1 Resistance to Fruit Rots

Especially for harvesting hull-less seeded pumpkins by machine, fruit rots can be the bane of successful production of hull-less seeds. Seed from a single rotten fruit can produce off flavors in a seed batch, rendering seed inedible. Three

types of fruit rots are most prevalent in north temperate regions of pumpkin culture, fusarium rots (*Fusarium solanum* and related species), bacterial leaf spot rot (*Xanthomonium campestris* (Pammel) Dowson pv. *cucurbitae* (Bryan) Dye), and black rot (*Didymella bryoniae* (Auersw.) Rehm). Although the fusarium fungus has a wide host range, including corn and many legumes, it does not appear to be a serious problem for hull-less genotypes (Loy, unpublished observations). However, hull-less seeded pumpkins in particular appear to be highly susceptibility to bacterial leaf spot. This disease is very subtle to detect because leaf symptoms, appearing as small necrotic spots with a yellow halo, are often fairly obscure. The lesions may coalesce and look similar to angular leaf spot (*Pseudomonas syringae* pv. *lachrymans*) (Zitter et al. 1996). Initial symptoms on fruit appear as small, raised tan dots, but can expand into slightly sunken tan spots, surrounded by a dark ring. Most of the lesions fail to penetrate the fruit, but eventually penetration into the seed cavity will occur on most fruit showing extensive symptoms. Tolerance of fruit to this disease has been observed among ornamental pumpkins (Loy, unpublished observations), and so attempts are being made to transfer this tolerance into hull-less seeded breeding lines.

Most breeding lines developed at the University of New Hampshire do not appear to be very susceptible to black rot. Recently, however, this disease has become a major concern of oil-pumpkin growers in Austria (Huss et al. 2007). Winkler (personal communication) has noted genetic variation for susceptibility for black rot among cultigens, so it appears that reasonable tolerance to this disease can be introgressed into hull-less seeded pumpkins from existing cultivars.

16.4.6 Expanding the Genetic Base in Oil Seed Pumpkins

Hull-less seeded pumpkin cultivars do not have natural resistance to the most serious disease pathogens; thus, it is necessary to expand the genetic base of oil seed pumpkins. According to Duchesne (1786) and Naudin (1856) *C. pepo* is the most polymorphic species in the plant kingdom. Its fruits occur 'in myriad of shapes, sizes, and colors' (Paris 1988). Genetic variation of Styrian oil-pumpkin is, however, restricted due to selection for its specific seed-type and because of its limited environmental range of cultivation. Because of its relatively simple inheritance, the hull-less trait can be introduced into any cultivar type of *C. pepo* (Paris 1986). The same might be true for the hull-less genotype found in *C. moschata* (Zhou 1987). In many instances, however, it may prove desirable to transfer useful genetic traits between species, especially those traits conferring disease resistance. Successful interspecific crosses between *C. pepo* and *C. moschata* are difficult but possible, especially with *C. pepo* as the maternal parent (Castetter 1930; Erwin and Haber 1929; Robinson and Decker-Walters 1997; Pachner and Lelley unpublished results). Powdery mildew tolerance was

transferred from the wild species, *C. okeechobeensis* to *C. pepo* by using *C. okeechobeensis* × *C. moschata* as a genetic bridge (Robinson and Decker-Walters 1997). This intermediate resistance to powdery mildew appears to be due primarily to one incompletely dominant gene and has been introduced into several squash and pumpkin cultivars. Loy (unpublished results) has introduced this source of PMR into several hull-less seeded pumpkin breeding lines that have been selfed to the F_4 and F_5 generations.

The first genes conferring tolerance in *C. pepo* to zucchini yellow mosaic virus (ZYMV) originated from the *C. moschata* genotype 'Nigerian Local' (Provvidenti 1997). Further resistance against ZYMV was introduced into *C. pepo* from the Portuguese landrace 'Menina' (Paris and Cohen 2000). Studying the inheritance of resistance to ZYMV in five different *C. moschata* genotypes of widely different geographic origin revealed at least five separate loci that confer high tolerance against the virus (Pachner and Lelley 2004). Via interspecific crosses these resistances are now available in *C. pepo* for further breeding (Pachner and Lelley unpublished results). The *C. moschata* cultivar 'Soler' from Puerto Rico, in addition to having resistance against ZYMV, has also provided resistance against powdery mildew to *C. pepo* (Pachner and Lelley unpublished results). The Chinese hull-less genotype described by Zhou (1987) also conferred resistance against cucumber mosaic virus (CMV). Thus, *C. moschata* seems to be one of the most important genetic resources for breeding of *C. pepo*.

While direct crossing of *C. moschata* with oil pumpkin has not been reported, successful crossing of *C. moschata* with zucchini has been repeatedly achieved (Robinson and Decker-Walters 1997). Crossing such an F_1 with oil pumpkin led to the introduction of several traits from *C. moschata* genotypes to oil pumpkin (Pachner and Lelley unpublished results).

16.5 Integration of New Biotechnologies in Breeding Programs

Recently the first consensus genetic map of *C. pepo* has been published (Zraidi et al. 2007). This map has been constructed using mainly RAPD and AFLP markers. Meanwhile, efforts to obtain SSR markers for *Cucurbita*, led to the development of 500 such markers (Gong et al. 2008). These markers have been used for updating the map of *C. pepo* as well as to develop a map for *C. moschata*. The high transferability of the markers between *C. pepo* and *C. moschata* (around 90%) and also to *C. equadorensis* (up to 70%) suggest that these markers will be very useful for a number of applications in the genus *Cucurbita*. Transferability to other genera of the Cucurbitaceae family is under investigation. Altogether 5 SSR markers have been found closely linked to the hull-less trait which could lead eventually to the cloning of the gene responsible for testa lignification. SSR markers can give a major boost for marker-assisted breeding in *Cucurbita*. One

SSR marker closely linked to resistance to ZYMV is now routinely used in selection work (Gong et al. 2008).

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Chapter 17
Maize for Oil

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17.1 Introduction

Maize is not traditionally viewed as an oilseed crop; actually maize oil is considered a 'minor' oil among the traditional vegetable oils, representing only about 3.4% of the 1998–1999 US market (Orthoefer et al. 2003). Maize oil is a co-product of the wet and dry milling industries, which essentially breaks down the maize kernel into starch, oil, protein, and fiber (for review see Johnson and May 2003; Duensing et al. 2003). The US is the largest worldwide producer of maize oil; Brazil, China, Romania, countries of the former Soviet Union, former Yugoslavia, and South Africa also produce maize oil (Orthoefer et al. 2003).

17.2 Maize Kernel Structure and Composition

The maize kernel is a mixture of maternal tissues (e.g., pericarp) and zygotic tissues (e.g., embryo, aleurone, and endosperm) (Fig. 17.1). The embryo is diploid (2n) while the aleurone (i.e., first cell layer of endosperm tissue) and endosperm are triploid (3n). The triploid nature of the aleurone and endosperm result from two identical maternal gametes (i.e., polar nuclei, the result of megasporogenesis) fusing with one paternal gamete (i.e., sperm nuclei, the result of microsporogenesis).

The typical maize kernel, on a dry weight basis, is composed of 61–78% starch, 6–12% protein, 3.1–5.7% oil, 1.0–3.0% sugar, and 1.1–3.9% ash (Miller 1958; Watson 2003). Most of the starch is associated with the endosperm (<95%), while the embryo contains high levels of protein (~26%), oil (~83%), sugar (~70%), and ash (~80%) (Earle and Curtis 1946; Watson 2003). The typical fatty acid profile of a maize kernel is 57.9% linoleic acid, <1% linolenic acid, 25.2% oleic acid, 11.6% palmitic

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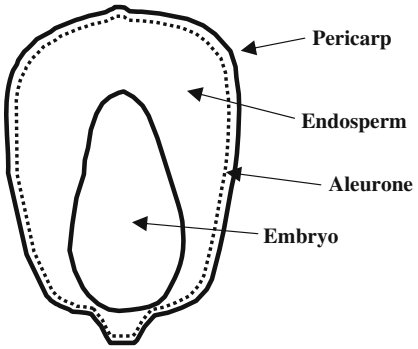

Fig. 17.1 Diagram of a maize kernel. The pericarp is maternal tissue, the endosperm and aleurone are triploid tissues, and the embryo is diploid

acid, and 1.8% stearic acid (Dunlap et al. 1995; White and Weber 2003). Most of the genetic research on maize kernel oil has focused on total content rather than composition, the inheritance of which is highly quantitative; however, several single gene mutations influencing fatty acid profile have been identified. Linoleic acid content is controlled by a single recessive mutation, *linoleic acid1* (*ln1*) (de la Roche et al. 1971). High oleic acid content (57% vs. 27%) is due to the recessive mutation *oleic acid content1* (*olc1*) (Wright 1995).

17.3 Modern Maize Breeding

Because of the hybrid nature of the crop modern temperate maize breeding in the US and Canada has evolved into two very distinct activities: inbred line development and hybrid commercialization (Duvick and Cassman 1999; Lee and Tollenaar 2007; Fig. 17.2). Maize breeding in North America, unlike breeding activities for many other crop species, has been done primarily in the private sector for the past 30+ years. Movement of this activity out of the public domain has been accompanied by legal protection of the commercial germplasm through legally binding 'use agreements', US patents, and the US Plant Variety Protection Act (PVP act).

17.3.1 Germplasm

The genetic base of North American hybrid maize industry represents only a small portion of the entire *Zea mays* gene pool. There are 250–300 races of maize (Brown and Goodman 1977), of which only one, the Corn Belt Dent (CBD), is the predominant source of commercial germplasm (Goodman 1985).

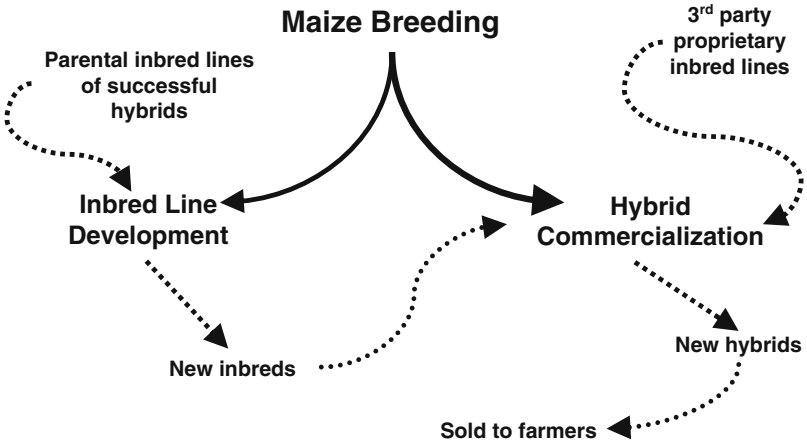

Fig. 17.2 Overview of a modern maize breeding program. (from: Lee and Tollenaar 2007)

Of the hundreds of open-pollinated varieties (OPVs) of CBD that were grown up to the 1940s, only half a dozen or so can be considered as significant contributors to current inbred lines. But the overwhelming majority of the inbred lines trace their pedigree back to only two OPVs, Reid Yellow Dent and to a far lesser extent Lancaster Surecrop (Goodman and Holley 1988; Tracy and Chandler 2006; Lee and Tracy 2009)

One of the consequences of the hybrid concept was the development of heterotic patterns, also referred to interchangeably as heterotic groups. These groups were 'created' by breeders as a means of maximizing the amount of hybrid vigor and ultimately grain yield in a more predictable manner (cf., Tracy and Chandler 2006). Modern hybrids then are the result of crossing an inbred line from one heterotic pattern with an inbred line from a different heterotic pattern. Today, inbred lines are classified into heterotic groups and are further sub-divided into families within a heterotic group. Classification of heterotic patterns is generally based upon several criteria such as pedigree, molecular marker-based associations, and performance in hybrid combinations (Smith et al. 1990), and has resulted in somewhere between two and seven distinct heterotic patterns being described (Smith and Smith 1989; Troyer 1999; Lu and Bernardo 2001; Gethi et al. 2002; Mikel and Dudley 2006). We have chosen to represent the germplasm as three main heterotic patterns 'Stiff Stalk', 'Lancaster' and 'Iodent', and a miscellaneous category of lines that do not fit into the three primary heterotic patterns in the northern and central US and Canadian corn growing regions. Most conventional inbred line development has focused on 'recycling' (i.e., intermating) lines within a heterotic pattern and even to within a heterotic family; however at least two novel heterotic patterns

have arisen: 'Maiz Amargo' and 'Commercial Hybrid' derived germplasm (Mikel and Dudley 2006; Lee and Tracy 2009).

17.3.2 Heterosis and the Inbred/Hybrid Concept

Hybrid maize traces its roots back to experiments on heterosis and inbreeding conducted by Shull (1908, 1909) at Cold Spring Harbor Laboratories in New York and East (1909) at Connecticut State College. They observed that when maize plants were self-pollinated (i.e., inbred) in successive generations, their vigor and grain yield rapidly deteriorated (Shull 1908; East 1909). However, when two inbred lines from unrelated populations were crossed, both vigor and grain yield of the F_1 hybrid often exceeded that observed for the original source populations (Shull 1908). It was these observations, made nearly 100 years ago, and methodology outlined by Shull (1909) that gave rise to the modern hybrid maize industry (cf., Crow 1998).

Since Shull's initial papers there has been debate about the underlying genetic mechanism of heterosis. The two main theories are the dominance theory and the overdominance theory. The dominance hypothesis attributes heterosis to the accumulation of favorable dominant genes or masking of deleterious recessive alleles in the hybrid (Davenport 1908; Bruce 1910; Keeble and Pellew 1910). In quantitative genetic terms, heterosis results when there is some degree of directional dominance (d) and the parents differ in gene frequency (Bruce 1910; Falconer 1981). The dominance hypothesis can be expressed in terms of a single-locus (B) with no epistasis as

$$\text{Heterosis} = \mathrm{d} - \{\mathrm{a} + (-\mathrm{a})\}/2 \tag{17.1}$$

where a and –a are the genotypic values of the parental genotypes (B_1B_1 and B_2B_2) and d is the genotypic value of the non-parental genotype (B_1B_2). The dominance theory is consistent with recent genomic evidence of differences in genic content between maize inbred lines (Fu and Dooner 2002; Song and Messing 2003; Brunner et al. 2005), and has been demonstrated as the underlying mechanism of a heterotic response for grain yield in a quantitative trait locus mapping study (Graham et al. 1997). The other hypothesis, over-dominance, argues that the heterozygous combination of the alleles at a single locus is superior to either of the homozygous combinations (Shull 1908; East 1908). The over-dominance hypothesis, unlike the dominance hypothesis, does not require the presence of either linkage or the involvement of multiple loci for heterosis to be expressed, nor is it necessarily based on classic Mendelian genetics. However, like the dominance hypothesis, it also requires that the parents differ in gene frequency. While there is no direct evidence in support

of this hypothesis in the literature, it has not been completely rejected as an underlying genetic cause.

17.3.3 Inbred Line Development

Most if not all inbred line development activities in North America involve the use of the pedigree method of breeding (Duvick et al. 2004; Mikel and Dudley 2006; Lee and Tollenaar 2007; Fig. 17.3). Pedigree breeding as it is structured in the commercial sector is akin to reciprocal recurrent selection (RRS) (Hallauer and Miranda 1988; Duvick et al. 2004). Breeding crosses tend to be made by crossing inbred lines within a heterotic pattern. Inbred lines from the other heterotic patterns are used to improve the heterotic pattern represented by the breeding cross. This type of breeding scheme (i.e., akin to RRS) allows maize breeders to improve both additive and non-additive genetic effects, resulting in greater overall genetic gains (Comstock et al. 1949).

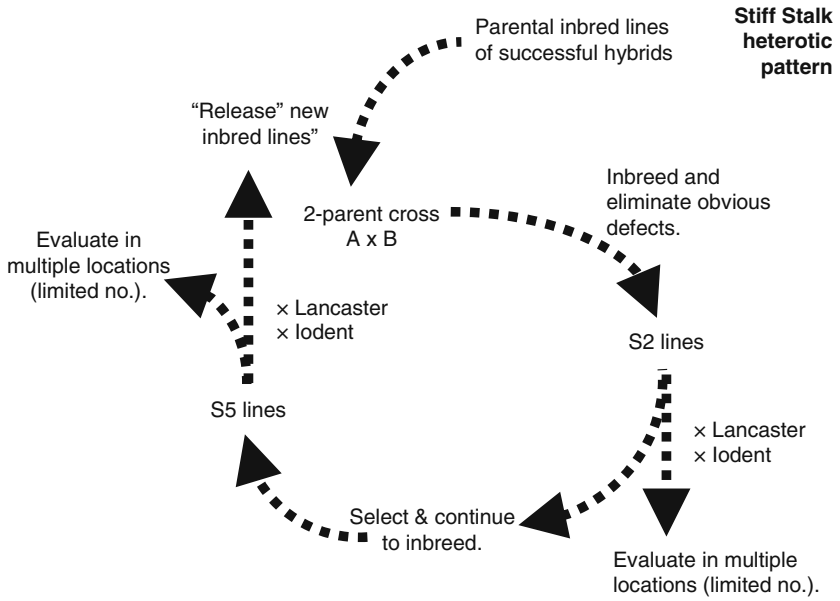

Fig. 17.3 Typical inbred line development scheme depicting a 2-parent breeding cross involving two inbred lines from the Stiff Stalk heterotic pattern. (from: Lee and Tollenaar 2007)

Marker assisted breeding, a variation of the pedigree method, is being used by some companies. Based on the phenotype data from early generation testing, molecular marker genotyping is used in conjunction with off-season nurseries to reduce inbred development time (cf., Lee and Tracy 2009). In recent years there has been a renewed interest in using doubled haploids in maize inbred line

development, reducing the time of the inbreeding process (cf., Lee and Tracy 2009).

Compared to other crops backcrossing has not been heavily used in maize breeding. Backcrossing has been used to incorporate disease resistance genes into elite inbred lines or to transfer inbred nuclear genotypes into male sterile cytoplasms (Hallauer et al. 1988). However, with the widespread use of transgenes in maize breeding (e.g., herbicide resistance genes, insect resistance genes), backcrossing coupled with molecular markers for rapid recurrent parent genome recovery has become a standard tool of the maize breeder (Lee and Tracy 2009). Most commercial maize breeding programs have adopted the process of developing new inbred lines from non-transgenic germplasm, and then as new inbreds show promise they will begin backcrossing in desired transgenes, at the same time maintaining the original non-transgenic inbred. Thus, if a particular transgene is removed from the marketplace, or the use agreements are no longer valid the new inbred is not lost. This also permits the sale and production of non-transgenic versions of the new hybrids in locations that disallow the use of transgenes.

17.3.4 Hybrid Development

Commercial maize grain yields during the hybrid era (1939 onward) have increased more than 6-fold (Lee and Tollenaar 2007). This increase is not exclusively due to genetic improvement, as there have been substantial changes to agronomic practices during this 65–70 year period (e.g., plant population density and distribution, herbicides, fertilizers). As the agronomic practices changed, breeders incorporated these changes into their testing programs, meaning that realistically 100% of the increase in grain yield is actually due to the interaction between genetics and agronomic practices (Tollenaar and Lee 2002).

In contrast to inbred line development, hybrid commercialization generally involves a multi-tiered testing system (Duvick and Cassman 1999). More hybrid combinations are tested in fewer environments during the early testing phase, while in the later testing phases fewer hybrid combinations are tested in more environments. Again, grain yield is the primary trait of interest, testing for grain yield is always done in hybrid combinations, and testing is done using the most current agronomic practices. The specific traits that are assessed are: (1) machine harvestable grain yield adjusted to 15.5% grain moisture (bu ac^{-1} or Mg ha^{-1}); (2) grain moisture at harvest (%); (3) test weight, a measure of bulk density (lbs bu^{-1} or kg hl^{-1}); (4) broken stalks, dropped ears, root lodging; (5) days to 50% anthesis, days to 50% silking, and the interval between silking and anthesis; (6) germination under cold, wet conditions (i.e., cold germ test); (7) disease resistance (ear, stalk, and leaf diseases); (8) general plant appearance (cf., Lee and Tracy 2009).

17.4 Recurrent Selection

Noticeably absent from the methods for inbred line development outlined above is recurrent selection (RS). While RS has been widely used in the public sector for germplasm improvement, it is not a method that is routinely utilized by the commercial maize breeding sector for inbred line development, circa 2007. However, RS was the breeding method used in the Illinois High-Oil/Low-Oil (IHO/ILO) long-term selection experiment and therefore warrants some general discussion regarding methods and philosophy.

The general goal of RS programs is to increase the frequency of desirable alleles while maintaining genetic variation in the population (Hallauer and Miranda 1988). In maize breeding, a population is a heterogenous mixture of heterozygous genotypes derived by intermating inbred lines and/or OPVs. Populations are maintained by randomly intermating individuals in the population or allowing open-pollination under isolation (i.e., at least 200 m from other maize). Recurrent selection programs are cyclical with a cycle consisting of a generation in which selection takes place followed by recombination of the selected individuals or families. In academic research the term RS generally indicates a closed population (i.e., no new germplasm being incorporated) unless otherwise specified. Hallauer and Miranda (1988) and Bernardo (2002) outline numerous RS programs.

Divergent recurrent selection is a form of RS in which starting from an initial closed population a trait is selected in opposite directions and populations divergent for that trait are developed. Divergent RS experiments can be useful in understanding the limits of selection and also the relationship between the selected trait and unselected traits that change in response to selection (cf., Lee and Tracy 2009).

17.5 The Illinois High-Oil/Low-Oil Long-Term Selection Experiment

The Illinois high-oil/low-oil (IHO/ILO) long-term selection experiment is perhaps the best known example of a divergent RS experiment in which there have been more than 100 generations of selection for kernel oil content (Dudley and Lambert 2004). G.C. Hopkins, a chemist by training, initiated the experiment in 1896 to determine if it was possible to change the chemical composition of a maize kernel (Hopkins 1899). Hopkins actually initiated two divergent selection experiments, one for oil and one for protein. Both selection experiments were started using an OPV called Burr's White. The selection experiment has continued since, except for 4 years during the Second World War. Several comprehensive summaries of the IHO/ILO experiment have been published that contain details regarding selection intensity, methods of chemical analysis, and breeding procedures (for details see Dudley et al. 1974; Dudley and

Lambert 2004). Seven researchers have shepherded the IHO/ILO experiment from cycle 0 through cycle 100: C.G. Hopkins, L.H. Smith, C.M. Woodworth, E.R. Leng, D.E. Alexander, R.J. Lambert, and J.W. Dudley. What essentially started out as a relatively simple experiment, to determine if it is possible to change to chemical composition of a maize kernel through selection, resulted in the development of a very unique set of germplasm for quantitative genetic studies. Results from these studies and their implications will be briefly summarized and discussed below.

Obviously the answer to C.G. Hopkins question was yes, that through selection the chemical composition of a maize kernel can be altered (Fig. 17.4). The oil content of Burr's White, as measured by Hopkins at the start of the experiment was 4.69%. After over 100 cycles of selection for oil content in the high direction, the mean oil content of the IHO population was 20.37%, which translated into a gain per cycle of selection of 0.17% (Dudley and Lambert 2004). Progress in the low direction, however, did not mirror that of the high. For the first nine cycles of selection, progress in the low direction proceeded at a rate similar to that of the high (-0.22% cycle^{-1}), however beyond cycle nine progress in the low direction ranged between -0.05 and 0.01% cycle^{-1} with progress essentially stopping after cycle 76 with a mean oil content of 0.35% (Dudley and Lambert 2004). Selection for low kernel oil content had reached a selection limit and one, which most likely, was

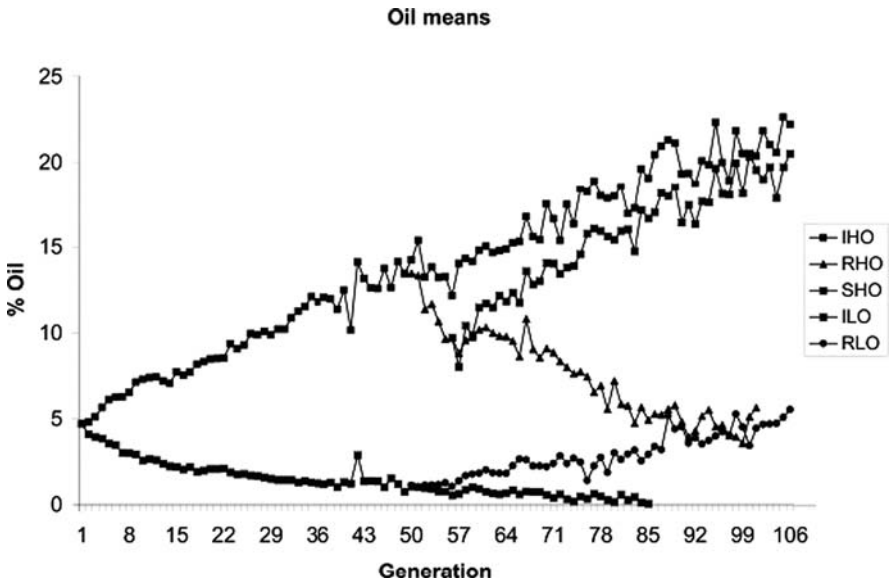

Fig. 17.4 Illinois long-term high-oil/low-oil selection experiment – 100 + generations. Plot of mean oil concentration against generation (i.e., cycle) for Illinois High Oil (IHO), Reverse High Oil (RHO), Switchback High Oil (SHO), Illinois Low Oil (ILO), and Reverse Low Oil (RLO). (from: Dudley 2007)

anticipated, based on biology and foreshadowed by several earlier quantitative genetic examinations of the selection experiment (Winter 1929; Dudley and Lambert 1969).

After 48 cycles of 'forward' selection for high-oil and low-oil, E.R. Leng initiated 'reverse' selection to determine how much residual genetic variation remained in the populations available for selection to act upon (Leng 1962). The reverse high-oil (RHO) population started with cycle 48 of ILO with selection proceeding in the high direction and the reverse low-oil (RLO) population started with cycle 48 of IHO with selection proceeding in the low direction (Fig. 17.4). After seven generations of selection in RHO, a fifth selection experiment was initiated called 'switchback' high-oil (SHO). Selection in RHO was immediately effective in shifting the mean oil content downwards, however, selection in the RLO had little effect in the first several generations, with marked progress being evident only after the third generation of selection (Leng 1962). The effect of selection in the SHO exhibited the same lag that was exhibited in the RLO, only after four generations of selection in SHO did the mean oil content shift upwards (Leng 1962). The two 'reverse' populations and the 'switchback' population demonstrated that there was still sufficient residual genetic variation upon which selection could act to drive the phenotype in the opposite direction. What was perhaps the most surprising was that as much genetic variability was present after long-term selection as existed in the original unselected population (Leng 1962).

Progress from selection in IHO and ILO was due to the accumulation of a large number of small favorable alleles in coupling phase linkage, that were present in the original Burr's White OPV at a low frequency (Dudley 2007). Accompanying these changes in kernel oil content, were changes in starch and protein composition of the kernels. During the first 70 cycles of selection in IHO and ILO, protein concentration increased from 109 to approximately 150 g/kg (Dudley et al. 1974). From cycle 65 to 100 of IHO oil content increased from 161 to 224 g/kg, protein concentration showed a significant increase from 148 to 158 g/kg, while starch content significantly declined from 435 to 336 g/kg (Dudley and Lambert 2004). The IHO/ILO germplasm has been used in several quantitative trait loci (QTL) studies, the results from which confirm the findings of the classic quantitative genetic studies and the correlated responses (cf., Dudley 2007).

17.6 Other Breeding Programs for High-Oil

The IHO/ILO long-term selection experiment was not the only breeding population developed for kernel oil content. In the 1950s, D.E. Alexander initiated several 'new' breeding programs for high-oil maize. There have also been several forays into developing commercial high-oil hybrids through various approaches.

17.6.1 'Other' Recurrently Selected High-Oil Populations

D.E. Alexander initiated five 'new' breeding populations for high-oil selection in the 1950s. The intent was not to duplicate the IHO selection experiment, but rather to develop other sources of 'high-oil genes' for breeders to use. The other compelling reason for creating these populations was to examine the negative association between grain yield and oil content that was present in the IHO population (note the decline in starch content in IHO mentioned above) (Lambert et al. 2004).

(1) Alexho Synthetic – served as the source for three additional selection experiments: Alexho Single Kernel, Alexho Elite (AE), and Ultra High-Oil (UHO).
(2) Disease Oil (DO) – developed to combine improved resistance/tolerance to leaf blights and stalk rots with high-oil.
(3) Arnel's Reid Yellow Dent (ARYD) – a selection of 363 ears from the OPV Reid Yellow Dent that was grown on the Arnel farm in 1964.
(4) Stiff Stalk Synthetic High-Oil (RSSSCHO) – comprised of intermating three improved versions of Stiff Stalk Synthetic.
(5) Iowa-2-Ear High-Oil (BS10HO) – a two-eared population developed by analyzing 10,000 kernels from BS10(FR)C_2.

Work with these populations showed similar responses to selection for oil content as the IHO population. However, unlike the IHO population, two kernel mutations (waxy and floury), which affect starch composition and protein content, respectively, arose in the later cycles of Alexho Synthetic.

17.6.2 Commercial Breeding Activities for High-Oil

The first major attempt at developing commercial high-oil hybrids was in the late 1940s by converting the widely grown double-cross hybrid, US13, into a high-oil hybrid. This involved backcrossing IHO genes into the four parental inbred lines: Wf9, 38-11, Hy2, and L317. Rather than measuring oil content during the backcrossing process, embryo size was used as an indicator of oil content. In the end, the converted US13 was ~5% lower in grain yield, ~8% higher in protein content, and ~30% higher in oil content (Jugenheimer 1961). Starting in 1946, Funk Bros. Seed Co. began a high-oil breeding program that continued until 1972. The initial breeding approaches involved both RS and backcrossing the IHO genes into conventional inbred lines. The RS efforts were mildly effective (58–63 g/kg oil content), while the backcrossing approach produced inbred lines in the 70–100 g/kg oil content range. Funk Bros. used these lines to produce three high-oil double cross hybrids. Unfortunately these hybrids were ~10% lower in grain yield than Funk Bros.'s widely grown hybrid (Lambert 2001; Lambert et al. 2004).

17.6.3 TopCross® Method for High-Oil Maize Hybrids

TopCross® and TC Blend® seed corn are proprietary technologies of DuPont Quality Grains for producing high-oil maize while minimizing the yield disadvantages that traditionally accompany high-oil maize production (Bergquist et al. 1998, 1999). The approach utilizes a blend of two genotypes in a unit (i.e., bag) of seed maize. One genotype, comprising 90–92% of the seed in the blend, is a hybrid that is designated as the 'grain parent' and is male sterile, meaning that it produces no pollen. The second genotype, representing 8–10% of the seed in the blend, is a called a 'pollinator'. Unlike the grain parent, this genotype does produce pollen and is solely the source of pollen within the TopCross® grain production field. The 'pollinator' is a high-oil genotype (generally a synthetic population) in the 120–150 g/kg oil content range (Lambert 2001). The resulting F_1 kernels on the 'grain parent' will have higher oil content due to the genetics of the 'pollinator', a phenomenon called xenia where the genotype of the pollen parent affects the phenotype of the grain. Because of the xenia effect it is important to isolate TopCross® grain production fields from other maize fields to minimize the amount of pollen contamination from non-high oil maize. Reports suggest that in general maize kernel oil contents between 60 and 80 g/kg can be achieved with this technology (Lambert et al. 2004), but there still appears to be a significant yield disadvantage (Thomison et al. 2002).

Epilogue

While maize is a 'minor' oil crop, the breeding efforts for high-oil maize have made contributions beyond maize. The long-term selection experiment for high- and low-oil content has permitted testing of fundamental quantitative genetic principals that pertain to selection in both plants and animals. Both the IHO/ILO experiment and other high-oil breeding efforts have contributed breeding approaches and analytical technologies to other oilseed breeding programs. The use of NMR technology for single kernel selection oil content (Bauman et al. 1963) and backcross conversions are just two examples.

Dedication The author would like to dedicate this chapter to John W. Dudley and Robert J. Lambert for their perseverance and commitment to seeing the Illinois High-Oil/Low-Oil long-term selection experiment through 100 generations of selection.

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Chapter 18
New Crops Breeding: Lesquerella

David Dierig and Dennis T. Ray

18.1 Introduction

Lesquerella species contain a seed oil which is approximately 55% lesquerolic acid, a 20-carbon long fatty acid with a single hydroxyl group and double bond, and has a similar hydroxy fatty acid (HFA) profile as castor oil. Large markets exist for hydroxylated oils as feedstocks for lithium greases, polymers in paints and coatings, base stocks for lubricants, nylon-11, hydraulic fluids, and applications in the personal care industry (Roetheli et al. 1992). The hydroxyl group of these oils makes them prime candidates as additives to diesel fuel to improve lubricity (Naughton 1992). Goodrum and Geller (2005) demonstrated that lesquerella oil has superior performance compared to castor, soybean, and rapeseed methyl esters at concentrations as low as 0.25% in reducing wear and damage to diesel engines, primarily with fuel injection systems. Castor oil also contains high amounts of HFAs, but the main HFA, ricinoleic acid, is two carbons shorter than lesquerolic acid, which imparts slightly different physicochemical properties to the oil. Lesquerella could be established as a reliable, domestic oilseed supply of HFAs for a variety of industrial applications (Roetheli et al. 1992) and at the same time provide an alternative crop for farmers and increase local profits. Lesquerella will not replace current commodity crops but instead will be placed in a rotation with these crops, e.g., a 2-year, 3-crop rotation of lesquerella, grain sorghum, and cotton.

18.2 Origin and Domestication

Lesquerella is a genus of the Brassicaceae family with species ranging primarily from the arctic to southern Mexico, with another dozen species occurring in South America. The greatest concentration of taxa are in the southwestern United States and Mexico, where *L. fendleri* originates, and in the Rocky Mountain and

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intermontane basin region of the western U.S. (Rollins and Shaw 1973; Rollins 1993). *L. fendleri* (Fig. 18.1) contains the hydroxy fatty acid lesquerolic acid (14-hyroxy-cis-11-eicosenoic acid, C20:1-OH) as the main component of its seed oil. Other species with a second type of HFA in their seed oil, densipolic acid (12-hydroxy-cis-9-cis-15-octadecadienoic acid, C18:2-OH) originate from the eastern U.S. A third type of HFA called auricolic acid (14-hydroxy-cis-11,cis- 17-eicosadienoic acid, C20:2-OH) is found primarily in one species from Oklahoma.

Fig. 18.1 Flowers (*left*) of *Lesquerella fendleri* (Brassicaceae), and a lesquerella production field (*right*) in Arizona

The genus *Lesquerella* was named in honor of paleobotanist Leo Lesquereux (1806–1889), who came from Switzerland to the U.S. in 1848, and settled in Columbus, Ohio. His specialty was the collection of fossil plants, especially in connection with coal deposits. The taxonomy of *Lesquerell*a, commonly called bladderpod, was studied by Payson (1921) and later revised by Rollins (1955). O'Kane et al. (1999), Al-Shehbaz and O'Kane (2002), and O'Kane and Al-Shehbaz (2002) proposed that 75 species in the genus *Lesquerella* be transferred to the genus *Physaria* and all of the annual species of *Lesquerella* from the southeastern U.S. be placed into a new genus, *Paysonia*. Their new synonym for *L. fendleri* is *Physaria fendleri* (A. Gray). Public literature still refers to this plant as lesquerella or *L. fendleri* and Rollins and Shaw (1973) noted difficulties in the similarities between the two genera but also stated the problem of merging them because of similarities with *Lesquerella* and another genus *Alyssum*. The distinction for agronomic purposes and crop development is that *L. fendleri* is a short-lived perennial that can be grown as an annual. Some other species of *Lesquerella* and *Physaria* are perennial and do not flower until the second year of growth. *Physaria* species are not as adaptable for domestication because of this, even though they can be very productive seed yielders.

The first interest in *Lesquerella* species for domestication came in the late 1950s. USDA-ARS began a massive screening of over 200 families of oilseed plants from the wild and described the new hydroxy fatty acids from this plant (Jones and Wolf 1960; Smith et al. 1961). Plant exploration trips to collect *Lesquerella* species were based on the fact that only occasionally does a plant with an unusual oil composition also have high crop potential with no real biological barriers for domestication. A series of articles *The Search for New Industrial Crops* and *The Search for New Industrial Oils* were published in Economic Botany and the Journal of the American Oil Chemists' Society between 1960 and 1984, and described lesquerella as well as other potential new crops. The second article in *The Search for New Industrial Crop* series was the first description of collections of *Lesquerella* made by USDA-ARS (Barclay et al. 1962). A breeding program by Dr. D. Rubis at the University of Arizona began in 1968 and continued until 1971, using the germplasm collected by USDA. Dr. Rubis generously contributed his germplasm to the USDA, ARS program in Arizona that began in 1986 by Dr. A.E. Thompson (Thompson and Dierig 1994). The breeding program is still ongoing at the ARS facility in Maricopa, Arizona.

L. fendleri is found in its native environment on calcareous soils in the southwestern states of Arizona, New Mexico, and Texas, with a few collections from southern Utah and Colorado by Rollins and Shaw (1973). Collections from the states of Coahuila, Chihuahua, Nuevo Leon, Zacatecas, and Durango, Mexico were made (Salywon et al. 2005; Rollins and Shaw 1973). These populations were usually associated with moisture availability in mixed, sparse vegetation, and were easily recognized by their glabrous siliques and fused trichomes which set *L. fendleri* apart from other *Lesquerella* species.

18.3 Genetic Resources

There are 233 *Lesquerella* accessions available in the National Plant Germplasm System (NPGS). One hundred and twenty of these are *L. fendleri*, and most of these accessions were collected during the period from 1993 until 2002 through trips supported by USDA-ARS (Dierig et al. 1996; Salywon et al. 2005). Prior to this there were only 17 species represented and 21 accessions of *L. fendleri* in NPGS (Thompson et al. 1992). In the USDA-ARS-ALARC working collection, there are now 413 accessions of 57 *Lesquerella* and 17 *Physaria* species. One hundred and thirty are *L. fendleri* accessions. A phenotypic evaluation of germplasm available in NPGS was completed by Jenderek et al. (2008). The curator within the NPGS for *Lesquerella* species is located at the USDA, ARS, National Arid Land Plant Genetic Resources Unit – Parlier, California.

In Rollins' review (Rollins 1993) of *Lesquerella* of North America, 83 species were included. Other species have since been discovered including four by Rollins (1997), Rollins et al. (1995) and Anderson et al. (1997) as

well as others by O'Kane (1999) bringing the total number of North American species to about 90.

Some *Lesquerella* species are on federal or state lists as rare, endangered, or threatened species. One of these species, *L. pallida*, has been valuable to our breeding program because of its high lesquerolic HFA content. This species is also autofertile compared to the self incompatibility and open pollination of *L. fendleri*. No collections have been made of this species since the original collection in the 1800s, until a report by Nixon et al. (1983) on the rediscovery of the species.

A number of *Lesquerella* and *Physaria* species have traits of interest for genetic improvement, although none have the productivity of *L. fendleri*. Apart from plant accessions our database contains over 10,000 records of germplasm lines including various traits such as yellow seeds, non-shattering selections, salt tolerance, male sterility, five petal plants, multi-locule silique selections and other traits and crosses.

18.4 Major Breeding Achievements

An important accomplishment has been to provide a large collection of genetic diversity through plant germplasm collections. The numerous collection trips throughout the U.S. and Mexico and subsequent evaluations provided valuable diversity for the plant breeding program. Within these accessions, traits such as flower color, autofertility, plant architecture, seed coat color, male sterility, and seed oil characteristics have been identified. The inheritance of some of these traits has been determined to allow their use in the breeding program (Dierig et al. 2001).

The improvement of oil content has made a significant contribution to the commercialization process of lesquerella. Unimproved accessions of *L. fendleri* have oil contents around 24%. The last germplasm line publicly released had 32% (Dierig et al. 2006a), and another line ready for release has 36% oil content. The difference in oil yield between the unimproved and improved accessions is 86 liters/ha (56 gal per acre) oil versus 146 liters/ha (84 gal per acre) based on current yields of 1800 kg/ha, respectively.

The oil profile of this species has been surveyed by Dierig et al. (2006b) and lesquerolic HFA was highly variable. *L. fendleri* appears to have a limit of around 67%. Hayes et al. (1995) reported that *L. fendleri* fills only 2 of the 3 triglyceride positions with HFA. The *sn* 2 position is left unfilled, which explains the upper limit of two-thirds or 67% for HFA content. A few other species of *Lesquerella* are able to fill all 3 positions and have HFA content of up to 89%, which is near the level of castor. Current lines contain 60% lesquerolic acid. A single gene mutation with high oleic and 0% lesquerolic acid was recently discovered (Dierig et al. 2008, in prep.). This germplasm provides a platform for examining a molecular approach for transforming lesquerella with genes in the HFA biosynthetic pathway to increase the HFA content.

Interspecific hybrids between *L. fendleri* and two other species have been developed, introgressing the high lesquerolic trait into *L. fendleri*. The hybrids are not ready for public release until further improvements are made but have over 75% lesquerolic acid (Dierig et al. 2004). Seeds per pod (silique) are fewer than *L. fendleri*, but this is as expected since the other parent species had larger but fewer seed, and the hybrid expressed mid parent values. The hybrids hold promise for providing non-genetically modified traits not currently available in *L. fendleri* such as high lesquerolic acid, autofertility, and an expanded geographical area for commercial production.

18.5 Current Breeding Goals

Lesquerella fendleri plants are open-pollinated, highly genetically diverse, and have no biological barriers to being a commercial crop. *L. fendleri* is highly productive and it is felt that by exploring other areas of research the crop has the potential to double in yields. A few traits that could be further exploited will be discussed below, although this is not a comprehensive list.

18.5.1 Oil Content and Fatty Acid Profile

There have been two plant germplasm releases with improved total oil and lesquerolic acid contents (Dierig et al. 1998, 2006a). Results from a recurrent selection program have consistently produced plants with oil content between 40 and 45%. This appears to be the upper limit for the oil content of this plant. However, this is the average value of seeds from a single plant. There has yet to be a screening of single seeds for this trait; a technique has just been developed, but will still be difficult because of the small seed size (Isbell et al. 2008). A single seed weighs approximately 0.0006 g. Screening germplasm via single seeds for this trait should allow faster progress in increasing oil content.

The upper limit for lesquerolic acid HFA content in *L. fendleri* appears to be about 67% (described above). Some market applications may require this to be kept at the limit if the material, such as nylon 11, requires a difunctional triglyceride. Lines are being developed through the development of interspecific hybrids with greater than 80% lesquerolic acid utilizing species with all three positions of the triglyceride (trifunctional) and introgressing that trait into the hybrids. An improved line with these traits would fit most of the same markets as imported castor. The other desirable goals would be to lower the unsaturated fatty acids linoleic and linolenic acids.

18.5.2 Seed Yield

The current lesquerella seed yields are approximately 1800 kg/ha, but it is felt that the plant has the potential of yielding 2500–3000 kg/ha. This increase will come through a combination of improved agronomic practice and breeding. Some agronomic issues include more precise plant spacing, better irrigation management, and more efficient harvesting. Developing more productive varieties will include selection based on harvest index and for specific environments. *L. fendleri* is the most productive of all species so far tested because of its extensive branching and subsequent flowering along each branch. Selection for branching at warmer or cooler temperatures will identify plants better adapted for growth in different climates.

Plants that reach maturity in a shorter time period will save production costs. This will require selection based on seed germination at cooler temperatures, or plants that will branch at lower temperatures.

18.5.3 Wider Adaptation and Shorter Growing Period

Since these two goals are related, they will be discussed together. These issues are also tied into the discussion above on improved seed yield. If plants are able to be developed that branch at lower or cooler temperatures, the planting date could be moved from October to February. Planting later than February in Arizona is not desirable because of the probability of summer rains that may occur during the dry down period causing seed shatter. Lesquerella does not normally shatter; however, when irrigation is terminated and a desiccant is applied 7–10 days before combining, the crop exposed to hard rains could lose its seed yield.

18.5.4 Autofertility

A few *Lesquerella* species are autofertile such as *L. pallida* and *L. mcvaughiana*. Both have the same chromosome number as *L. fendleri* with potential for use as a source of germplasm to introgress this desirable trait. Within *L. fendleri* there may also be potential for variation in autofertility, but it has not been looked for in a thorough manner. The advantage of autofertility would be that pollinators would not be required to increase seed yield. Plants may begin flowering in February or earlier in warmer years but these temperatures are still too cool for pollinators to be present in the field which causes a yield reduction. There are also years when feral bee populations are not as abundant resulting in lower yields, unless managed bees are used. The cost of bees for pollination obviously results in higher production costs and can be significant due to problems beekeepers are experiencing such as colony collapse disorder. Self pollination

might prove to be detrimental due to inbreeding depression, but the trait still warrants investigation.

18.6 Breeding Methods and Techniques

Lesquerella, as well as other oilseeds in the Brassicaceae family, are generally cross pollinated and self incompatible. A single flower produces up to 30 seeds inside a silique (pod) and each seed has the potential to originate from a different pollen source causing each seed to be genetically different. Although more genetic variability for selection is generated, a desired trait is more difficult to select because the seeds from a single plant are half instead of full siblings, as self pollination rarely occurs (Dierig et al. 2004). Half seed selections and inheritance studies have allowed selection of mutants in the fatty acid profile instead of through half sib selection. The ability to analyze a half or a single seed of *L. fendleri* for fatty acid and oil content has greatly improved breeding of this crop (Isbell et al. 2008). Since the seed is very small, this has proved to be a greater challenge compared to other oilseed crops where it has been accomplished. The seed of *L. fendleri* weighs about 0.6 mg, which is less than half the size of alfalfa seed.

Interspecific hybrids have been a focus of our program to utilize traits not found in *L. fendleri* but that occur in other *Lesquerella* species. Eastern species hybridize in nature but species native to the western U.S. do not hybridize readily. The current method used for producing these hybrids is described in Dierig et al. (2004). This includes use of ovule culture and colchicine treatment. Cytological information has also been essential to determining the behavior of parents and progeny in these crosses. One example of this was in a cross of another species with *L. fenderi* that was thought to have the same chromosome number. When some of the progeny failed to have the high HFA trait of this parent, cytological examination, using pollen mother cells, found that the seed which had originated from an exploration trip and was thought to be a single species, was actually a mix of two different species (D.T. Ray, personal data). Bud pollinations, where buds are manually opened before anthesis to pollinate, are routinely used in both inter- and intra-specific crosses to overcome incompatibility. Other breeding methods include those used for cross pollinated crops such as recurrent selection.

Mutation breeding has been utilized in our program using ethyl methane sulfonate (EMS). We have found that *L. fendleri* has a tremendous amount of variability and many of the mutants we have in our collection such as fatty acid mutants (0% lesquerolic acid + high oleic; 0% linolenic; 0% linoleic), cream flower color, male sterility, yellow seed coat; a 5-petal flower, and multi-locule silique, also occurred without EMS treatment.

18.7 Integration of Information Technology and New Biotechnologies

Information technology has aided the progress of lesquerella breeding by integrating data management and creating a more efficient use of plant germplasm. White et al. (2007) described the importance of this especially with new crop development where the scientific input is much reduced compared to established, traditional crops. New crop development is especially prone to fluctuating efforts over time due to the limited available financial resources. Often times research results do not get published because funding ends or a researcher retires. Intellectual Property Rights can also be an issue for exclusive licensing of products from new crops or Plant Variety Protection and germplasm used in developing the variety along with breeding methods. The goal of developing an information technology system for lesquerella has been the documentation of germplasm and collection information, maintaining careful records of genealogy and population structures, and developing a network available to interested users. This information system keeps careful documentation of all the available phenotypic and molecular documentation. The system we have utilized is the International Crop Information System (ICIS, www.icis.org).

SSR markers have been developed for lesquerella (Salywon and Dierig 2006). These SSRs along with other molecular markers have value in association mapping of traits, such as lower linolenic acid content. The association mapping methodology calculates linkage disequilibrium and is superior to quantitative trait loci (QTLs) mapping in determining genetic variances (Zhao et al. 2007). Bioinformatics approaches such as searching the EST libraries for DNA sequences with similarity to important genes are now being used in lesquerella. Necessary genes to incorporate HFA at the *sn*-2 position of *L. fendleri* seed oil are being examined (Dyer and Mullen 2005).

18.8 Seed Production

There is currently no lesquerella commercial seed production. Technology Crops International (TCI, www.techcrops.com) is currently working on a marketing strategy to begin production. They plan beginning commercial production of lesquerella seed in areas of the southwestern U.S.

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Chapter 19
Cuphea

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19.1 Introduction

Temperate plant species whose seed oils are rich in medium-chain fatty acids (MCFAs) are relatively rare (Wolf et al. 1983). One species of particular interest is cuphea, a temperate annual oilseed crop with high levels of MCFAs such as capric and lauric acid (Graham et al. 1981; Graham and Kleiman 1985; Graham and Kleiman 1992). These fatty acids are highly valued as feedstocks in manufacturing cosmetics, soaps and detergents, pharmaceuticals, and industrial lubricants (Wolf et al. 1983; Thompson et al. 1990; Cermak and Isbell 2004). Additionally, new uses for MCFAs have the potential to significantly replace petroleum-based products like motor oil, hydraulic fluid, and diesel fuel (Geller et al. 1999; Geller and Goodrum 2000; Leroux et al. 2006). This chapter primarily covers the advances of cuphea research since the previous reviews of cuphea breeding presented by Knapp (1990a, 1993) with the main focus on oilseed production in the cuphea species of *C. lanceolata*, and *C. visscosissima* which are targeted for commercialization in the US.

Research interests have centered on cuphea primarily for its seeds which have a high content of unique fatty acids (Earle et al. 1960; Graham et al. 1981; Wilson et al. 1960). The predominant fatty acids include: caprylic acid (C8), capric acid (C10), lauric acid (C12) and myristic acid (C14). Lauric acid has been the primary fatty acid of interest in US breeding programs. Lauric acid is used in foods, mostly vegetable shortenings, and in soaps and detergents as a defoaming agent and booster (Thompson 1984; Babayan 1981). Traditionally, the tropical oil crops such as coconut (*Cocos nucifera* L.) and oil palm (*Elaeis guineensis* Jacq.) commercially supply these acids. Currently the US soap and detergent industry gets half of these fatty acids from the petroleum industry and the other half from imported coconut and palm kernel oils (Hardin 1991). Coconut oil is 45–50% lauric acid, while some undeveloped lines of cuphea can produce oil that contains nearly 80% lauric acid (Graham

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1989b). To date, there are no temperate oilseed crops that can supply these lipids (Ignacio 1985; Arkcoll 1988). Many of the fatty acid-rich species of cuphea are summer annuals and through crop development programs could potentially become domestic sources of fatty acids.

The cuphea breeding line, 'PSR23' (*Cuphea viscosissima* Jacq. × *C. lanceolata* W.T. Aiton) is the only domesticated cuphea in the US and is the current focus of production and breeding programs in Illinois, Iowa, North Dakota, and Minnesota. Although rich in capric acid, PSR23 serves well as an agronomically sound plant for agronomic and physiological studies and as a foundation for breeding programs in the US.

Breeding and research efforts on other *Cuphea* spp. continues throughout the world. Research in India is focused primarily on *C. procumbens* for seed oil production (Pandey et al. 2000; Rameshkumar et al. 2002; Singh and Singh 2002; Singh and Rameshkumar 2003; Singh et al. 2007). South American researchers are looking at *C. carthagenesis* (Mathioni et al. 2005; Dezanet et al. 2007) and *C. glutinosa* (Yagueddu et al. 2006) for medicinal purposes. Japanese researchers are investigating food uses with *C. leptopoda* (Saikusa et al. 2001).

19.2 Domestication and Breeding History

The genus *Cuphea* (Lythraceae) contains approximately 260 species that are native to the area from Mexico through Brazil, with one species native to the eastern United States (Graham and Kleiman 1985). Several are adapted to temperate agriculture and have seed oils rich in capric and lauric acid. Initial domestication programs began in Germany to evaluate different species and to determine the feasibility of domesticating cuphea (Hirsinger 1980; Hirsinger and Röbbelen 1980; Graham et al. 1981; Hirsinger and Knowles 1984; Hirsinger 1985; Röbbelen and von Witzke 1989). At Oregon State University (OSU), Steven Knapp began an intensive breeding program to domesticate cuphea for US production. Knapp worked extensively with *C. viscosissima*, the only species native to the United States, and *C. lanceolata*, a species native to the Sierra Madre of Mexico (Graham 1988). The essential agronomic traits and seed oil yields of these species were sufficient for competition as oilseed crops in the US. Seed shattering and seed dormancy were the major domestication barriers within the genus at the time (Knapp 1990a,b).

In the mid to late 1990s, important breakthroughs were made towards eliminating seed shattering and seed dormancy by exploiting interspecific diversity. Non-shattering phenotypes within the *C. viscosissima* × *C. lanceolata* f. *silenoides* population and an autofertile, non-dormant, and non-shattering *C. viscosissima* × *C. lanceolata* f. *silenoides* cultivar have all been developed (Knapp and Crane 2000a,b,c). Knapp was also successful at developing other

elite lines of cuphea that are non-shattering, auto-fertile, and have reduced levels of sticky hairs.

Sticky or glandular hairs covering stems, leaves, and flowers are characteristic of most cuphea species (Graham 1988; Amarasinghe et al. 1991). These glandular hairs have been cited as a negative trait primarily due to the difficulty in harvesting the crop (Hirsinger 1980; Hirsinger and Röbbelen 1980; Thompson 1984). The stickiness of cuphea is certainly difficult when working by hand, but it is not a limitation to the commercialization of cuphea. Recent large scale production of several hundred acres in 2005–2007 in Minnesota and Illinois were not impeded by the stickiness when production combines were used (Fig. 19.1). Although some of the sticky residue from cuphea chaff accumulates in harvesting equipment, it does not seem to hinder harvesting. The indeterminate nature, high levels of moisture, and shattering levels of cuphea are seen as the current barriers to successful and consistent harvests.

Fig. 19.1 Direct combining of PSR23 cuphea in Illinois

19.2.1 Oil Crop Breeding

Studies conducted in the early 1980s on cuphea focused primarily on characterizing fatty acid profiles of various cuphea species collected from the wild (Graham et al. 1981; Wolf et al. 1983; Hirsinger 1985). These studies helped direct early breeding programs to focus on lauric acid accumulating species, *C. wrightii*, for the soap and detergent industry (Thompson and Kleiman 1988). Unfortunately, domestication of this species proved to be extremely difficult

due to seed shattering, open pollination, and poor agronomic traits. However, recent efforts with the high capric acid species of *C. viscosissima* and *C. lanceolata* have been successful in improving many of the agronomic traits necessary for cuphea domestication (Knapp 1993). Several early breeding programs employed mutation breeding to address fatty acid accumulation (Hirsinger 1980; Hirsinger and Röbbelen 1980; Campbell 1987; Röbbelen and von Witzke 1989; National Botanical Research Institute 2003); however, they all experienced limited success. No newer reports of mutation breeding have been reported in the literature. Non-shattering and determinacy still pose difficult hurdles in cuphea species. To date, variety trails of cuphea accessions have yet to identify a completely non-shattering or determinate accession. With efforts continuing on improving agronomic traits, altering total oil and fatty acid content will soon become a priority once again.

19.3 Genetic Resources

The USDA-ARS National Plant Germplasm System (NPGS) cuphea collection is currently maintained at the North Central Regional Plant Introduction Station (NCRPIS) in Ames, IA under the curation of L. Marek. As of January 2008, 649 accessions of cuphea are in the NCRPIS collection; however, only 499 are available through the Germplasm Resources Information Network (GRIN) for distribution (a few as vegetative cuttings only, the rest as seed only). The accessions represent 67 different taxa, plus seven accessions that have not been identified to species, and 30 accessions that are hybrids of various combinations. The available accessions represent 38 different taxa, plus 2 unidentified to species, and 26 that are hybrids.

Over the past 10 years, the NCRPIS has distributed approximately 4,250 cuphea items in 167 requests. Nearly 80% of all requests were sent within the US. International distributions were to Japan, the Netherlands, Germany, France, China, England, Brazil, Greece, and Canada. Cuphea seed requests are distributed evenly between ornamental and oil related uses (breeding, enzymes, development, and evaluation) (L. Marek, personal communication).

The cuphea genetic resources collection was recently assisted by the identification of protocols to help remove dominancy issues of wild type accessions (Widrlechner and Kovach 2000; Crane et al. 2006). It was also found that the current storage protocols were detrimental to seed germination (Volk et al. 2006). *C. wrightii* A. Gray, *C. laminuligera* Koehne, *C. carthagenensis* (Jacq.) J.F. Macbr., and *C. aequipetala* Cav are considered sensitive to low temperature storage. The seeds of these species have triacylglycerols that are crystalline at –18°C and melt when the seeds are warmed to >35°C (Volk et al. 2007). Cuphea seeds imbibed while the triacylglycerols are crystalline fail to germinate and exhibit visual damage. However, germination proceeded normally when

dry seeds were warmed adequately to melt any crystalline triacylglycerols before imbibition.

Although most of the available accessions have basic descriptors, the majority of the collection has limited information regarding fatty acid content and yield. Several studies have screened portions of the collection, but very little consistency exists between them (Graham et al. 1981; Wolf et al. 1983; Hirsinger 1985). Extraction method, location and year where seeds were produced, and gas chromatography analysis have all led to variations in total oil and fatty acid composition being reported for individual accessions. Many of the early studies often involved complicated and difficult extraction and analysis procedures requiring hours of preparation. These methods are impractical for supporting high throughput breeding programs aimed at developing new varieties. A recent study by Phippen et al. (2006) developed a reliable and efficient method for determining total oil and fatty acid content in cuphea seed and evaluated 185 cuphea accessions.

19.4 Advances in Cuphea Production

Knapp, while at OSU, essentially maintained the only cuphea breeding program in the US from the mid 1980s to 2004. Many of his breeding advancements were summarized in Knapp (1993) and more recently in Knapp et al. (2004).

Knapp's more recent efforts were directed towards: the development of fully self-pollinated, partially non-shattering cultivars; breeding for high oil, high capric acid, and semi-determinant flowering; and strategies for developing high lauric and fully non-shattering cultivars (Knapp et al. 2004). The development of high capric cultivars is underway using high capric acid (85–89% C10) germplasm sources. As reported by Knapp et al. (2004), the molecular breeding program was restarted to build the foundation for genetic analyses and marker-assisted selection in cuphea. More than 200 sequence-tagged-site (STS) markers have been developed. The molecular genetic diversity in *C. viscosissima* and *C. lanceolata* has already been surveyed, along with the development of segregating populations and near-isogenic lines for mapping and manipulating phenotypic loci. Quantitative trait loci for seed dormancy, seed shattering, self-pollination, and other economically important traits continue to be identified. This advancement effort is aided by a genetic map of cuphea developed by Webb et al. (1992).

19.4.1 'PSR23' Cuphea

One of the first successful releases of a cuphea breeding line was 'PSR23' (PI 606544) released by Knapp and Crane (2000c). PSR23 was the first line to

introduce the critical 'Partial Shatter Reduction' (PSR) trait into cuphea. This trait inhibits the rotation of the placenta from the capsule thereby leading to an increase in seed retention (Fig. 19.2). Typical wild type cuphea populations have a near 100% seed loss due to shattering. The PSR trait reduces this seed loss to only 20–30%. Other traits of interest in PSR23 include relatively high oil content of 295 g kg^{-1}, high capric acid content of 72%, self-compatibility, and non-dormant seed. Although still largely open-pollinated, PSR23 was selected as the first cuphea line to begin commercialization.

Fig. 19.2 Partial shatter reduction trait in PSR23 cuphea with reduced placenta rotation (**A**). Wild type shattering trait with increased placenta rotation (**B**)

PSR23 was first distributed to research programs in Illinois (IL) and Minnesota (MN) in the summer of 2000. The original population was heterogeneous and clearly demonstrated potential for environmental selection. Over the next 7 years, selections were increased in IL and MN, remained separated and developed unique phenotypes for each region. Under the drier and longer IL growing season, a larger, erect, and less vegetatively pronounced phenotype was favored. Unfortunately, this phenotype lost the essential PSR trait. In the cooler shorter season of the northern corn belt, a smaller, more vegetatively pronounced, and more compact phenotype prevailed. With this phenotype, the reduced shattering trait remains intact.

A recent environmental adaptation study of the PSR23 ecotypes was conducted in IL and MN in 2007. Preliminary results from the Illinois field studies indicate that IL grown PSR23 selected in 2006 produced the least amount of seed in 2007 (210 kg ha^{-1}), which is not surprising due to the lack of the PSR trait. However, due to the small plant size of the MN grown PSR23 selected in 2006 its seed production under IL growing conditions was limited to 240 kg ha^{-1}. When compared to the original PSR23 with a seed yield of 350 kg ha^{-1}, both the MN and IL ecotypes fared worse under IL conditions. Under MN growing conditions, all cuphea lines performed much better than in IL with the original PSR23 reporting 830 kg ha^{-1}. Interestingly, only the first year selection in MN performed as well as the original line. After 7 years of selection at both

locations, most lines significantly decreased in seed yield when compared to the original line. This suggests the original PSR23 is still heterogeneous and that the current selection protocols are not adequate for maintaining high seed yields.

19.4.2 Commercialization

With PSR23 being the only cuphea line available in adequate volumes, many of the more recent agronomic, plant physiological, and product development research reports in the US are based on this line. Although two ecotypes of PSR23 exist, they do not differ significantly in total oil yield and fatty acid constituents.

This first major breakthrough in producing cuphea on a large scale was the identification of herbicides that could control broadleaf weeds in production fields. Fortunately, cuphea demonstrates tolerance to several soil-applied herbicides including ethalfluralin, isoxaflutole, and trifluralin, and one postemergence herbicide, mesotrione (Forcella et al. 2005a). Repeated studies in IL and MN have enabled researchers to secure a registered 24C herbicide label for the mesotrione herbicide for application on cuphea in 2005. This helped facilitate large scale seed increases by contracted producers. To help control biennial wormwood (*Artemisia biennis*) and Canada thistle (*Cirsium arvense*) in MN, clopyralid could also be used safely in conjunction with soil-applied isoxaflutole (Papiernik et al. 2006).

The summer of 2004 marked the first year for an experimental commercialization trial focused on developing an agricultural management strategy for cuphea utilizing conventional technologies to minimize the need for specialized equipment. Technology Crops International, in cooperation with the USDA Agricultural Research Service, grew 18.6 ha of PSR23 within a 32 km radius of Morris, Minnesota (45.35°N, 95.53°W). Although not all hectarage was successful, the harvestable plantings produced seed yields ranging from approximately 78–744 kg ha^{-1} at 12% moisture (Gesch et al. 2006). Being the first large scale production of a partially domesticated breeding line, valuable knowledge was learned through this experience that might not have been gained by plot-scale experiments alone. PSR23 remains indeterminate displaying a wide range of seed maturities at time of harvest. Gesch et al. (2006) indicated post-harvest management of seed on a large-scale (e.g., drying, cleaning, and storing) was problematic, suggesting further need for introducing determinacy into the current cuphea breeding lines.

Much of the success of the 2004 commercialization trial was due to the many research projects focused on improving the cultural practices of producing cuphea. Recent studies have addressed seed germination response to temperature (Berti and Johnson 2008), row spacing (Gesch et al. 2003; Sharratt and Gesch 2004), sowing dates (Gesch et al. 2002; Forcella et al. 2005b), temperature sensitivity (Gesch and Forcella 2007), nutrient requirements

(Olness et al. 2005), irrigation studies (Gesch et al. 2004), seed physiological maturity (Berti et al. 2007) seed drying (Cermak et al. 2005) and harvesting methods (Berti et al. 2005; Forcella et al. 2007). Tisserat et al. (2008) have indicated that applications of ultra-high CO_2 treatments accelerated cuphea PSR23 growth and development and aided in seedling establishment. The basic knowledge gained by the previously mentioned experiments has been the single greatest advancement towards breeding and ultimately commercializing cuphea. Although significant progress has been made in production protocols for cuphea, the lack of any significant breeding effort to develop auto-fertile, non-shattering, and determinate plant lines still limit the success of cuphea as a commercial crop.

19.4.3 Product Development

With the success of the 2004 commercialization trial and the following growing seasons, several thousand pounds of seed were made available to the USDA-ARS in Peoria, IL for product development. Some of the more recent advances can be found in modifying the fatty acids of cuphea to meet current industrial needs as lubricants (Evangelista and Cermak 2007), fuels (Leroux et al. 2006) and cosmetics (Cermak et al. 2007).

A recent study even characterized the proteins in cuphea (PSR23) seed to provide fundamental information on their size, amino acid profile, solubility classes, and solubility behavior (Evangelista et al. 2006). The seed contained 32% (dry basis, db) oil and 21% (db) crude protein. Glutelins and albumins accounted for 83.5% and 15.4%, respectively, of the total protein extracted. PSR23 has been further investigated to determine the effects of oil processing conditions on functional properties of the seed proteins to evaluate their potential for value-added uses (Hojilla-Evangelista and Evangelista 2006).

19.5 Current Breeding Goals

19.5.1 Lauric Acid Accumulation

The original US cuphea breeding programs focused on cuphea species rich in capric acid, with the intent of crossing in lauric acid once an agronomically sound plant had been obtained. Current breeding lines are all progeny of *C. viscosissima* × *C. lanceolata* f. *silenoides,* which are diploid with six chromosomes ($n = 6$) and rich in capric acid. Attempts have been made in the past to create high lauric acid accumulating cuphea types, however, much of this early work was on non-adapted cuphea species. Koehne (1903) described five cuphea interspecific hybrids that were documented from herbarium samples in Europe. Röbbelen and Hirsinger (1982) observed spontaneous outcrossing among

species in their collection and were able to produce colchicine-induced hybrids of several cuphea species. However, the only successful crosses were between species with similar fatty acid profiles. Lorey and Röbbelen (1984) were also able to create hybrids, but lacked any success in altering fatty acid content. More recently, Ray et al. (1988) developed 18 interspecific hybrids between eight different species by hand emasculation and controlled pollination. They are the first to report a successful cross between a capric and lauric acid accumulating cuphea species. Unfortunately, the crosses between *C. leptopoda* and *C. llaminuigera* were conducted on species not suitable for commercial production in the US. These results demonstrate the feasibility of developing a lauric acid accumulating interspecific hybrid using the capric species.

In 2001, a new cuphea breeding program was established at Western Illinois University (WIU) to address the introgression of lauric acid accumulation into the progeny lines for PSR23. The breeding program was initially supported by private industry and was highly encouraged to only employ traditional breeding methods for fear of losing certain commercial markets. This approach was supported by the promising results from Ray et al. (1988) which suggested a more traditional approach was warranted to help create an agronomically sound high lauric cuphea plant.

Cuphea species have a tremendous variation in chromosome numbers and ploidy levels (Graham and Cavalcanti 2001). The original PSR23 is an autogamous diploid with six chromosomes ($n = 6$). Selected wild accessions suitable for agronomic production are mostly diploid, but vary in number of chromosomes from 8 to 14. *C. lutea* ($n = 14$), *C. viscosissima* ($n = 6$), *C. tolucana* ($n = 12$), *C. wrightii* ($n = 22$), and *C. carthagenesis* ($n = 8$) are the autogamous species (Graham 1989a). *C. lutea* and *C. wrightii* are undoubtedly allotetraploids (Campbell 1987).

To help overcome the barriers in creating hybrids between the wild accessions and the PSR23 line, various breeding methods are being employed. Initial inter-specific crosses were conducted between PSR23 (PI 606544) and *C. lutea* accessions utilizing hand emasculation techniques and pollen mixing procedures as described by Ray et al. (1988) and Fehr (1987). Thirty two crosses were successful and were evaluated in the field in 2005. From these crosses, four progeny lines were selected for the 2006 growing season. Fatty acid profiles of these progeny indicated a slight increase in lauric acid (C12) from 2.9 to 4.9%, but a much larger increase in C14 (2.9–23.5%) and C16 (4.1–11.9%). Unfortunately, all four progeny lines were unstable and reverted to the original profiles.

To improve success of the interspecific crosses, seedlings of PSR23 were treated with a meiotic inhibitor, colchicine, to create colchiploids. The use of colchicine and other meiotic disruption chemicals have been shown to be successful in creating hybrids in cuphea and other species (Przybecki et al. 2001; NBRI 2003). Large scale plantings of PSR23 are mainly pollinated by resident bumblebees. The long floral tubes prevent honeybees from gaining access to the nectar. Commercial production of PSR23 will remain limited without solving the insect pollinator problem. It is hypothesized that by

increasing the ploidy level of the PSR23 not only will self-fertilization be increased (Barringer 2007), but the chances of developing interspecific crosses with the high lauric acid accessions would be improved. Successful ploidy level changes were identified by increased vigor and cytologically confirmed using pollen mother cell analysis techniques as described by Ray et al. (1989). Confirmed polyploid PSR23 plants were found to be fertile and were later crossed with high lauric acid accumulating accessions of *C. lutea*, *C. tolucana*, and *C. carthagenesis* in 2006. Seeds collected from each cross were excised and germinated to avoid any seed dormancy issues (Mathias et al. 1990). S_1 progeny were evaluated under field conditions in 2007. Fatty acid profiles indicated once again a significant increase in myristic acid (C14) with no increase of lauric acid (C12). Selection criteria for the S_2 progeny will be for self-pollination and increased lauric acid content. Hybrids with the highest seed and oil yield will be further selected to improve seed weight and total yield in subsequent years.

Along with developing lauric acid accumulating PSR23 progenies, the WIU program continually focuses on developing new varieties utilizing mass and recurrent selection for improving agronomic traits including increased vigor, stem rigidity, drought tolerance, and self-pollination.

19.5.2 Insect Resistance

With cuphea production limited in the US to the Midwest and upper Midwest, the adaptability of cuphea to the current crop rotation and production protocols of the region play a critical role in the success of growing this new crop. Proposing cuphea as a new broadleaf crop may provide an undesirable habitat for corn, soybean, and wheat pests. Research conducted by the USDA-ARS recently investigated the potential of utilizing cuphea in crop rotations to provide cultural control of Western corn rootworm beetles (*Diabrotica virgifera virgifera* LeConte). It was hypothesized that cuphea, because of its sticky surface, would reduce or prevent oviposition in these fields. Unfortunately after an intensive 4 year study with seven rotation programs, it was concluded that a crop rotation with cuphea would not provide any consistent, economical cultural control of corn rootworm (Behle and Isbell 2005).

From 2000–2005, no major insect pests were recorded as feeding on cuphea vegetation. The only known insect pests were flea beetles (*Altica* spp.) which could cause early season damage to young seedlings if not controlled. During the 2006 growing season, elevated corn earworm (*Helicoverpa zea* Boddie) populations were experienced in much of the Midwest including cuphea production research sites in central and west-central Illinois. Corn earworm larvae were observed feeding on cuphea leaves during August and early September and feeding shifted to flowers and seed pods as larvae counts increased through September. By crop harvest time in October, seed losses to corn earworm in IL were estimated at 90%, while sites elsewhere in the state experienced more than

50% seed loss. Minor losses were reported in Iowa, with only occasional sightings occurring in North Dakota, and no presence was reported in Minnesota. Effective insecticides are available, but generally require repeated applications and entail substantial monetary and environmental costs. Use of resistant crop varieties is generally acknowledged as the best core strategy for avoidance of corn earworm damage.

Evaluation plots of novel cuphea genotypes located adjacent to production fields in IL displayed a wide range of severity and timing of feeding losses; suggesting that genetic sources of elevated corn earworm feeding resistance may be available. A 2007 breeding project evaluated forty accessions representing 16 different species of cuphea for reduced larval feeding. The accessions were selected based on their agronomic potential in the Midwest, self-pollination, and diverse sticky hair structures. Preliminary results indicate larval insects have a preference for certain cuphea species and that several species demonstrated no larval feeding damage throughout the entire growing season. It is believed that the variations in the sticky hairs are contributing to the effective defense against insect pests. Aphids and many other insects are typically immobilized by the sticky hairs. A breeding program is already initiated to cross the identified resistant accessions with the original PSR23 line. In contrast, non-sticky mutants have been reported for *C. lanceolata* (Hirsinger 1980; Hirsinger and Röbbelen 1980; Knapp 1993). Although a non-sticky trait might prove useful in aiding harvest, the role of sticky hairs as a defense mechanism certainly outweighs the convenience in handling the crop.

19.5.3 Anthocyanin Mutants

During the summer of 2004, variations in flower pigments were noticed in PSR23 production fields in both MN and IL. PSR23 is characterized by deep purple flowers with red pigmented stems. Several novel plants were collected including a completely all white flower and variations of pink flowers.

The all white flower phenotype is devoid of any anthocyanin or red pigments throughout the entire plant (Fig. 19.3). Even under cold and nutrient stress conditions, the all white phenotype will remain. Named as ‘Snowflake’ in IL and ‘Blizzard’ in MN, both all-white lines are exhibiting inbreeding depression. After five self-pollinated generations in a growth chamber, the anthocyaninless phenotype appears to be stable. However, this line clearly demonstrates diminished vigor and seed set after each cross. When grown under field conditions, Snowflake performs well early in the growing season but soon collapses under environmental stress. Anthocyanins have been shown to serve as antioxidants and enable plants to better deal with environmental stresses (Chalker 1999). Efforts are currently under way to backcross Snowflake to the original PSR23 to develop an all white flower plant with anthocyanin production limited to the vegetative tissue. The all white flower is not only

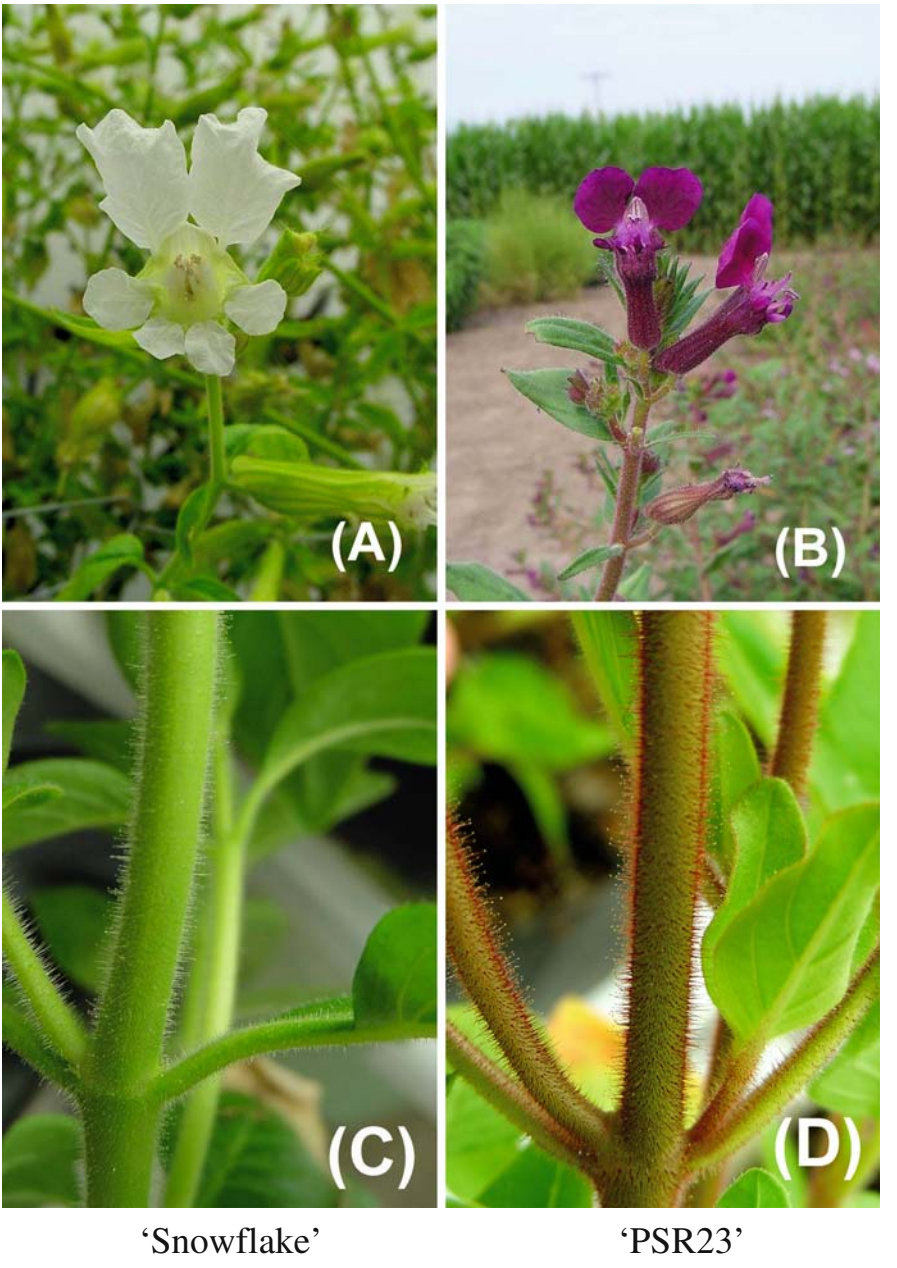

Fig. 19.3 Comparison of the all-white ‘Snowflake’ breeding line flower (**A**) and stem (**C**) to the anthocyanin rich ‘PSR23’ breeding line flower (**B**) and stem (**D**)

unique for ornamental applications but also appears to have improved seed retention and produces seed coats devoid of anthocyanin pigments. This could potentially play a role in developing seed oils with less pigment contamination, thus diminishing production costs.

19.6 Breeding Methods

19.6.1 Genetic Engineering

As with the development of any new crop, the time required to domesticate and develop new varieties utilizing traditional methods is extremely slow. Several research programs have investigated developing tissue culture methodologies for propagation and perhaps engineering new traits. Early work focused on explant propagation methodologies for *C. wrightii* (Janick and Whipkey 1986) and *C. tolucana* (Przybecki et al. 2001). Other studies began screening a wider variety of species for the potential of utilizing engineering techniques (Millam et al. 1997). However, it is currently unrealistic to pursue genetically engineering cuphea for large scale agronomic production. The most likely arena cuphea can serve is as a rich source of genes encoding enzymes specialized for seed-specific synthesis of short- and medium-chain fatty acids (Filichkin et al. 2006). If cuphea species fail to be commercialized, they can still potentially provide a diverse source of seed-specific genes for manipulating fatty acid content in other oilseed plants.

19.7 Concluding Remarks

Although the advancement of cuphea breeding has been slow, tremendous progress has been made in developing the basic understanding of cuphea physiology and agronomic production guidelines. The identification and development of new potential industrial products and biofuels from cuphea oils are also helping to increase the awareness of this unique oilseed crop. However, for cuphea to truly be successful as a new commercial crop grown on large scale volumes, continued industry support and the development of auto-fertile, non-shattering, and determinate plant lines are needed. I would certainly like to invite fellow breeders to take up the challenge and assist in advancing the breeding efforts to locate or create these much needed traits.

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