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Ajay Kumar Thakur
Pankaj Kumar *Editors*

Agricultural Biotechnology: Latest Research and Trends

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Dr. Prashant Bhatt

The book is dedicated to Dr. Prashant Bhatt, a renowned plant biotechnologist and mentor to many students, including one of the authors (DKS) and several leading contributors in plant biotechnology. Prashant's humility, kindness, empathy, and optimism were exemplary; his innovation and vision were inspirational; and his laughter and love of life were infectious and hard to ignore.

Born into a family rich in moral values and Ayurvedic knowledge, Prashant had a lifelong thirst for exploration and overcame all financial limitations. During his Ph. D. (1976) in Plant Developmental Biology from Gujarat University, he assembled a cytophotometer along with his junior colleagues, which won him the Hari Om Ashram Award for Young Scientists. During this time, he forged a lifelong partnership in science and at home with his classmate Daksha Mehta, who with her quiet, reasoning, calming presence countered Prashant's excitable personality.

Prashant was awarded the National Merit Scholarship from the Government of India to pursue studies abroad (1977–1981). He worked with an eminent developmental biologist Ian Sussex at Yale University, who planted the seeds of biotechnology in him. He later served as a Visiting Scientist at the USDA's Vegetable Breeding Laboratory in Charleston, South Carolina, for developing in vitro selection methods for herbicide resistance.

During his tenure as an Associate Professor at the MS University of Baroda, he teamed with Professor Atul Mehta making it a centre of excellence for plant tissue culture. Funding from CSIR and UGC, Government of India, attracted workshops at an international level and inspired young Indian scientists to pursue careers in plant biotechnology. He also conducted multidisciplinary research in collaboration with experts in Biochemistry and Horticulture, and taught courses at the Department of Microbiology and Genetics, introducing new curricula of somatic cell genetics.

With award of a Senior Fulbright Fellowship by USEFI, in 1986, he joined the laboratories of Professors David Hildebrand and Joe Chappell at the University of

Kentucky, with significant work on the regulation of HMG CoA Reductase, the key enzyme in the sterol biosynthetic pathway.

In the late 1980s, several tissue culture laboratories began opening up all over India, encouraged by the emerging need for quality products and new developments in horticulture. The Bhatt's joined one such company. Dr Bhatt, as a Vice President of Unicorn Biotek in Andhra Pradesh, set up the first commercial tissue culture company built with indigenous equipment and know-how in India. Under Prashant's expertise, tissue culture crops other than banana were introduced. Tissue culture Strawberry—limited to the cool and hilly areas could now grow anywhere: in non-hilly, non-temperate parts of India such as the arid regions of Gujarat.

Dr Bhatt's set up Sun Agrigenetics in 1999 in Vadodara, with the vision and mission to bring biotechnology to farmers' benefit in Gujarat. Sun Agrigenetics introduced tissue culture plants of a variety of crops beyond banana. Some of the 'firsts' tissue culture plants developed and commercialised were *Coccinea, Trichosanthes,* watermelon, lemon, Giant Reed. With his vision, Sun Agrigenetics became the sole company with dual recognition: NCS-TCP recognition as a commercial unit, as well as a DSIR recognised research centre from the Government of India. Development commercial protocols for elite date palm and red sandalwood were Dr Bhatt's dream projects, research grants from the Department of Biotechnology bringing them to reality. While Sun Agrigenetics was being set up, farmers in Gujarat were hardly aware of tissue culture and its benefits to the agriculture sector. With Prashant's efforts in directly interacting with farmers' groups on the field, 'tissue plants' soon became a colloquial term in the farmer community. Even while working in a commercial environment, Prashant emphasised education and continued to guide students through lectures, field visits, and advising research dissertations through Universities. Several research projects, a number of research articles of national and international repute, and elected life membership to the Plant Tissue Culture Association of India speak of his achievements. In 2015, he was awarded the Professor A.R. Mehta Memorial Lecture by the Indian Botanical Society, a fitting acknowledgement to his and Professor Mehta's long-term collaboration and friendship.

Prashant's legacy consists of the legion of students he trained, who now have their own trainees, as well as his two daughters and a grandson.

Preface

Biotechnology has emerged as an interdisciplinary science amalgamating molecular biology, genetics, biochemistry, microbiology, and chemical and process engineering. Advances in biotechnology in the last decade have resulted in the development of a number of powerful techniques, which have enhanced the well-being of human, environment, and society by targeting the use of various biological entities to result in useful products. The motivation to edit a comprehensive volume on current trends in biotechnology arose from the growing awareness of the recent advances in plant tissue culture, cell and molecular biology, genetics and applied breeding, biochemistry, food processing, and various "omics" technologies that all have boosted the pace of growth of this vibrant science. This book covers a wide range of applications of biotechnology. These include discussions on in vitro propagation technology, encapsulation technology, in vitro production of secondary metabolites, applications of somaclonal variations, and doubled haploids in agriculture, molecular breeding, and application of nanobiotechnology in agriculture. Separate chapters are dedicated to discussing the impact of various "omics" technologies including phenomics, genomics, proteomics, metabolomics, and bioinformatics in biotechnology research and their role in crop improvement. Some chapters describe the development perspective and acceptance level of transgenic crops worldwide. Separate chapters on RNA interference technology and miRNA-mediated regulation of biotic and abiotic stress responses in plants have also been discussed. Genome editing technology along with its probable applications has been discussed in detail.

This book is intended as a reference for plant/agricultural biotechnologists and plant molecular biologists. Also, it will serve as a comprehensive text for graduate and postgraduate level students in the department of plant biotechnology and plant molecular biology.

This book is the culmination of the efforts of several dignified researchers, scientists, and postdoctoral fellows who are very well-known and reputed figures in different frontiers of biotechnology research sector. We sincerely believe that this book will prove to be a useful contribution not only to science but also to the general public interest. We express our gratitude and appreciation to all the contributing authors who helped us tremendously with their time, critical thoughts, and suggestions to put together this peer-reviewed edited volume. While every effort was made to reach uniformity in style, the presented results and ideas and

organizational details of the chapters reflect the preferences of the respective authors. The editors are also thankful to the Springer Publication group and their team members for giving the opportunity to publish this book. Lastly, we thank our family members for their consistent support, understanding, encouragement, and patience during the entire period of this work.

Solan, India — Dinesh Kumar Srivastava
Bharatpur, India — Ajay Kumar Thakur
Solan, India — Pankaj Kumar

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About the Editors

D. K. Srivastava has retired as Director Extension Education, prior to this he worked as Professor and Head in the Department of Biotechnology, Dr. Yashwant Singh Parmar University of Horticulture and Forestry, Solan, Himachal Pradesh, India. He did his post-doctoral studies in the field of Plant Molecular Biology at the Institute of Molecular Genetics, USSR Academy of Sciences, Moscow, USSR and at Washington University, St. Louis, USA. He has 32 years of research experience and 29 years of teaching experience in the field of Plant Biotechnology and Molecular Biology. He had guided 32 students of M. Sc. and 9 students of Ph.D. for their research work. He has published 110 research papers in the journals of national and international repute, 38 research articles and contributed 12 chapters in the edited books, and also authored a book on biotechnology. He has delivered 45 invited lectures and attended 47 national and international conferences. His main area of research includes Plant tissue culture, Genetic transformation and Molecular characterization of plants. He is life member of various National and International Academic bodies/societies. He has received many awards for his scientific contributions including 'MS Swaminathan Award' by the Society of Plant Research, Meerut, India.

Ajay Kumar Thakur is presently working as Senior Scientist (Biotechnlogy) at ICAR-Directorate of Rapeseed-Mustard Research, Bharatpur, Rajasthan. He has obtained his graduation, PG and Doctoral degrees from Dr. Y.S. Parmar University of Horticulture & Forestry, Solan, H.P. He got induced into Agricultural Research Services (ARS) in 2008. He has published 40 research/review papers in various journals of International and National repute, authored one book 'Agricultural Biotechnology at a Glance', contributed 8 book chapters and 22 popular articles. He has developed high efficiency plant regeneration and genetic transformation protocols in a number of crops including *Populus ciliata*, *P. deltoides*, *Punica granatum*, *Capsicum annum* and *Cucumis sativus*. Dr. Thakur is associated with *Brassica juncea* improvement programme using biotechnological interventions from last 11 years. He has developed a core set of SSR markers for *B. juncea* genomics and is presently working on germplasm characterization and association mapping of various agronomically important traits in this oilseed crop. He has been granted with one Indian patent and associated in the development of a high yielding Indian mustard variety Giriraj, a white rust resistant Indian mustard genetic stock DRMR MJA 35, which is a *Moricandia* system- based cytoplasmic male sterile line of *B. juncea,* and a multiple disease (Alternaria blight, white rust and powdery mildew) resistant Indian mustard genetic stock, DRMRIJ 12-48. Dr. Thakur has received many awards from various societies and scientific organizations for his scientific contribution. He is also an elected Member of Plant Tissue Culture Association of India.

Pankaj Kumar is presently working as Assistant Professor (Biotechnology), Department of Biotechnology, Dr. Yashwant Singh Parmar University of Horticulture and Forestry, Solan, Himachal Pradesh, India. Formerly, he has worked as SERB- National Post Doctoral Fellow (DST Young Scientist Scheme) at Council of Scientific & Industrial Research - Institute of Himalayan Bioresource Technology, Palampur, Himachal Pradesh, India. Dr. Kumar is working on plant secondary metabolite enhancement through cell and tissue engineering

approaches; Identification of molecular cues linked with enhanced metabolites using comparative transcriptomics approach and also have experience on hydro-aero cultivation of medicinal plants for industrial importance. During Doctoral Studies; he (research team) has generated technology at Dr. Yashwant Singh Parmar University of Horticulture & Forestry Nauni, Solan for developing insect pest-resistant transgenic plants in economically important vegetable crops of cauliflower cv. Pusa Snowball, cabbage cv. Pride of India and broccoli cv. Solan Green Head (with *cryIAa* gene) for insect pest resistance. He has been awarded DST INSPIRE JRF/SRF Fellowship, Department of Science and Technology, Ministry of Science and Technology, Government of India for Ph.D. full doctoral program. He has qualified ICAR AICE-SRF (PGS), ICAR ASRB National Eligibility Test (NET). He has published 35 research/review papers in various journals of International and National repute, contributed 13 book chapters and 5 popular articles. Dr. Kumar has received many awards from various societies and scientific organizations for his scientific contribution i.e. Young Scientist Award Biotechnology-2018 by Society for Plant Research, Young Scientist Award -2019 by the Society of Tropical Agriculture, New Delhi, India, Excellence in Research Award -2019 by Agro Environmental Development Society, India. etc.

Commercial Micropropagation of Some Economically Important Crops

1

Daksha Bhatt

Abstract

Commercial tissue culture commenced in India in 1984 subsequent to the laudable classic work at University of Delhi using basic tissue culture for studies in developmental biology commenced way back in the 1950s, and its importance gradually recognized for application in crop improvement. Since then it has been a trend of 'rise and fall, and rise again' till date. The total tissue culture plant production in India is currently estimated to be 300 million plants per annum, of which banana shares approximately 50%. Scores of species in fruit, vegetable, floriculture, forest, plantation, medicinal, and aromatic plant categories are now produced through micropropagation. Next to banana, sugarcane and ornamentals, other crops such as pineapple, pomegranate, lemon, ivy gourd (*Coccinea indica*), pointed gourd (*Trichosanthes dioica*), and teasle or spine gourd (*Momordica dioica*), turmeric, and ginger are now commercially produced through micropropagation in India. There are challenges for the micropropagation industry producing banana and other plants in general, with many tissue culture units facing severe economic loss ending up in non-performance or closure. Some aspects of the problems are discussed in detail.

Keywords

Commercial micropropagation · Banana · Cucurbits TC · Micropropagation challenges

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1.1 Introduction

The laudable efforts by Late Professor PM Maheshwari in year 1950 for using tissue culture techniques for basic studies are the keystone for modern plant biotechnology in India. The discovery of haploids in tissue culture by Guha and Maheshwari (1964) became a global landmark in the field of developmental biology. It was later in the 1960s when another tissue culture laboratory was set up at the MS University of Baroda, where apart from developmental studies, practical aspects of tissue culture began to be considered under the able guidance of Late Professor Mehta. Plant tissue culture remained limited to an academic interest until its commercialization was incepted in early 1984, with *A.V. Thomas Co.* beginning cardamom tissue culture (TC) for domestic use. The stage was set by them for ornamental crops, produced for export purpose. Very soon other companies like *Indo American Hybrid Seeds* joined the scene to produce tissue culture plants of ornamentals and also initiated work on banana tissue culture. Ornamental plants remained at the fore-front then with an idea to reach the European export markets. No one would have perhaps had the hint at that time that horticulture crops were beckoning, calling for a very different picture decades later for commercial micropropagation in India.

1.2 The Beginning

Several commercial plant tissue culture (PTC) units were soon set up in the later part of the 80s, with imported equipment and technology collaboration. The banana was the first candidate among the many horticultural crops that were identified. The first indigenous commercial laboratory was set up in Hyderabad with in-house technology development at *Unicorn Biotech Ltd* in 1987 under the guidance of Late Dr. P. Bhatt. During this period, more than 50 commercial laboratories were set up, with banana and sugarcane as the main crops and a high total installed capacity of around 210 million plants per annum. Department of Biotechnology (DBT), Government of India (GOI), set up two Tissue Culture Pilot Plants in 1989, one of them being at TERI's in Haryana. Within the next 2 years, the number of commercial units went up to 74. In Maharashtra, 25 units were set up. There were nine PTC units each in Karnataka and West Bengal, 6 units in Andhra Pradesh, 4 each in Tamil Nadu and Kerala, 3 each in Gujarat, Haryana and UP (Govil and Gupta 1997). At that time around 75 species in ornamental (foliage plants, flowering plants), fruit, vegetable, plantation crops, and forest tree species were produced by these laboratories.

Resistance to accepting tissue culture plants by small growers, inconsistencies of production know-how resulting in poor performance, narrow product range, lack of trained manpower and infrastructure were the chief constraints that these leaders initially faced resulting in fluctuated outputs and capacity utilization gradually dropping to around 50%. For them, there were no guidelines to follow. The scenario gradually changed in later years after 1991 when GOI identified micropropagation of plants as an industrial activity under the Industries (Development and Regulation) Act of 1951, which gave a boost to the commercialization of plant tissue culture

(PTC). In 2005, only 45 PTC units were functioning with production volumes of 30 million plants per annum (Shukla et al. 2012). GOI, through DBT, formed the National Certification System (NCS-TCP) in 2006 to establish a structured system for production and distribution of quality planting material, and to regulate its commercial production. Since its inception, there is a significant impact on the quality of plants produced, as evident from the fact that there has been no major virus outbreak (DBT 2016). With these guidelines and intervention by the GOI, again there was a rise in the production of tissue culture plants (TCP) mounting to a capacity to 300 million plants per annum in the year 2012. In 2016, there were 100 NCS-TCP recognized units; with units not yet recognized, the aggregate score was 200. The actual production of TC plants was 350 million in total installed capacity of 500 million (Shukla 2017). Thus, the history of commercial tissue culture in India is a story of 'rise and fall, and rise again' as quoted by Prakash (2006).

1.3 Present Scenario

In the past two decades, the plant micropropagation industry has made a very strong presence in line with other biotech industries. As of date, there are 83 NCS-TCP recognized production units spread across the country. Out of these, the first four states: Gujarat, Maharashtra, Tamil Nadu, and Karnataka house 51 units, that is, 61% of total PTC units (Fig. 1.1). Capacity wise, some PTC units are large with installed capacities of around 1–3 million plants/annum. Jain Irrigation Ltd. set up their PTC unit in 1995—today it is the largest PTC unit in the world with a production capacity of 70 million plants per annum producing banana, pomegranate, and strawberry. Others have smaller capacities between 0.5 and 1 million. In 2016, the gross installed production capacity was about 500 million plantlets per annum, as of the date this number would have increased by 25%. The industry witnesses an actual production of approximately 350 million plants (DBT stakeholders meet 2016). Banana, potato, sugarcane, apple, pineapple, strawberry, gerbera, anthurium, lilium, orchids, bamboo, date palm, teak, and pomegranate are some of the major plants tissue cultured in India. Cardamom TCP covers 500 ha and vanilla covers 50 ha among the spice group plants (Anis and Ahmad 2016). Apart from this, turmeric, ginger, lemon, pointed gourd, ivy gourd, spine gourd, and papaya are new additions to the product list of TCPs.

The role of various plant tissue culture techniques in new opportunities for its contribution towards crop improvement, productivity, and food security has been reviewed by several workers. In the earlier years, ornamental plants were the main TC product of some of the commercial companies in trend with the global market, and this was reviewed in detail by Govil and Gupta (1997). Prakash (2000) enumerated the factors influencing the development of the micropropagation industry in India and explained some management and planning issues that hampered further growth of the industry. Later on, they analysed the opportunities and

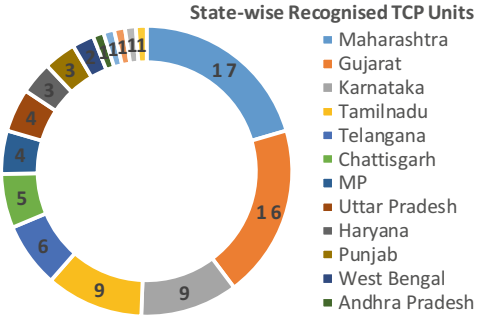

Fig. 1.1 Present state-wise distribution of NCS-TCP recognized PTC units (DBT 2020)

problems faced by the industry (Prakash 2012). Recently, Ragavendran and Natarajan (2017) presented an extensive review of TC in all crop species for fast multiplication, the introduction of new traits in crop plants and the development of crops resistant to biotic and abiotic stress, signifying the value of this technology in food security at a global level. Anis and Ahmad (2016) highlighted that in order to make commercialization successful for any crop, the technology should be reproducible and the system must have the least chances of genetic instability. Kumar (2017) in his review has focused on banana PTC industry in particular, mentioning the need to reduce the demand–supply gap with better marketing policies and better distribution of certified quality plantlets to farmers. Gulzar et al. (2020) described in detail various applications of plant biotechnology for genetic improvement and illustrated the viability of transgenic methods for clonal production of valuable germplasm. Whereas many of the reviews and reports signify opportunities and applications of PTC and results are mainly limited to laboratory, pilot-scale or field trials, a clear picture does not emerge on its further application by the PTC industry in terms of absolute volumes produced for each crop and what is the current trend in commercialization, barring the TC banana. In the absence of authentic information, it is only an estimate that can be made on total production for each type of product based on market information and personal communication with concerned people. Based on this, the major producers (albeit not limited) of PTC in the country are shown in Table 1.1.

Among banana, Grand Naine, a popular variety grown mostly in all export-oriented countries of Asia, Africa, South America, is a superior selection of Giant Cavendish which is introduced in India since 1990. Due to many desirable traits like excellent fruit quality, immunity to fusarium wilt, it has proved a better variety (Singh & Chundawat 2002). India being the largest producer of banana, and the need for large volumes of planting material, it is not surprising that it was the first horticultural crop to be commercially produced through TC. The first PTC banana was incidentally sold at a price twice the current rate in the 1980s by Indo American Hybrid Seeds when the very first TC banana was produced. As evident from

Table 1.1 List of NCS-TCP recognized units (DBT 2020), with total production volumes (wherever available) in the past year, and some non-recognized units (personal communications)

State	Total recognized units in state	S. no.	Name of unit	Products	PTC units not recognized	Products
Andhra Pradesh	1	1	Micco Laboratories Pvt. Ltd.			
Bihar	1	2	Hecure Agro Plants Pvt. Ltd.			
Chhattisgarh	5	3	Aditya Biotech Lab & Research Pvt Ltd.	Banana, Jatropha, Acacia, Eucalyptus, gerbera, sugarcane, bamboo		
		4	AKF Plant Sciences Pvt Ltd.	Banana, ginger, pomegranate	Mahaveer Iron and Steel Ltd. (plant tissue culture division)	Banana, gerbera, ornamentals, strawberry
		5	COE AIB Tissue Culture Lab, IGKV			
		6	Devleela Biotech	Banana, bamboo		
		7	Yash Biotech, Raipur	Banana, bamboo, sugarcane		
Gujarat	16	8	ABC Agrobiotechnology Pvt. Ltd.	Banana, lemon, teak, guava, pomegranate, potato, bamboo	Vasundhara biotech	Banana
		9	Aranya Agri Biotech LLP	Banana, pointed gourd, ivy gourd, teasle gourd, lemon, pineapple, fig, pomegranate	Kalpataru Agro Biotech	Banana
		10	Greenfield Biotech	Pointed gourd, banana, *Coccinia*	Sri Ratnam Biotech,	Banana
		11	IRM Enterprises Pvt. Ltd. (formerly Cadila		Shri Abhimanyu Biotech	Banana

(continued)

Table 1.1 (continued)

State	Total recognized units in state	S. no.	Name of unit	Products	PTC units not recognized	Products
			Pharmaceuticals Ltd.-Agro Division)	Banana, potato, lily bulb, foliage, pointed gourd, teak, sugarcane, pomegranate		
		12	Kutch Crop Services Ltd.	Banana	Ambika Agro	Banana, pointed gourd
		13	Metrogen Biotech		Gangamani Agri Biotech	Banana, pomegranate
		14	Natural Life Sciences	Banana, potato, sweet potato, fig, lemon, pineapple, pointed gourd, ivy gourd		
		15	PAC Bio Fungbact Pvt. Ltd.	Banana		
		16	Palaj Agrotech	Banana		
		17	Sarjan Biotech Pvt. Ltd.	Banana, pomegranate		
		18	Shaili Biotech (P) Ltd.	Phormium, dracaena, banana, sarracenia, alpinia dionaea, lily nepenthes		
		19	Siddhi Plantek	Banana, bamboo pomegranate, potato, pointed gourd, lemon		
		20	Shree Ganesh Khand Udyog Sahakari Mandali Limited	Sugarcane, Banana		
		21	UMA Biotech Industries	Banana		
		22	Vasundhara Agribiotech	Banana		
		23	Vitrigold Biotech Pvt. Ltd.	Banana		

Haryana	3	24	Sheel Biotech Ltd.	Banana, citrus, apple, gerbera,, lilium, anthurium, gladiolus, bamboo, eucalyptus, poplar, Yucca		
		25	Technico Agri Sciences Ltd.	Potato		
		26	Tata Energy Research Institute (TERI)	Sandalwood plants, teak (Sagwan) plants, red sandalwood, bamboo, banana, sugarcane, potato, papaya, pineapple, strawberry, turmeric, ginger, syngonium, ficus, spathiphyllum, anthurium, cordyline		
Himachal Pradesh	1	27	Nishant Biotech	Apple, cherry and pear rootstocks, kiwi		
Karnataka	9	28	Biotechnology Centre, Dept. of Horticulture		EK Plant technologies	Ornamentals
		29	H.U. Gugle Biotech Pvt. Ltd.	Banana	Excel Plant Link Pvt. Ltd.	Banana, pineapple, ornamentals, carnation, gerbera, gypsophila, strawberry, phalaenopsis
		30	Hybrid Agri Biotech Pvt. Ltd.	Banana	Meghana Tissue Culture Nursery	Banana
		31	Jagadamba Bio Plants	Banana, teak, pomegranate, date palm, potato, ornamentals, medicinal, bamboo. cardamom, vanilla	Unique Plant Tech and Nursery, Karnataka	Banana, ornamentals, medicinal plants

(continued)

Table 1.1 (continued)

State	Total recognized units in state	S. no.	Name of unit	Products	PTC units not recognized	Products
		32	K F Biotech Pvt Ltd.		Aditya biotech	
		33	Mysore Organic Farms Pvt. Ltd.	Banana		
		34	Novel Biotech			
		35	Shanthi Agrotech	Banana		
		36	Sree Adithya Biotech			
Maharashtra	17	37	Ajeet Seeds Ltd.	Banana	A1 Biotech	Banana and house plants
		38	Beejsheetal Research Pvt Ltd.	Banana		
		39	Biosis Plants Pvt. Ltd.	Banana, strawberry	Bhoomiputra Biotech	Banana
		40	Geeta Agro Biotech	Banana	Jain Irrigation Systems Ltd.	Banana, pomegranate, strawberry
		41	H.U Gugle Agro Biotech Co.	Banana		
		42	Ishved Biotech Pvt. Ltd.	Horticultural, ornamental and floriculture plants	Ecofriendly Biotech and Bioplants Pvt. Ltd.	Banana, gerbera, carnation
		43	Janani Biotech and Tissue Culture Lab		Shri Ganesh Biotech	Banana
		44	K.F. Bioplants Pvt. Ltd.	Gerbera, rose, carnation, lily strawberry, limonium, alstroemeria, gypsophila, rananculus, phalaenopsis	Kisan Agri Biotech	Banana
		45	Kimya Biotech Pvt. Ltd.	Gerbera, carnation	Nirmal Seeds P.Ltd.	Banana, pomegranate

		46	Kshitij Biotech Corporation	Banana, ginger, date palms, turmeric	Patil Biotech	Banana
		47	Mahabeej Biotechnology Centre			
		48	Namo Bioplants			
		49	Ram Biotech			
		50	Rise N Shine Biotech Pvt. Ltd.	Banana, gerbera, carnation, orchid, chrysanthemum	Callus Biotech	Banana, Santalum
		51	Seven Star Fruits Pvt. Ltd.			
		52	Thopte Biotech Pvt. Ltd.			
		53	Vasant Tissue Culture Laboratory	Banana		
MP	4	54	Reva Flora Culture	Bamboo, Banana, pomegranate, teak, lemon, etc.	ITI Biotech Tissue Culture Lab	*Banana, Roses, Stevia, Gerbera, Anthurium, Parwal*, guava, Citrus, strawberry
		55	Sachdev Nursery	Pomegranate, lemon, guava, orchid, banana, sugarcane	Bhoomi Biotechnology Venture for Research and Development	Pomegranate, Banana, bamboo
		56	Shri Mukund Biotech			
		57	Tirupati Fresh Agro Crop Science Pvt. Ltd.	Banana, pomegranate, sugarcane		
Odisha	1	58	Excel Plant Link Pvt. Ltd. (UNIT-II)			
Punjab	3	59	Bhatti Tissue Tech.	Potato	Bharat Agritech	Potato
		60	Mahindra Hzpc Pvt. Ltd.	Potato, banana		

(continued)

Table 1.1 (continued)

State	Total recognized units in state	S. no.	Name of unit	Products	PTC units not recognized	Products
		61	PepsiCo India Holding Pvt. Ltd.			
Rajasthan	1	62	Atul Rajasthan Date Palms Ltd.	Date palm		
Tamil Nadu	9	63	Annai Meenashi Biotech			
		64	Genewin Biotech	Syngonium, banana, philodendron xanadu, orchids, vanilla, gerbera, bamboo, aloe, pomegranate		
		65	Growmore Biotech Ltd.	Over 50 crops: fruit crops, ornamentals, commercial, trees and medicinals		
		66	Hi-Fi Biotech India Private Limited	Banana		
		67	Hosur Hortitech	Banana		
		68	Jay Blossoms Bio Tech	Banana		
		69	Jayasree Biotech, Hosur	Banana		
		70	SPIC Agro Biotech Centre	Banana, gerbera		
		71	Sree Visal Biotech			
Telangana	6	72	ACE Agro Technologies	Banana, date palm	Greeno Agrotech India	Banana, pomegranate, fig philodendron, xanadu, cordyline, teak, bamboo
		73	Agri Vitro Tech Laboratories	Banana, Gerbera		Teak and others

					Shivshakti Biotechnologies Ltd.	
		74	Atlantis Phyto Tech	Banana	Sri Soam Biotech	Banana
		75	Kisan Agri Biotech, Hyderabad		Sri Venkateshwara Agro Technologies	Banana
		76	Microsun Bioplants (India) Pvt. Ltd.	Banana	Tulasi BIotech	
		77	Rodasy Biotechnologies	Banana	Visal Agri Biotech	
Uttar Pradesh	4	78	Dr. MC Saxena Group of Colleges	Banana		
		79	Hindustan Bioenergy Ltd.			
		80	Sagar Agrisciences Pvt. Ltd.	Banana		
		81	Sagar Agrisciences Pvt. Ltd. (UNIT-II)	Banana		
West Bengal	2	82	Elegant Flower Company Pvt. Ltd.	Potato, banana	Sristi Agro Biotech Pvt. L. (W Bengal)	Banana, house plants, flowers, strawberry, bamboo, vanilla, cardamom, rhododendron
		83	Pallishree Ltd.	Banana, pineapple, gerbera, orchid, syngonium, philodendron, Xanadu	Synergy Agri Products P. Ltd. (W Bengal)	Banana, alocasia, calathea, yucca Cordyline australis atro, elephantipes
Kerala					AVT Biotech	Banana, leek, wasabia, ornamentals
					Greenvalley Biotech	Banana, sugarcane, ornamentals

statistical data of the past 5 years, the area under production of banana is on a continuous rise (Tables 1.2 and 1.3).

The estimated production of banana in year 2016–2017 was 31.08 million tons (NHB 2017), which rose to 3.7% higher to 31.17 million tons in the subsequent year, and 10% higher than the previous 5 years' average (Press Information Bureau 2019). Thus, there is a steady increase in production as well as productivity within the country. India contributes up to 26% of total world production of banana having higher productivity than the world average, but stands lower compared to the productivity of Indonesia (Fig. 1.2). Within the country, six states (Gujarat, Tamil Nadu, Andhra Pradesh, Gujarat, Maharashtra, and UP) contribute 2/3 of the production from ½ of total area (Fig. 1.3). Commercial cultivation of banana is not only limited to the plains in the major banana growing states. TC banana has also reached the States of Jammu (Jeet et al. 2019), as well as in Meghalaya (Fig. 1.4a), from where the local clones of Hill banana were procured, multiplied through micropropagation and cultivated (Meghalaya SFAC 2014); Dir of Horticulture Meghalaya, personal communication).

With total area under banana cultivation of 859,000 ha, 2.546 billion plants are required at the rate of 2964 plants/ha. The current total annual TC banana plant production is estimated (Table 1.1) to be around 150 million. Out of this, the four states in South India- Telangana, AP, Karnataka, and Tamil Nadu produce 11–14 million banana each year (Rao TVLN, Mane R, personal communication). Report from Rabobank (2019) gives an official estimate of TC banana production in India up to 80 million plants which would be about 3% of total requirement of planting material. In another publication (APAARI 2019), it is stated that 17% of banana plantations in India are TC derived. In this case, TC banana produced would be 432 million. If this estimate is correct, the un-recognized labs are producing a much larger volume of TC banana than those that are DBT recognized. All these are calculated estimates, but it is clear that the market share of TC banana plants stands in-between 3 and 17%. The Department of Biotechnology in India expects the annual growth in demand for tissue-cultured banana plants to increase at a rate of 25%.

Whatever are the statistics, being no more than estimates or assumptions, it is very clear that we are still lagging in productivity and the need for quality planting material inter alia *is* still looming over on modern horticulture, as brought to our attention from frequent reports from National Research Centre for Banana and other publications. The productivity of banana in India is higher than world average, like in few other crops (Fig. 1.2), but still lower than world's best productivity. A significant control has been brought over due to disease free TC banana and the dominance of Cavendish banana which are resistant to *Fusarium oxysporum* f. sp. *cubense*. A new threat has arisen since the past couple of decades from a new race of *F. oxysporum, called* TR4. Its presence is confirmed in Asia, South Africa, and Australia. FAO (2020) goes to state that further spread of TR4 would have a significant impact on global banana production and business. It can infect and destroy plants that were resistant to the older races of *Fusarium.* This includes Cavendish and other varieties as well as plantains. So far, no methods of eradication

Table 1.2 The area under total cultivation of some crops (only those crops where commercial TC production is done are included) in India (NHB 2018)

Area of cultivation (million ha)	Year				Production ('000 MT)			
crop/year	2014–2015	2015–2016	2016–2017	2017–2018	2014–2015	2015–2016	2016–2017	2017–2018
Banana	0.822	0.841	0.860	0.884	28.1	29.1	30.4	30.8
Potato	2.14	2.12	2.18	2.14	51.3	43.4	48.6	51.3
Pineapple	0.10	0.11	0.11	0.103		1924	1861	1706
Lemon	0.268	245	248	286	2950	2438	2364	3148
Papaya	0.115	0.132	0.134	0.138	4913	5667	5940	5989
Pomegranate	0.181	0.197	0.209		1789	2306	2613	2845
Turmeric	0.184	0.197	0.216	0.234	830	943	1052	
Parwal	NA	0.018	0.018	0.020	NA	18	18	20
Other gourds				0.110				660

Table 1.3 Estimates of production and productivity in various crop categories for 2018–2019 and advance estimates for 2019–2020 (GOI Press Release 2020)

Crops	2018–2019		2019–2020 (first advance estimate)		2019–2020 (second advance estimate)	
	Area (in '000 ha)	Production '000 MT	Area (in '000 ha)	Production '000 MT	Area (in '000 ha)	Production '000 MT
Fruits						
Apple	308	2316	308	2734	308	2734
Banana	866	30,460	875	29,649	878	31,504
Citrus						
Lime/lemon	305	3482	314	3547	317	3717
Guava	276	4253	286	4345	287	4304
Pineapple	104	1711	108	1781	107	1799
Pomegranate	253	2915	264	2329	261	2315
Strawberry	1	5	1	8	1	8
Others	248	2298	255	2281	255	2268
Total fruits	6597	97,967	6660	95,743	6664	99,069
Vegetables						
Parwal/pointed gourd	55	757	55	741	56	760
Potato	2173	50,190	2149	51,947	2158	51,300
Sweet potato	110	1156	116	1194	116	1186
Tapioca	163	4976	139	4046	164	5043
Others	1441	21,118	1519	21,156	1504	22,962
Total vegetables	10,073	183,170	10,292	188,009	10,353	191,769
Aromatics and medicinal	627	795	634	822	628	798
Flowers cut		647		688		762
Flowers loose	303	2263	294	2186	305	2301
Total flowers	303	2910	294	2873	305	3063
Total plantation	3872	16,350	3866	16,412	3887	16,240

Spices						
Cardamom	81	23	79	26	79	26
Garlic	358	2910	356	2862	363	2917
Ginger	164	1788	168	1805	172	1844
Vanilla	0	0.1	0	0	0	0
Saffron	3	0	3	0	4	0
Turmeric	253	961	239	913	246	939
Total spices	3960	9428	3866	9372	3824	9420
Total	25,433	310,738	25,611	313,351	25,661	320,479

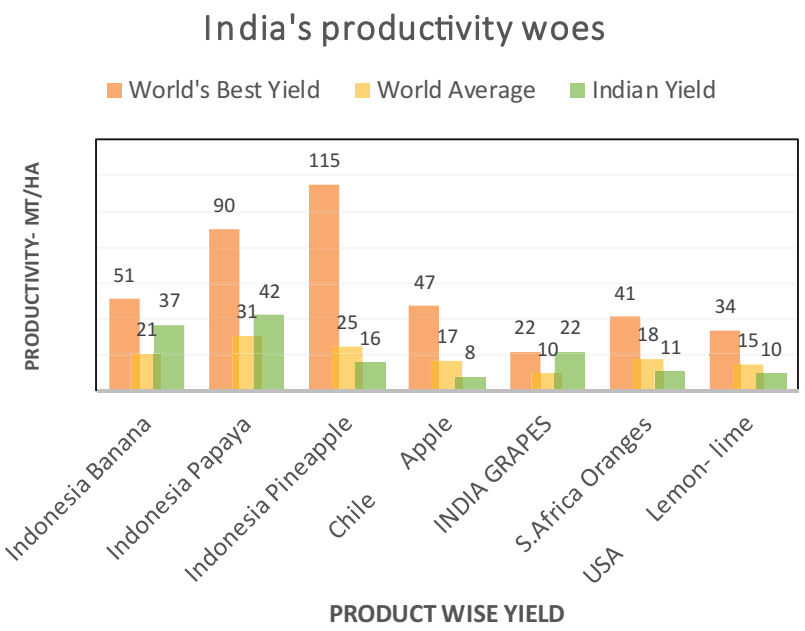

Fig. 1.2 Crop yields in major countries with maximum and average yields

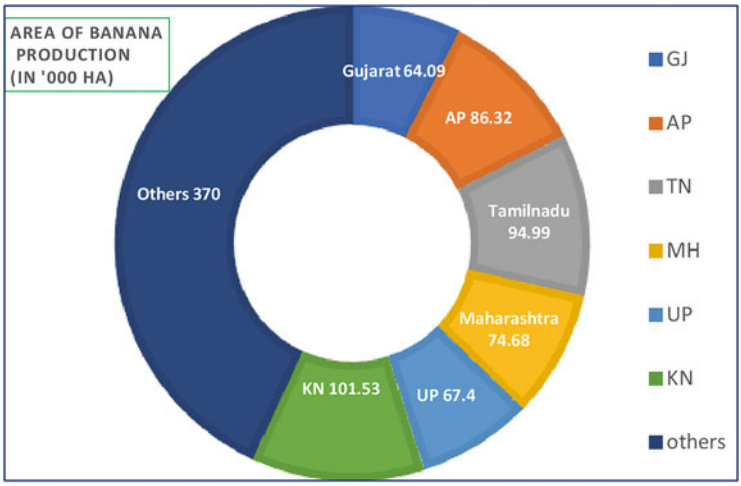

Fig. 1.3 State-wise area under cultivation of banana (in '000 ha) in different states of India (Data from NHB 2017)

are available (Hwang 2001). Approaches have been made to look for resistance in wild genotypes or by mutation breeding with some success (Smith et al. 2006). Somaclonal variation has been exploited in micropropagated plants for various

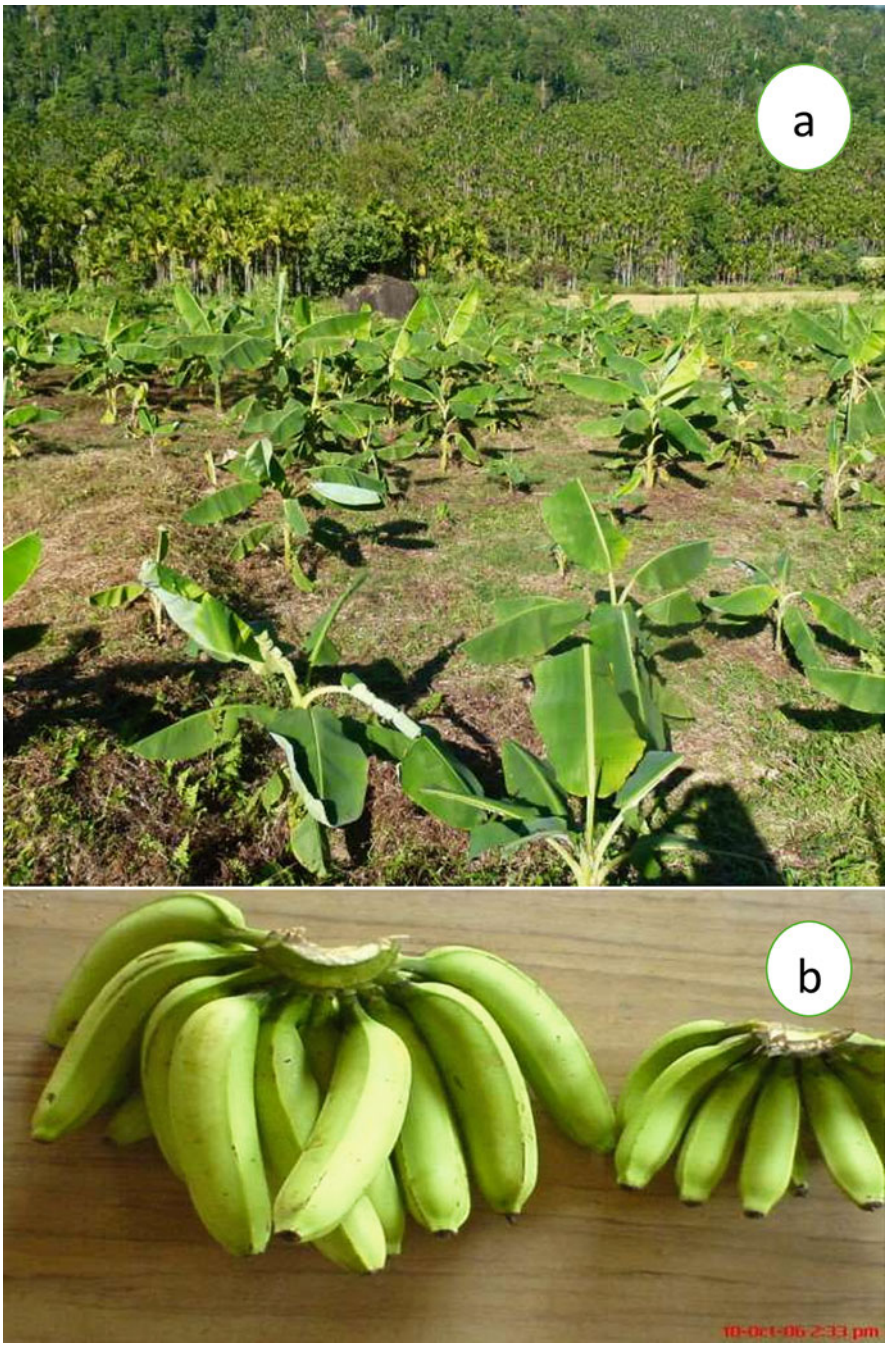

Fig. 1.4 Commercial micropropagation of selected banana varieties: (**a**) TC Hill banana plantation in Meghalaya; (**b**) Grand Naine Banana fruits from salt tolerant selected plants (right) and normal plants grown under 1400 ppm saline conditions (left)

useful characters by several groups. For example, Hegazy et al. (2012) screened 40,000 in vitro G9 banana plants reporting 12 off-type groups with different morphological characters. The role of commercial micropropagation units would be now to look for such variants showing high degree of resistance to TR4 among our local genotypes and ensure its commercialization via micropropagation. In absence of any effective genes available for routine genetic transformation for fusarium wilt resistance in banana, the selection of somaclonal variants or mutants from breeding work is the only way to improve banana varieties. Such work is already under way in Australia with extra-dwarf Cavendish cultivar called Dwarf Parfitt (Smith et al. 2006). A unique example of somaclonal variation from TC plants is development of TR4 resistance in plants distributed by TBRI in Taiwan and registered under the name GCTCV-218. This variant is now commercialized under the name *Formosana* (Vézina and Van den Bergh 2017*)*. In India, the occurrence of TR4 fusarium wilt has already been found in Bihar, UP, MP, and Gujarat. (Agri Business 2018). Hence it is an urgent need for researchers in our country to work on available methods and develop super clones that would be TR4 resistant and make them available for commercialization.

Apart from TR4 resistance, there is a scope for selecting plants for tolerance or resistance to abiotic stresses such as frost, drought, and salinity tolerance, so that banana can be cultivated with higher productivity in the diverse climatic conditions within the country. Banana germplasm from the North East States of India had been suggested as a source in breeding programmes as it is rich in B genome, which is the source of resistance to different diseases (Mustaffa and Sathiamoorthy 2003), and for same reason, it could be a good source of mother plant selection in micropropagation programmes pertinent to local region, rather than the introduction of commercial variety like G9. An early effort by Ulisses (2000) for in vitro selection for salt-tolerant banana variety Nanicao did not go beyond selection methods using a range of salt levels and survival rates of cultures. Similarly, Bidabadi et al. (2012) screened EMS-treated banana mutants for stress tolerance using PEG, but results were reported only up to culture level for various levels of stress tolerance.

Recurrent selection for salt tolerance using a combination of natural selection and somaclonal variation was attempted in vitro (Bhatt and Bhatt 2007a, b) in banana variety G9. Selected plants were grown in traditional banana growing areas of central Gujarat where there is recent ingression of seawater increasing the soil salinity between 1400 and 1600 TDS. Here the normal plants either do not survive or they yield poorly, producing a very small bunch (Fig. 1.4b). Successive trials for three consecutive years in the farmer's field gave very satisfactory results in this region with normal fruit quality and yield. Such efforts for resistance or tolerance to abiotic and biotic stresses pertinent to our nation should be taken up more aggressively using any of the modern crop improvement methods. Most commercial PTC companies neither have the requisite allocation of funds for R&D work nor do they have expert team of scientists. The research institutes through valuable research generate plants in few numbers with unique or improved characteristics but mass scale production is not in their purview. As a result, the potential remains unexploited. A fruitful collaboration between research institutes and industry has been

suggested since long and it is high time now that they bring their heads together to take useful research to commercial heights.

1.4 Challenges for Commercial Micropropagation Companies

The rapid rise in a number of PTC labs and a substantial upsurge in production volumes has not occurred without any challenges and risks faced by the commercial PTC laboratories (Table 1.4).

The initial idea of setting up large micropropagation units somehow has not gained pace. Excepting a few, several large labs downsized their production or have shut down. Smaller new PTC units spring up and even before they appear on the marketing map, their existence is wiped off. The trend of small and medium sized

Table 1.4 Challenges in commercial micropropagation industry and methods to overcome the risks (Bhatt 2018)

Challenges	Measures to overcome the risks
High cost of setting up and functioning of a PTC unit	Must work in a step-up phase wise manner to avoid high initial investments
Lack of trained manpower for production at several levels	Skilled workers, trained supervisors, managerial staff are mandatory
Long term crop	Be prepared for a long journey, have a good product mix
Technical issue like contamination/spoilage	Stringent monitoring of production with proper standards of clean room facility
Breakdown of machinery/power supply	Continuous power supply with backup is needed. Although manpower is the main machinery, proper maintenance of all equipment should be done
Sustainability of business will be at stake due to lack of innovation	In situation where same product, variety or process continued leads to failure, a strong research/innovative effort has to be made
Unassured market	A thorough study of market requirement needed
Long gestation period for returns	Keep in mind long gestation period while doing financial planning, and make provisions accordingly
Expiry of TC plants, unsold volumes, dependence on season and weather vagaries is a challenge	Proper business planning, flexibility, and prompt decisions are required by management to make production and sales happen on time
Market entry and brand creation, novelty of product	Be there when and where needed and create a brand image of product. Novelty or value addition to a product requires strong R&D plus marketing support to production team
Quality products for best field performance	Comply and adhere to production standards recommended by DBT and do in-house QC measures

production units with production capacity of 1–5 million plants was set beginning this millennium. Looking at the number of DBT recognized units over recent years, we found that present number of 83 is not much more than 70 labs in 2008. The current scenario is again changing. The large and medium production units are downsizing their capacity, but associating with smaller TC units for contract production or trading plants from other laboratories to meet with their sale targets. This is the reason why the official figures of volumes produced and sold by the NCS-TCP recognized companies, particularly in case of banana stand to be much lower than the actual and total production volumes. Hundreds of small 'homestead' TC labs have sprung up across the country, run mostly as a family business. One may feel that without the official gear of quality monitoring, the quality of plants that they produce would be inferior. The fact is that hidden in remote areas and unknown to media, their performance may not be underrated. With low profile, minimum running costs and an assured outlet to larger corporate labs, they flourish. This is a classic example of hi-tech cottage industry for banana which can be compared with similar role played by the farmers in Vietnam in the tissue culture production and supply of potato seed plantlets. This is frequently cited as a model of effective, decentralized, small-scale biotechnology (Joffe 1993). As long as the mother plant selection is ideal, and the production procedures are correct, the bigger units will have no issue in having quality plants delivered to the growers.

As is evident from the available data (Table 1.1), majority of the commercial units except those producing potato and ornamentals have their focus only on banana. This is a seasonal crop and traditionally its major planting is done during June to August each year using suckers during the favourable monsoon period to achieve high survival rates. Theoretically, it is true that TC plants can be planted round the year, yet it has not changed the farmer's mindset significantly and there remains a high demand for plants during monsoon. The maximum market demand of this fruit during July to September corresponds to high consumption of banana among certain communities and religions in the festival season. This is another reason why the farmer is tempted to stick to traditional planting schedules such that the crop can be harvested during the period of heavy consumer demand. With this market scenario, a typical TC lab targets to produce over 80% of its banana volume such that it can be sold within 3 months during the pre-monsoon and peak monsoon period between June to August. Due to perishable nature, nurseries cannot stock the plants for more than several weeks, as over-grown plants are not acceptable. This leads to much pressure for space and availability of manpower in the laboratory during the final stages of rooting and culture growth before they all end up for hardening stage, lagging behind in their target. As a result, a substantial fraction of plants remains unsold at the end of the season in many nurseries as they are not ready for field planting on time as 'Finished Goods'. Even with the huge demand of banana plantlets during the planting season, many banana TC producers cry their woes 1 year or another.

The optimum growth conditions required during primary and secondary hardening are another hurdle and an expensive affair during the soaring summer temperatures and low humidity during summer months in the plains across most

of the country. The period of maximum activity in the laboratory also clashes with production of mother cultures for the next season, adding more burden for the laboratory. Most laboratories face shortage of manpower during this peak period of production between February and June. This becomes aggravating especially in the laboratory culture production since it is dominated by the trained workers. This is an industry where the trained hands and a high level of patience is required to do repetitive cuttings and only trained workers can achieve the targets. It does not give you a choice of 'extras or replacements' who can be picked up at the factory gate each morning like in other industries. A production manager's role becomes very crucial in maintaining a steady attendance of skilled workers with overtime hours, extra shifts in production, offer for production incentives and many a times he plays a role also as a counsellor to retain these workers since he is pressed for delivering the plants in their entire quantity by a certain week. Accomplishing this with the best of his efforts, there is a temporary relief to the production manager but not for long.

The next perplexing question for many laboratories producing large volumes of only banana is how to engage all these skilled workers during the lacuna for several months before there is build-up of cultures ready for the next season. Unless a more or less uniformly spread demand of banana plants round the year works out, a micropropagation unit will always face this issue, and when the production machinery does not function on time, there will be losses accumulated. A different strategy chosen by few companies as a remedy to this issue is product diversification. Many tissue culture crops propagated commercially at present have a different or an extended planting season. They even can be allowed to grow few weeks more without any losses in productivity. For example, pineapple, lemon, pomegranate, guava, fig, jamun, and papaya are some of the tropical horticulture species which are good candidates for commercial production. Among temperate and soft fruit, apple as well as strawberry are commercially produced through TC. There is ample selection from other crop groups—spice crops such as turmeric, ginger; an array of medicinal, ornamental, and forest species. Such a product mix ensures round the year production and revenue generation, with worker and space occupancy utilized at maximum efficiency round the year. This is an ideal model adopted by many middle and high-level companies since the 1990s. The first successful demonstration of commercial production of strawberry in India was from day neutral and higher temperature tolerant varieties introduced under the names Sujata and La Bella in 1990 by *Unicorn Biotek* in an effort to have a good product mix. In those years, micropropagation was commercialized for another crop-turmeric, using the varieties Suguna, Suvarna, and Duggirala (Bhatt, unpublished). Another example for a middle level TC company, as mentioned in Government of Gujarat Magazine in 2012, '*Sun Agrigenetics*' has defined a niche for itself, continuously innovating and bringing to market a variety of new TC plants over and above several varieties of banana. Some examples of its 'firsts' are Tindora, Parwal, Lemon, Fig, Sweet potato, etc. (Anonymous 2012). Careful planning and marketing, and technical expertise for handling a variety of crops will be crucial for this type of business and will call for a strong team effort. It is apparent that such a scenario is not possible with the small laboratories

having limited knowledge and expertise on these new crops, hence a technical collaboration with academic institutes or other companies would be required.

1.5 Cost Minimizing Efforts in TCP Production

Discussing economics of PTC business, many scientists state that if cheaper alternatives are sought for production, they could provide plants at a lower cost to benefit the growers, and work in a profitable business. Mention is made that media is the main contributing cost in TC plant production (Pant and Mehta 2016). A careful study from commercial point of view presents a different picture.

About 48% of expenses are allotted to salaries, and efficient utilization of their capacity is the crux of cost-effective production (Table 1.5). It is evident that more effort should be put into reducing unnecessary manpower and focusing on energy conservation measures. Improving worker efficiency and minimizing losses would result in more production, while not compromising on raw material quality. In PTC labs all over the country, there is a rapid turnover of trained workers replaced by newer workers who need longer time to achieve their optimal production capacity. This scenario is practically more severe considering the fact that young female workers or 'nimble fingers' as Reddy (2007) puts it, are participating in PTC labs, thus changing the horticulture scenario. With their 'nimble finger' quality bringing in maximum efficiency, in our social setup of gender inequality, they also bring in instability of jobs. Among other things, this makes it more challenging for any PTC unit where there is a majority of female skilled workers. Though there is no statistical data available, in general it is a case for most commercial labs not only in India but at global level too.

An equal contribution is enjoyed by energy and raw material costs put together. Cheaper sources of raw material may lead to ebbing of quality, albeit any effort put into minimizing the costs would not bring about substantial change in production costs. With a standard media recipe used in PTC, cost of agar agar used for gelling contributes about 54% of total raw material cost. Looking for cheaper alternative to make media cost-effective, gellan gum appears to be best without compromising culture quality as it contributes to only about 21% of the raw material cost on a unit volume of media made. Looking for alternatives of other chemicals is a futile effort particularly when static and non-automated system is used. Energy costs do not run exactly in parallel with production except for direct consumption of energy for

Table 1.5 Running cost analysis in a typical PTC unit (Bhatt, unpublished)

Major running cost factors in TC plant production for a one million plant capacity	Rs. (in million)	% of total running cost
Salaries (ten workers, three supervisors, one manager)	1.4	48.7
Laboratory raw materials	0.75	26.1
Energy cost	0.72	25.1
Total major running cost in Rs. (million)	2.87	100

operating sterilizers and laminar work stations. Cooling of growth rooms and inoculation rooms would require more or less same energy, hence full capacity utilization of both areas need to be considered. The energy tariff shows a great inequality for PTC units in different states in the country. It is likely that the cluster states enjoy better rates for power supply attracting setting up of larger numbers of PTC units and hence a bigger volume of production is possible. In other states, the industry growth is incumbered as they pay high commercial tariffs which are way beyond their production capacities.

1.6 Commercial Production of Lemon

Among the citrus species, acid lime or lemon is cultivated in 317,000 ha contributing to 30% of total citrus growing area. Development of commercial TC technology has been a call for lemon to make available improved, high yielding and plants tolerant to biotic and abiotic stresses. Seed derived plants begin fruiting after 7 years and it would be economical to have an early fruiting crop. It is not possible to estimate current volumes of micropropagated lemon in commercial production, but several laboratories have been producing seedless and kagzi lime. Major problem faced during micropropagation of lemon is inconsistencies in multiplication rates and low rate of in vitro or in vivo rooting. The cost of production thus offsets the returns gained in commercial sale. Another alternative to TC propagation is micrografting which has been standardized at research level, and field trials are required before it can be applied commercially as is done in solanaceous vegetable crops.

Even though the cost of TC production is high, it is worthwhile to multiply elite plants to produce sufficient planting material with better performance and yield as discussed above. In this direction, an effort was made early in year 2005 in Gujarat. A high temperature tolerant kagzi lime clone was procured from Rajasthan and commercial micropropagation was done to produce around 1 lac plants (0.1 million) which were distributed to farmers across Gujarat during next several years (Fig. 1.5a). Reports received during personal communication with growers on performance of these plants suggested that there was a significant higher yield from TC derived plants. The fruiting continued for a longer period and yield was also improved (Bhatt, unpublished).

1.7 Commercial Production of Cucurbit Vegetables

Pointed gourd (*Trichosanthes dioica*) commonly known as parval, or patal and teasle gourd, (*Momordica dioica*) commonly known as kakrol or kantola, and ivy gourd (*Coccinea indica*) known as Tondli, tindora, and dondakaya in local languages are three of the several cucurbit vegetables grown in India. So far considered minor and underutilized crops, there is very little systematic effort on improving their agronomic practices and making available quality planting material for these cucurbits.

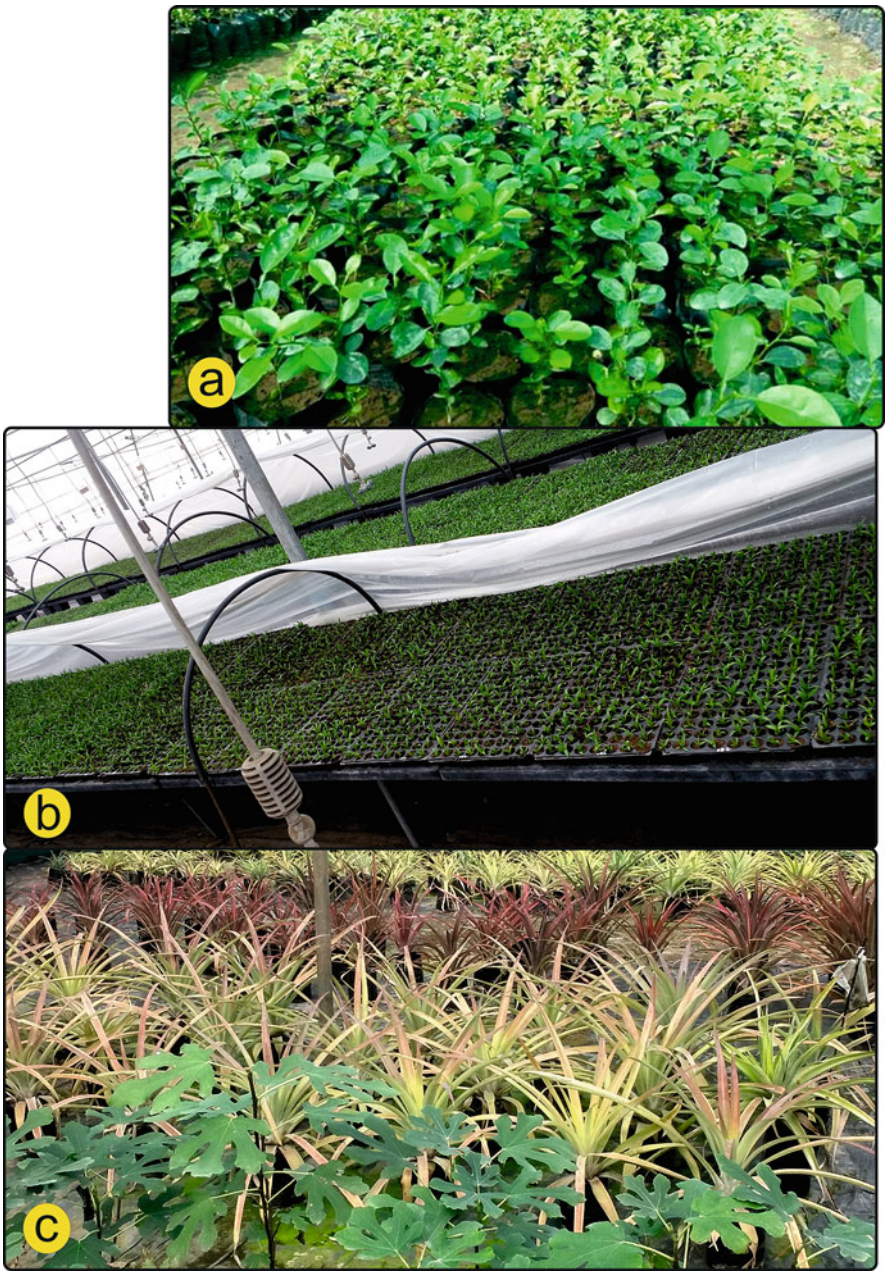

Fig. 1.5 Commercial micropropagation of lemon and pineapple (Bhatt and Bhatt, unpublished): (**a**) TC Kagzi lime in secondary hardening; (**b**) Pineapple var. MD2 in culture; (**c**) MD2 pineapple in secondary hardening

1. *Kakrol or teasle gourd*: For use as a vegetable, fruits had been so far mostly collected from random sources of plants naturally growing over hedges and fences, and was enjoying the status of a delicacy among many communities. The plant is dioecious and insect pollination being not very efficient, it was not a popular vegetable to cultivate among farmers due to difficulty of cultivation. With a rise in market demand, it is grown through vegetative propagation by cuttings, which turns out to be having a low survival rate. Plants grown from seeds segregate out and it is hard to identify the sex before they reach flowering stage, making rouging cumbersome or impossible. There are records that plantations raised turn out to having 70% of male plants that are more vigorous, outgrowing and masking the female plants restricting their growth, resulting in very low outputs per acre (personal communication with local farmers).
   Medicinal properties of *M. dioica* have been well enumerated since long (Sharma 2004) and recent times, its value as a phototherapeutic and pharmacological candidate is well recognized (Jha et al. 2017). They concluded that the plant contains significant amount of antioxidant, vitamin, secondary metabolites, and other important ingredients, and these may be helpful to fight against several diseases including neurodegenerative diseases, diabetes, and cancer. With increased market demand locally as well as internationally, and due to a lucrative market price, farmers have now been attracted for its systematic cultivation. W. Bengal, Assam, Odisha, and Gujarat are the main areas where it is cultivated and the planting material demand is arising from the southern states of India. As of now, commercial micropropagation has not been done on a large scale or its information is not available. It is surprising that though the tissue culture micropropagation has been standardized in laboratories in India and Bangladesh since long (Hoque et al. 2007; Karim and Ahmed 2010; Karim and Ullah 2011; Shekhawat et al. 2011; Patel and Ishnava 2015a), it has received attention from the commercial industries only recently. Among other reasons, a realization of its value as a nutraceutical brings about a sudden surge in demand of the crop.
2. *Pointed gourd* (*Trichosanthes dioica*) commonly known as parval or patal is reported to be used in Ayurveda and Unani medicine. Ayurveda refers parwal as *'the king of all vegetables'*. Pharmacological properties of pointed gourd are highlighted by Shah and Seth (2010) and shown to be antihyperglycemic, anthelmintic, antioxidant, antipyretic, hepatoprotective and useful in reducing low density lipoprotein (LDL) cholesterol.
   Pointed gourd is cultivated mainly in Bihar, Uttar Pradesh, West Bengal, Orissa, Assam, Madhya Pradesh, and Gujarat. There is some cultivation in Andhra Pradesh and Tamil Nadu, and recently in Telangana and Karnataka. The area covered under its cultivation is 56,000 ha, (GOI Press release 2020). In absence of low seed viability and segregation of resultant progeny into male and female, vegetative propagation is practised using cuttings taken from the base of female plants, thus planting material is of limited availability. Once again there is an issue of maintaining a good ratio of females over male plants as the latter always take over the open space on the trellis much faster. The area under cultivation has

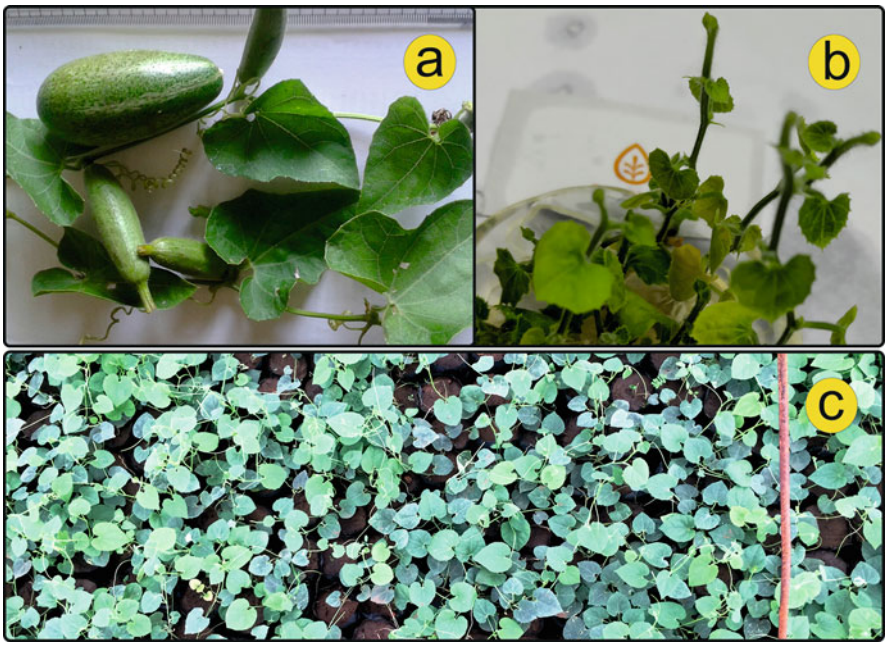

Fig. 1.6 Commercial micropropagation of pointed gourd (Bhatt and Bhatt, unpublished): (**a**) fruits of TC plants; (**b**) multiplying cultures; (**c**) plants in secondary hardening

not increased to match with its high demand in the local and export markets due to non-availability of sufficient cuttings from female plants.

Micropropagation of pointed gourd was reported as early as 1999 (Mythili and Thomas 1999), followed later by Kumar et al. (2003), Lal and Das (2009), Komal (2011). Albeit there was a lacuna in developing its micropropagation technology. Gujarat took the lead in commercial production of parwal in early 2005 (Sun Agrigenetics) (Fig. 1.6a, b, c) and couple of companies began sale of TC plants. Today several companies are doing commercial production of parwal but volumes are still low. Having a TC derived production using careful selection of mother plants, a precise and optimum ratio of male to female plants of 1:9 can be easily maintained in the field. This technology provides plants of good quality and in quantity, and assured for female or male plants. The plantations from TC plants have demonstrated better productivity at field level with early flowering, prolonged season and higher yield of 2.22 tons/ha *vis a vis* 672 1.66 tons/ha (personal communication with farmers). This is 33% increase in yield.

The business of production of TC plants in such cases is though not fool-proof. Reckless maintenance and little bit of neglect can result in mixing up of male and female plants at production level. At the onset of flowering, a farmer may realize that the 1:9 ratio of male: female is deviated significantly. By that time, it is already 4 or 5 months into the season where he has invested money into buying

expensive TC plants and in agronomy practices. The company would have to end up in compensating the grower for his losses. It may sound easy since plant propagates at a very fast rate in vitro, but the hardening of plants in case of parwal is another stumbling block for its large-scale production, and some research inputs are required to optimize plant survival during the entire hardening process. In addition to less requirement in the market due to less area of cultivation, above two difficulties may be the reason for many companies not to take up its production. Two local varieties are in production at present in Gujarat which are traditional clones. Known and improved varieties Swarna Rekha, Swarna Alaukik, CHES elite, CHES hybrid, and Rajendra hybrids released by different Agriculture Universities should be included in the micropropagation programmes, so that growers in these areas which are outside the range of present supplies can benefit from the TC plants.

3. *Ivy gourd* (*Coccinea indica*) is another underexploited cucurbit having an aggressive climbing behaviour. According to Ayurveda, there are two varieties, wild or bitter, and sweet or cultivated. The former is used in folklore medicine. The sweet or cultivated forms, although not having defined genotypes or varieties, have been found to have two distinct type of fruit characters. The first one with short and oblong fruits having striated dark green rind and producing more and hard seeds. The second type fruit has light green rind without striations, fruits are slenderer and with less seeds.

   The cultivated or sweet *C. indica* is asexually propagated from cuttings of woody stems during monsoon, since the seeds if produced at all are not viable. Commercial cultivation is done by training plants on a bower system. Planting material is usually scarce, and the fruits are distorted in shape due to infection, presumably carried forward from previous source due to its susceptibility to powdery mildew caused by species of *Erysiphe* (Imbumi 2004). As a result, farmers are reluctant to cultivate this easy to grow crop.

Ivy gourd is estimated to be cultivated in about 60,000 ha area mainly in Gujarat, Telangana, Karnataka, and Maharashtra. After the first scientific study by Chopra and Bose (1925), several workers have done pharmacological studies implicating the role of this plant in diabetes mellitus (Mukherjee et al. 1972; Hossain et al. 1992; Venkateswaran and Pari 2003). Pharmacognosy in this plant is extensively reviewed recently by Tamilselvan et al. (2011). The young stems and leaves are eaten as food and also used as medicine, and reported to having hypoglycaemic, hypolipidemic, and antioxidant properties. Our notion about ivy gourd is limited only to use of the unripe fruit as a vegetable. But the direct use of fresh plant parts such as young leaves and stem is practised traditionally in many other countries. Use of young vegetative parts directly as a food supplement has been evaluated and recommended in Taiwan as a source of folate, beta carotene and protein. Mixed with another species, the fresh plant parts supply between 30 and 90% of safe intake of provitamins recommended by WHO/FAO (Lin et al. 2007). Srivastava et al. (2016) have reviewed its pharmacological properties and signified its use recommended in Ayurveda as being analgesic, antipyretic, anti-inflammatory, antimicrobial, antiulcer, antidiabetic,

Table 1.6 Development of commercializing ivy gourd TC plants domestically and for export (Bhatt and Bhatt, unpublished)

Year	Number of plants sold	Area of sale	Cultivation method
2009–2010	100 plants field trial	Own plot	Open
2010–2011	50,000	Gujarat	Open
2011–2015	2,24,000	Gujarat and export	Open
2015–2019	1,48,000	Gujarat, Maharashtra, Telangana, AP, Rajasthan, Uttar Pradesh	Open and shade house

antioxidant, hypoglycaemic, hepatoprotective, antimalarial, antidyslipidemic, anticancer, and antitussive.

There have been many research reports on micropropagation of ivy gourd (Sundari et al. 2011; Gulati 1988; Sarker et al. 2008; Josekutty et al. 1993; Ghantikumar et al. 2013; Patel and Ishnava 2015b; Borah et al. 2019). None of this basic research culminated into its application or extended field trials despite its importance and need recognized. The first effort (Anonymous 2012) to develop commercial-scale micropropagation technology in these crops was done in year 2009 in Gujarat, and tremendous success was gained (Table 1.6, Fig. 1.7a, b, c) over the years (Bhatt et al. 2019). Plants could be planted round the year by farmers with close to zero mortality during transplanting. Fruiting commenced earlier than in cutting derived plants with a several-fold yield increase based on the agronomic practices followed by the farmers. Quality-wise, there was an improvement in fruit shape (Fig. 1.7e). It is generally observed that the fruits of plants derived by cuttings are distorted in shape due to infection, presumably by physical damage to the ovary by fruit fly (Table 1.7, Fig. 1.7f). While harvesting, the farmers grade the fruit based on its size and uniformity in shape, and 'A' grade would be in a lesser percentage. With TC derived plants, fruits are cylindrical and regular in shape (Fig. 1.7e), classifying more into 'A' Grade.

Since its first commercial cultivation in Gujarat on a small scale, area of cultivation is expanded and also extended to several states of India. In fact, in some very hot climate in Telangana, progressive farmers have started its cultivation under shade house (Fig. 1.7d); (personal communication with grower). Eyes are turned to plant sources in present days when people across the globe are looking for botanicals providing antioxidants, as well as hypoglycaemic properties and a demand has increased for ivy gourd from clients abroad who cater to Asian communities (Bhatt, unpublished). Unlike conventionally propagated plants, TC produced mother plants are easily propagated by cuttings, and hence the direct planting of TC plants has recently declined. On an average, during the monsoon season, a farmer will multiply his stock 100 to 200-fold by vegetative cuttings from TC plants so they are ready for sale in planting season of September to April (Personal communication with local farmers). Hence the area under cultivation of tissue derived first or second generation plants in on the rise. Similar results have been achieved for pointed gourd

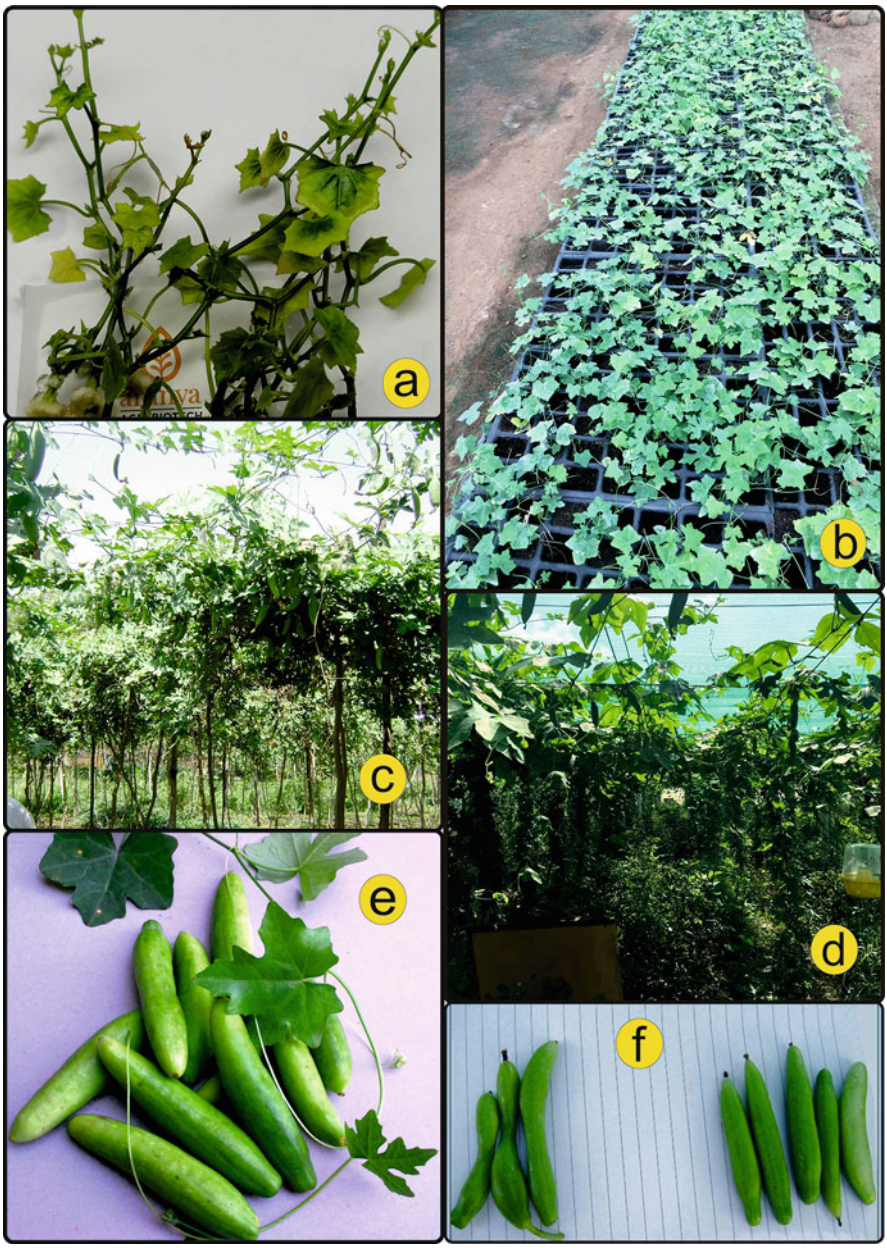

Fig. 1.7 Commercial micropropagation of ivy gourd (Bhatt et al. 2019): (**a**) multiplying cultures; (**b**) plants in secondary hardening; (**c**) plants in farmer's field; (**d**) protected cultivation of tindora (courtesy Sai Krishna); (**e**) fruits of TC plants; (**f**) fruit comparison between non-TC plants (left) and TCP (right)

Table 1.7 Comparison of plant performance of ivy gourd in conventionally grown vs Tissue culture produced plants in Gujarat. Data from several farmers' field (Bhatt et al. 2019)

Plant source	Conventionally grown	TC produced
Plant habit	Plants grow up to support in 6–7 months	Plants grow up to support in 2–3 months
Yield/acre	100–250 kg	1000–2500 kg
Harvesting frequency	Once a week	Every third day
Duration of fruiting	Poor or no yield in cooler months (December to February)	Continuous fruiting even in cooler months
Fruit quality	'A' grade fruit 20–30%	'A' grade fruit 70%
Fruit characters	Fruits with more seeds, less pulp and less flavoured	Fruits tender with more pulp, less seed and taste better

and teasel gourd. Only a few companies are commercially producing these three crops through TC.

1.8 Commercial Production of Pineapple

Among tropical fruits, pineapple contributes to more than 20% of the world's production of tropical fruits and is the next most widely cultivated tropical fruit crop next to banana. Most of the fruit is consumed fresh in whichever country or area it is grown, with about eight varieties commercially cultivated over the world. Several laboratories commercially produce pineapple through TC. Mauritius and Kew are the two commercial varieties cultivated in Kerala, former occupying 80% of the area under pineapple cultivation. Major pineapple growing states are Kerala, Meghalaya, Manipur, W. Bengal, Tripura, Karnataka, Goa and to some extent in other states. Pineapple Research Station at Vazhakulam, Kerala, provides technology, products, and services to growers in the State, who mostly grow pineapple and banana. Over the years, these growers have developed expertise in pineapple cultivation and hence the product rightly came to be known as Vazhakulam pineapple, which is a Mauritius variety. Queen and Kew varieties are cultivated in Tripura and Meghalaya. MD-2 is the most successful variety globally in recent years and preferred for commercial production due to its exceptional sweetness, less fibre and acid content, rich flavour and colour. Due to its consistency in cylindrical shape, it is ideal for processing. Being spineless leaves, it is easy for handling during cultivation, especially when there is no full mechanization. This variety was developed in 1961 in Hawaii, and the first commercial cultivation was taken to be done in Costa Rica.

Earlier studies on the performance of tissue culture pineapple suggested that there were more incidences of somaclonal variation, and the occurrence of such variations limited the application for large-scale commercial micropropagation (Salvi et al. 2001). Many reports on tissue culture of different species are available in which authors state that the performance and genetic fidelity of resultant plants are related

to the type of explant source, the protocols used to involve plant grown regulators, and the number of subculture cycles (Bairy et al. 2006). Thus, in absence of such optimization, commercial micropropagation may not have been attempted.

With more research into methodology for micropropagation of pineapple, like for any other species, it is now possible to eliminate the chances and monitor somaclonal variation in TC resultant plant batches. Comparison of TC derived pineapple with traditionally grown ones is shown to have no variation in terms of physical and nutrient parameters (Jackson et al. 2016). At global level, several laboratories now produce and supply mother stock of this variety and others are engaged in its commercial production. In many countries where pineapple is the major crop, MD-2 has become the first choice. Within India, a few laboratories produce TC pineapple of Queen and MD-2 varieties. In Gujarat, TC pineapple variety MD-2 was first introduced as commercial production in year 2014 from mother plants procured from the Pineapple Research Station (Fig. 1.5b, c) and since then about 5 lac TC plants have been planted on commercial scale in Gujarat, Bihar, and NE States (Bhatt, unpublished). There is some degree of resistance in adopting the improved MD-2 variety in Kerala. North and NE States show better acceptance to this variety and the results being very satisfactory, demand continues ever year. Many of the plants are supplied through Horticulture Departments to small growers.

1.9 Commercial Production of Turmeric and Ginger

Turmeric cultivation in India is in 3,29,722 ha with rhizome requirement of 3.3 lakh tons (Prasath et al. 2017), at 2.5 tons of rhizomes required per ha. Converting an average 50 g rhizome into number of plants, approximately 41.22 million plants are required for this area. Medicinal uses of turmeric have been long known (Roses 1999) and is used in many Ayurveda medicines or formulations used in our day-to-day lives. Curcumin, the crystalline orange yellow substance, which is the main active principle, has been the focus of attention for its effect as an immune-enhancer in life threatening viral diseases, especially during the COVID19 pandemic. Techniques for TC derived microrhizomes for several varieties of turmeric have been optimized in various laboratories as early as in 1978 in India (Nadgauda et al. 1978; Balachandran 1990) and reviewed by Kumar et al. (2017). Comparative studies have been extensively done using conventional and TC derived plants of turmeric (Chitra 2019). Field performance of microrhizomes derived turmeric (*C. longa*) was found to be better than conventional by Nayak and Naik (2006), and they suggested that microrhizomes derived plants can be used by growers for commercial production. In spite of such long history of studies on tissue culture, large-scale commercial production of turmeric plants or microrhizomes has not taken up so far in India. There are about eight laboratories known to sparingly produce them. PTC efforts are needed also for rapid multiplication of selected elite clones. According to a recent TNAU report (2020), the main limitation is high initial investment by any particular organization, but smaller commercial units will soon take up this work on a large scale as is already done in other countries. Ginger is also

prized for its medicinal value like turmeric. Several laboratories in India produce TC ginger (personal communication with R Mane).

The future of commercialization of PTC lies in innovation, more mechanization using plant bioreactors and efforts to increase productivity at micropropagation levels. In this direction, Lu et al. (2019) in sugarcane demonstrated rapid micropropagation using sugar-free medium, used CO_2 gas as carbon source and natural compounds having antimicrobial activity. Such systems should be applied to commercial production of sugarcane and other products in our country. The use of embryogenic cell suspension cultures makes mass clonal propagation of banana much more rapid and current research is focusing on scalp technology (Strosse 2017). In this system, embryogenic competent scalps from meristematic tissue with a small amount of corm tissue are used to generate highly efficient embryogenic cell system. This model can be applied in genetic engineering as well as disease resistant selection. Commercial PTC has gone a long way in India, albeit with just a few limited crop species. There is much more room for indigenous varieties and species to be included in our production portfolio, and bringing the national productivity at par with global averages.

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Clonal Propagation, a Tested Technique for Increasing Productivity: A Review of Bamboos, Eucalyptus and Chirpine

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Abstract

Bamboos, *Eucalyptus* and chir pines are the three most important trees in terms of their commercial and socio-economic values. All these genera have demands for agricultural, artistic, cultural, industrial, construction and household purposes. Besides these, they also hold their essentiality in traditional medicinal practices. Because of their high needs in daily human life, it is required to mass propagate them. This paper reviews the technique of successful clonal propagation on these three plants using various parts as explants. It was reviewed that micropropagation in various economically valuable species of bamboo such as *Bambusa arundinacea*, *B. vulgaris, B. tulda*, *Dendrocalamus asper*, *D. membranaceus*, *D. strictus*, *D. giganteus*, *D. hamiltonii*, *Drepanostachyum falcatum*, *Melocanna baccifera,* etc. was succeeded using seeds, nodal segments, immature inflorescences, etc. as explants. In the case of *Eucalyptus*, *E. cloeziana* and few other immensely important hybrids like, FRI-5 and FRI-14 were clonally propagated using the nodal shoots as explants. Chirpine (*Pinus roxburghii*) was successfully propagated using seeds, shoot apices as well as the megagametophyte as explants. In each case, the surface-sterilized explants were cultured on

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Murashige and Skoog's medium containing varied concentrations of plant growth regulator.

Keywords

Micropropagation · Bamboo · ChirPine · Eucalyptus · In vitro rooting

2.1 Introduction

Agriculture has emerged since the beginning of human civilization, initially as a means to guarantee food supply and later as a source of family income and improved profitability (Budd 1993). Domestication of animals and plants, which were found in wild, along with the gradual and long-term variations in their quantity and quality, were the first indication of what is combinely called as "agriculture" now (Colwell and Sasson 1994). Progressive improvements in agricultural techniques, domestications of animal and plant species from the wild, sequential selection of well-performing and well-adapted genotypes, along with visceral breeding, advanced at a slow pace (Budd 1993). The main challenges faced by the humans in the twenty-first century are food, energy and environment, specifically climate change and land degradation caused by pollution and habitat loss. Since land and water required in agriculture are diminishing, the only option left is to produce food crops and other agricultural goods from less cultivable land and with less irrigation water (Persley 2000).

Agricultural biotechnology, commonly known as agritech, is a field of agriculture science which involves the application of various scientific tools and techniques, including genetic engineering, molecular markers, molecular diagnostics, vaccines and tissue culture, employed in modifying various life forms, such as plants, animals and microorganisms. Crop biotechnology is an important aspect of agritech, which has been developed enormously in recent times. In vitro cultures provide an alternative to produce clones in a specific period of time (Goyal and Sen 2016). In vitro technologies include the techniques of micropropagation, somatic cell genetics and generation of transgenic plants.

2.2 Micropropagation

Clonal, true-to-type propagation of plants using various methods of tissue and cell culture, better known as micropropagation is the commercially effective and practically oriented plant biotechnology now (Altman 1997). Propagation of plants in tissue culture is used to produce high-quality clones of standard plants (de Kleijn et al. 1992; Smith 1996). Such plants are then selected on the basis of their horticultural traits, resistance to pests, quality of crops or suitability for environmental stresses. There are many advantages of micropropagation over the traditional propagation techniques, some of which include: (1) collection of explant at any time

irrespective of the flowering season (assuming that seeds are not required), (2) elimination of virus using meristem cultures, (3) maintenance of elite genotypes by the production of clones, (4) rapid multiplication irrespective of time, (5) germination of immature or difficult seeds for the breeding programmes, (6) distribution of germplasm in the form of in vitro cultures across the border are safer in terms of their health status (Sharma and Thokchom 2014).

Clonal propagation technique relies on the approach of totipotency, i.e. the regeneration of whole plant from a single cell or a group of cells from within a tissue or an organ, expressing the full plant genome (Altman 1997). The evidences from early experiments which support the concept of totipotency came from the works of Vöchting (1878), who successfully maintained the viability and growth of plants and plant parts dissected in small fragments (Vasil and Vasil 1981). This is accomplished after the excised tissue or organ has been inoculated on the culture medium, which involves the following consecutive events: (1) dedifferentiation of the explant [source tissue or organ], which results in the activation of physiological processes that lead to cell division under suitable endogenous and exogenous conditions; (2) active cell division often results in the proliferation of callus tissue; (3) organization of defined meristems in the zones of active cell division resulting in shoot and/or root meristem; (4) regeneration and differentiation of new organs, namely organogenesis when formation of new shoot buds or new roots, or somatic embryogenesis leading to bipolar differentiation of somatic embryos (Altman 1997). The successfully regenerated plantlets are hardened and acclimatized in a greenhouse before the field transfer.

2.3 Clonal Propagation of Bamboos

Bamboos are woody perennials of family *Poaceae*, belonging to the sub-family *Bambusoideae* (Clark et al. 2015). Bamboos are versatile forest trees having enormous commercial and eco-sociological importance. Being a vital resource, the demand of this "Green Gold" is ever increasing (Goyal and Sen 2016). Various techniques are available for the propagation of bamboos, such as seed propagation, clump division, rhizome and culm cuttings, but these are the classical techniques that face crucial drawbacks for mass scale propagation. Hence to overcome the drawbacks of these insufficient and inefficient classical propagation techniques, micropropagation is the only viable method of mass production in bamboos. The promising micropropagation techniques for large-scale propagation of bamboos have raised hopes and initiated research focussing on the development of protocols for mass and rapid-scale propagation (Mudoi et al. 2013).

In 1999, Arya et al. reported the technique of high-frequency direct shoot proliferation in aseptic seed cultures of *Dendrocalamus asper* on modified MS medium supplemented with 1.0–10.0 mg/l benzyladenine (BAP). Multiple shoots were produced within 5 weeks which were subcultured in propagules of three shoots each. The proliferation rate was found to be 15–16 folds on MS medium containing 3.0 mg/l BAP. Excised propagules were rooted on medium supplemented with

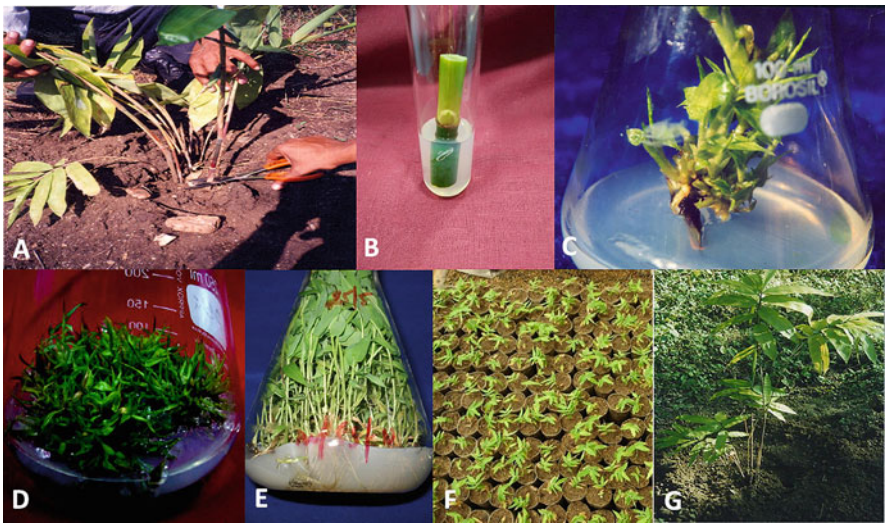

Fig. 2.1 Micropropagation of *Dendrocalamus asper*: (**a**) plant in natural habit, (**b**, **c**) axillary bud culture, (**d**) shoot multiplication, (**e**) *in vitro* rooting, (**f**) plantlets in greenhouse, (**g**) one year old plant

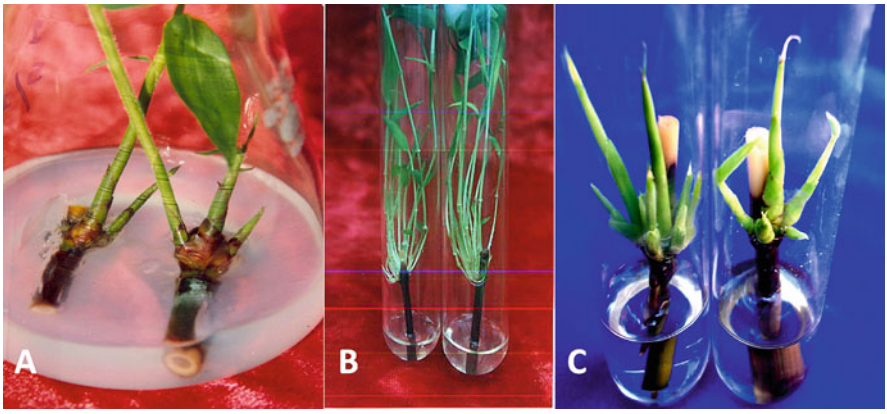

Fig. 2.2 Axillary bud culture: (**a**) *Dendrocalamus giganteus*, (**b**) *Dendrocalamus falcatum*, (**c**) *Melocanna baccifera*

10.0 mg/l indole-3-butyric acid (IBA) or 3.0 mg/l naphthaleneacetic acid (NAA). The plantlets were hardened, acclimatized and successfully transferred to soil. Furthermore, Arya et al. (2001) studied rapid micropropagation of *D. asper* (Fig. 2.1a) using nodal explants (Fig. 2.2b) and seeds culture. Ninety-five percent of nodal shoot explants of juvenile primary and lateral branches that were induced through forced axillary branching produced multiple shoots within 2 to 8 weeks on Murashige and Skoog's (MS) medium supplemented with 0.1–10 mg/l BAP

(Fig. 2.1c, d). Besides these, the seeds cultured on this medium produced approximately 1–20 multiple shoots with 6 weeks. It was found that concentration of BAP in the medium is the influencing factor in multiple shoot differentiation. The excised in vitro shoots were subcultured on MS medium containing 3.0 mg/l BAP which further multiplied. The process was continued for 3 years without any loss of vigour and the 95% multiplied shoots were rooted on MS medium containing 3.0 mg/l NAA or 10 mg/l IBA (Fig. 2.1e). These plants were successfully hardened, acclimatized and transferred to field (Fig. 2.1f, g).

Arya et al. (2002) described a complete protocol for the micropropagation of six economically valuable bamboo species, namely *Dendrocalamus asper*, *D. membranaceus*, *D. strictus*, *D. giganteus*, *Bambusa arundinacea* and *B. vulgaris* ev. Green. Multiple shoots were induced in all these species aseptically by in vitro cultures of nodal shoot segments via forced axillary branching. Nodal shoot segments collected from young primary and lateral branches, when subjected to MS medium supplemented with various concentrations of BAP (1–10 mg/l), produced axillary shoots from axillary buds within 2 weeks. These axillary shoots so produced were then subcultured on MS medium enriched with defined plant growth regulators for further multiplication, which is then followed by a regular subculture in every 4 weeks that increased the multiplication rate, reaching the maximum after 4–6 subculture cycles. More than 85% of these in vitro developed shoots were rooted successfully on MS medium containing NAA (2–3 mg/l) or IBA (10–15 mg/l) in the propagules of three to five shoots. These plantlets were hardened, acclimatized and transferred to field successfully.

In another study, Arya et al. (2008a, b, c) reported that flowering in bamboos is a tragic event which leads to many post-flowering consequences and is dangerous to plants. To overcome this loss caused by unpredictable flowering, direct shoot regeneration was practiced, where the immature inflorescences of *Dendrocalamus asper* were inoculated on MS medium supplemented with BAP at the concentration of 7 mg/l. Immature inflorescences (0.5–1.0 cm) produced the best regenerated shoots, which were subcultured on MS medium supplemented with 3 mg/l BAP for further multiplication and development. 10–15 folds shoot multiplication was observed among which 90–95% shoots were successfully rooted under in vitro conditions on MS medium containing 10 mg/l IBA. The regenerated plantlets were successfully transferred to fields after hardening and acclimatization, which showed 80–90% survival rate.

The technique of somatic embryogenesis was tested on *Dendrocalamus asper* for the large-scale production of this edible bamboo. Embryogenic callus was produced from the excised explants of nodal tissues and basal part of leaves that were grown from in vitro shoots. Induction of somatic embryos was performed on MS medium supplemented with 30μM 2,4-D and 2% sucrose in dark conditions. The callus proliferated 3–5 folds in every 4 weeks of subculture on medium enriched with 9μM 2,4-D + 2.85μM IAA + 0.88μM BAP, with efficiency of 21.7–32.8 globular somatic embryos per embryogenic callus. Maturation of globular somatic embryos to the scutellar and coleoptillar somatic embryos was achieved on medium enriched with 6% sucrose and 5μM ABA within 8 weeks. Plantlets were developed from

somatic embryos on MS medium supplemented with 4.4μM BAP and 2.8μM GA_3 with conversion rate of 70%. 30% embryos developed only in shoots on germination medium and these shoots were multiplied by subculture on MS medium containing 13.2μM BAP. 98% rooting was induced in the multiplied shoots on MS medium supplemented with 16μM NAA. The regenerated plantlets were hardened, acclimatized and transferred to fields successfully (Arya et al. 2008a, b, c).

Similar study was conducted on *Drepanostachyum falcatum*, a multipurpose bamboo having high economic value. Here the excised nodal segments having single axillary bud were cleaned with cotton soaked in 70% ethanol, followed by surface sterilization with 0.1% $HgCl_2$ for 12 min. After rinsing the axillary buds 3–4 times with autoclaved distilled water, they were transferred to liquid MS medium enriched with different concentrations of BAP (0.5 mg/l – 12.0 mg/l) which was found to be the influencing factor in multiple shoot proliferation (Fig. 2.2b). The multiple shoots so developed were then transferred to the medium containing 3.0 mg/l BAP for subculturing, which showed 9–11 fold shoot multiplication rate when subcultured in propagules of 4–5 shoots each in 4 weeks. In vitro developed shoots were rooted in MS medium containing 7 mg/l IBA or 5 mg/l NAA, followed by their hardening, acclimatization and field transfer, all showing normal growth (Arya et al. 2008a, b, c). Kant et al. (2009) suggested a complete protocol for micropropagation of *Melocanna baccifera* where nodal segments from clumps were used as explant (Fig. 2.2c). Single nodal segments of the 18 years old clumps were initially treated with a mixture of bavistin and streptomycin which was followed by surface sterilization with the solution of 0.1% mercuric chloride for 10–12 min. The pretreated explants were then inoculated on liquid MS medium enriched with cytokinin. Highest bud break along with maximum multiple shoots was observed on liquid MS medium containing 20μM BAP, followed by their further multiplication on liquid MS medium enriched with 15μM BAP and 3μM kinetin, at a rate of 2.99 folds, every 4 weeks. About 65% of in vitro developed shoots were subjected to rooting on MS medium of half strength supplemented with 25μM IBA. Finally the in vitro developed plantlets were hardened and acclimatized.

Another bamboo species *Dendrocalamus hamiltonii* was mass propagated under aseptic conditions, where the seeds disinfected with 4% sodium hypochlorite were inoculated on MS medium supplemented with cytokinins. Multiple shoots were produced in 3–5 weeks and it was found that at the concentration of 35μM BAP, 7–8 shoots were obtained. When the excised multiple shoots were subcultured on MS medium supplemented with plant growth regulators for 3–4 weeks, it was observed that BAP concentration at 10μM was best suited for increasing the rate of propagation. A pulse treatment in a two-step procedure was given to induce rooting, where 3–4 excised shoots were inoculated on MS medium supplemented with high concentrations of IBA (auxin) for 7 days, followed by the transfer of shoots to half strength MS medium without auxins for 10–15 days. The obtained plantlets were successfully hardened, acclimatized and transferred to soil for normal growth (Arya et al. 2012). Arya and Arya (2015) suggested a protocol of tissue culture for three economically important bamboo species, viz. *Dendrocalamus asper* (edible bamboo), *Drepanostachyum falcatum* (hill bamboo), *D. hamiltonii*, where

the multiple shoots were induced from nodal shoot segments and proliferated on MS medium enriched with various concentrations of BAP (1–10 mg/l). A 14–16 fold shoot multiplication was observed after 4 weeks subcultured cycles in *D. asper*, whereas it was six to ten fold in case of *D. hamiltonii* and *D. falcatum* which was followed by successful rooting on MS medium supplemented with 1.0–5.0 mg/l NAA or 5–10 mg/l IBA. Nodal tissues and basal part of leaves from in vitro developed multiple shoots were induced to form embryogenic callus. Induction of somatic embryos was performed on MS medium containing 20–30μM 2,4-D and 2% sucrose under dark conditions. Embryogenic callus of *D. asper* proliferated 3–5 folds on MS + 9μM 2,4-D + 2.85μM NAA + 0.88μM BAP medium in every 4 weeks, *D. hamiltonii* on MS + 10μM 2,4-D + 5μM BAP and *D. falcatum* callus multiplied two to three folds on MS + 10μM 2,4-D + 0.88μM BAP. Maturation of somatic embryos to scutellar and coleoptillar embryos was achieved on 5μM ABA medium. Within 30 days on MS medium containing 4.4μM BAP and 2.8μM GA_3, the somatic embryos of *D. asper* developed into plantlets with a conversion rate of 70%. Germination of somatic embryos of *D. hamiltonii* and *D. falcatum* was achieved on MS medium containing 5μM BAP. The plantlets so developed via in vitro proliferation as well as through germination of somatic embryos were hardened, acclimatized and transferred to the fields successfully.

Similar study was conducted on species of bamboo named *Bambusa vulgaris* where rapid clonal propagation was done using nodal shoot segments from a 3-year-old plant, cultured on MS medium supplemented with BAP ranging in concentration from 0.5 to 2.5 mg/l. BAP concentration in the medium influenced the number of shoots and its length. Kinetin incorporated at the concentration of 0.5 mg/l further helped in increasing the number of shoots or length. Furthermore, the root development was influenced by IBA and charcoal concentrations in the liquid MS medium where IBA concentration ranged between 1.0 and 2.5 mg/l and charcoal concentration at 2.5 mg/l. Best growth was observed at BAP concentration of 1.0 mg/l which improved growth to two–four folds in 4 weeks. 95% rooting was observed at IBA concentration 2 mg/l and charcoal concentration 2.5 mg/l (Bhariya et al. 2017). An improvised in vitro vegetative propagation technique was tested for *Bambusa tulda* Roxb. to study the influence of season, sterilization and hormones on the growth of cultures. The nodal segment for explant collected from different seasons was treated with varied concentrations of $HgCl_2$ which showed prominent variation in the establishment of aseptic cultures and bud break. Highest (78%) aseptic cultures were recorded in rainy season (July–August), whereas the lowest, i.e. 46% was reported in autumn. Summer and winter seasons were found to be best period reporting >60% of in vitro bud break, whereas autumn season recorded the lowest value of bud break, i.e. 42%. Different concentrations of sterilizing agents were tested, of which 0.1% $HgCl_2$ was found to be the most effective for maximum aseptic culture establishment (66%) as well as bud break (59%). When tested for combinations, 0.1% $HgCl_2$ in summer collected samples reported maximum (73%) response for both aseptic culture establishment and bud break. Maximum in vitro shoot multiplication of 4.75 fold was observed in liquid MS medium supplemented with 5.0μM BA + 5.0μM kinetin along with 100μM glutamine + 0.1μM IAA.

Successful rooting was achieved in the proliferated shoots on liquid MS medium supplemented with 40μM coumarin and 98% survival rate was reported when transferred in polythene bags (Bhadrawale et al. 2018).

Another economically important bamboo species *Bambusa balcooa* was tested for morphological responses using nodal cutting on MS medium supplemented with a range of combinations and concentrations of 6-furfuryl amino purine (Kinetin) and BAP. Approximately 96% of sprouting was observed on medium containing 4.0 mg/l BAP, 50 mg/l ascorbic acid and 25 mg/l each of L-arginine, citric acid and adenine sulphate. Further shoot proliferation was done efficiently on MS medium containing 4.0 mg/l BAP and 1.0 mg/l NAA which was followed by a transfer on basal medium of ½ strength containing 6.0 mg/l NAA and 100 mg/l activated charcoal for in vitro rooting. Well rooted plantlets were hardened in greenhouse for 4 weeks which showed 100% survival rate when transferred to field. Genetic stability of in vitro raised plantlets with its mother plant was confirmed using inter simple sequence repeats (ISSR) and start codon targeted (SCoT) markers which showed monomorphic patterns (Rajput et al. 2020).

2.4 International and National Status

At the International level, many genus and species of bamboos have been tested for the technique of clonal propagation, some of which are as follows. Prutpongse and Gavinlertvatana (1992) have successfully micropropagated 54 species of 15 genera of bamboos using axillary buds on stem node segments as explant; Gielis and Oprins (2002) have succeeded to develop micropropagation protocol for about 60 temperate and tropical bamboos via axillary branching; Jimenez and Guevara (2007) successfully propagated *Guadua angustifolia* and *Dendrocalamus giganteus* through axillary shoot proliferation; Islam et al. (2011) successfully performed clonal propagation in *Bambusa vulgaris* by leafy branch cuttings.

Most of the work on the clonal propagation of bamboos was conducted in India. Das and Singh Samanta (2000) clonally propagated ornamental bamboo, *Bambusa vulgaris* by modifying culture media components; Das and Pal (2005) clonally propagated genetically uniform regenerant from axillary meristems of adult bamboo (*Bambusa tulda* and *Bambusa balcooa*); Anand et al. (2013) propagated an edible bamboo, *Bambusa bambos* by forced axillary branching (Arya et al. 1999, 2002, 2008a, b, c, 2009, 2012; Arya and Arya 2015; Bhariya et al. 2017; Bhadrawale et al. 2018; Rajput et al. 2020 and so on as mentioned above).

2.5 Clonal Propagation of Eucalyptus

Eucalyptus is a diverse genus of flowering trees and shrubs belonging to the myrtale family, *Myrtaceae* (Nagpal et al. 2010). *Eucalyptus* is one of the most valuable genera as it is the source of some of the heaviest, hardest and most durable hardwood of the world. It is used as a main source of biomass for paper pulp, fibre board,

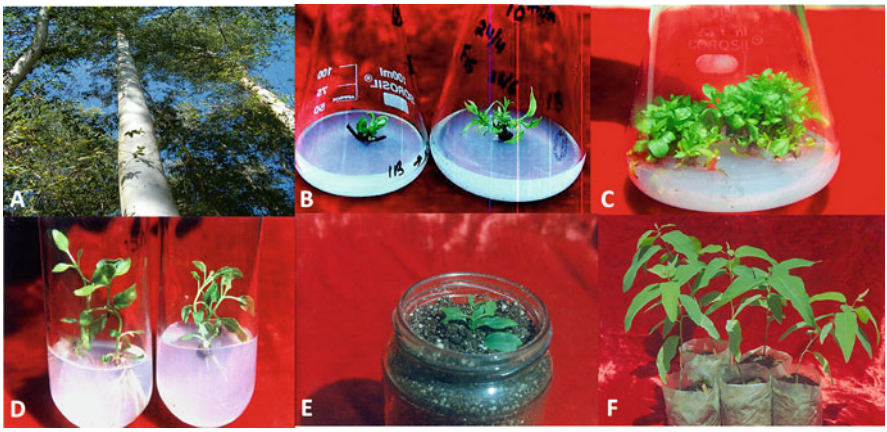

Fig. 2.3 Micropropagation of *Eucalyptus* FRI 5 hybrid: (**a**) plant in natural habit, (**b**) axillary bud break, (**c**) shoot multiplication, (**d**) *in vitro* rooting, (**e**, **f**) hardening and acclimatization

industrial charcoal and fuelwood (Turnbull 1999). To cope with their growing demands in the world, fast growing eucalyptus plantations are necessary (Arya et al. 2009). In the year 2009, Arya et al. developed protocols for the multiplication of two immensely important hybrids of eucalyptus, namely FRI-5 (*Eucalyptus camaldulensis* Dehn x *Eucalytpus tereticornis* Sm) (Fig. 2.3a) and FRI-14 (*Eucalyptus torelliana* F. V. Muell x *Eucalyptus citriodora* Hook). Nodal segments from 30–32 years old mature tree were selected as explants and were inoculated on MS medium supplemented with various concentrations of BAP, either alone or in combinations with auxins, i.e. IBA or NAA (Fig. 2.3b, c). MS medium was found appropriate for all the experiments conducted during the course of production and 85–92% rooting was achieved on MS medium of half strength supplemented with IBA (Fig. 2.3d). After hardening and acclimatization, 90–98% rate of survival was achieved (Fig. 2.3e, f). The parameters like height, DBH, clear bole length and self-pruning capability of both the hybrids were recorded for 3 years so as to estimate the suitability of these hybrids in various climatic zones of Uttarakhand.

In another study conducted on *Eucalyptus cloeziana*, the shoots of adult trees were used as explants for the in vitro establishment of cultures. Shoots were induced to multiply on Woody Plant Medium (WPM) supplemented with BAP and NAA at different concentrations. The proliferation promotion of shoots using optimum concentrations of plant growth regulators was found to be genotype dependent. The multiplication results were best achieved at BAP concentrations greater than 0.60 mg/l along with NAA at concentration 0.10 mg/l for the adult tree genotypes numbered 03 and 13; while for the genotype number 05, best results were achieved with WPM devoid of NAA. Shoot elongation was observed in WPM containing 0.1 mg/l BA. There was no effect observed of the pulse treatment of gibberellic acid (GA_3) provided. Ex vitro rooting and acclimatization of the regenerated plantlets was achieved successfully in mini-incubators and the adventitious roots arose

directly on the explants, providing propagules for establishing clonal micro-garden of selected adult trees of *E. cloeziana* (Silva de Oliveira et al. 2015).

2.6 International and National Status

Worldwide, the studies on clonal propagation of *Eucalyptus* were successfully achieved by Mehra-Palta in 1982, using cotyledons, shoot tips and nodal stem segments as explants; Trindade and Pais (2003) induced morphogenesis in *E. globulus* seeds, cotyledons, hypocotyls and leaves from in vitro clonal plantlets; Silva de Oliveira et al. (2015) propagated *E. cloeziana* under in vitro conditions as mentioned above.

In India, such clonal propagation studies were conducted by Gupta et al. (1983) on *E. citriodora* in 1981, using terminal buds as explants and on *E. torelliana* and *E. camaldulensis* in 1983 using nodal stem explants. Sita and Rani clonally propagated *E. grandis* in 1985 by employing nodal segments as explants. Kapoor and Chauhan micropropagated hybrid of *Eucalyptus* in 1992 using nodal stem explants. In 2009, Arya et al. developed a protocol for the micropropagation of a *Eucalyptus* hybrid as mentioned above. Girija Shankar regenerated *Eucalyptus camaldulensis* in 2012 using their nodal segments as explant under in vitro conditions (Girijashankar 2012).

2.7 Clonal Propagation of Chirpine

The genus *Pinus* belongs to the family *Pinaceae* and comprises approximately 94 species distributed in the northern hemisphere. *Pinus roxburghii* Sarg syn. *P. longifolia,* which is commonly known as long leaf pine or chir pine is the most valuable pine among the six indigenous pines found in Indian subcontinent (Kalia et al. 2007). It is used for the ornamental purposes. Besides this, the turpentine oil distillates from the oleo-resin leaves are utilized in manufacturing fireworks, disinfectants and insecticides and enter in some lubricating composition, hair fixing and nail polishing preparations (Anonymous 2003). It is used in preparation of ointments and plasters and in many products like chewing gum, varnishes and polishes, but is a common cause of contact allergy. The resin is applied to treat boils (Rajbhandari 2001) and taken orally to combat gastric troubles (Manandhar 2002).

In vitro proliferation of *Pinus roxburghii* Sarg was attempted using mature seeds of chirpine where the seeds were surface sterilized with 0.1% $HgCl_2$, followed by the treatment with 3.0% H_2O_2 (w/v) for 20 and 10 min, respectively. After each treatment, the seeds were rinsed with sterilized distilled water. The megagametophytes were cut out and sterilized with $HgCl_2$ for 5 minutes before the excision of embryo. These cut zygotic embryos were then induced on MS medium containing 3% sucrose and 0.8% agar at pH 5.8. BAP treatment was provided in two ways: (1) Continued exposure to lower BAP (5–25μM) containing

culture medium for 5 weeks. (2) Irregular exposure to high BAP (125 or 250μM) concentrations for 1, 2 or 4 h which was followed by culturing on MS basal medium for 5 weeks. The explants having induced adventitious buds were transferred to MS basal medium of ½ concentration for elongation and rooting was induced on ½ MS incorporated with 2.5μM NAA. Pulse treatment of BAP was found better in which the buds developed within 3–4 weeks, whereas in case of continuous exposure, buds developed in 4–5 weeks. After serial subcultures, the plantlets were hardened, acclimatized and transferred to fields successfully (Kalia et al. 2001). In year 2007, Kalia et al. cultured the shoot apices of *Pinus roxburghii* Sarg on MS medium supplemented with cytokinin [BAP, kinetin and N-benzyl-9-(2-tetrahydropyranyl) adenine (BPA)] alone, as well as in combination with auxin NAA. Among the three cytokinins tested at different concentrations, it was found that BAP at the concentration 10μM was optimal in terms of explants responsiveness, which was observed to be 97.22% and numbers of buds per explants were 7.42 approximately. The rate of elongation of induced buds on MS basal medium containing 0.5% activated charcoal was affected greatly by the concentration of BAP in medium. 2.4 times increase in length was observed within 4 weeks when the shoots were induced in lower concentrations of BAP and decapitated explants enhanced the rate of elongation of axillary buds. Subculturing the axillary buds established proliferating shoot cultures and reported that 2–3 cm length of shoots was more appropriate for culturing. 70.83% shoots could be induced to rooting when transferred on MS medium of half strength supplemented with 0.5μM NAA followed by its elongation in liquid ½ MS basal medium. Hardening and field transfer was successfully achieved after 20–22 weeks of shoot buds initiation.

In another study, the technique of somatic embryogenesis was performed on *Pinus roxburghii* Sarg, where megagametophyte containing different developmental stages of zygotic embryos were collected and inoculated on different media. Embryogenic explants of *Pinus roxburghii* with female gametophytes (Fig. 2.4a) containing immature pre-cotyledonary embryos were used to initiate embryogenic calli. Megagametophyte containing zygotic embryos at various developmental stages were collected and cultured on different media. Megagametophyte containing pre-cotyledonary zygotic embryos of 0.1–1.2 mm embryonal head when cultured on Douglus fir cotyledon revised (DCR) medium containing 2,4-D or NAA and BAP, initiated the embryonic calli. Callus development was initiated from the suspensor region of immature embryos. The technique of immature embryo culture was prominent as the callus development took place in the megagametophytes where the suspensor was extended onto the medium from the excised micropylar end (Fig. 2.4b, c). A pro-embryo of 6–8 meristematic cells and suspensor of 6–10 cells long (Fig. 2.4d), vacuolated cells dominated in the early stages of callus development. Cleavage polyembryony was observed in the proliferating callus and the somatic embryos was developed up to stage-1 and stage-2 embryos (Fig. 2.4e, f) on DCR medium enriched with 5μM 2,4-D or 10μM NAA (Arya et al. 2000).

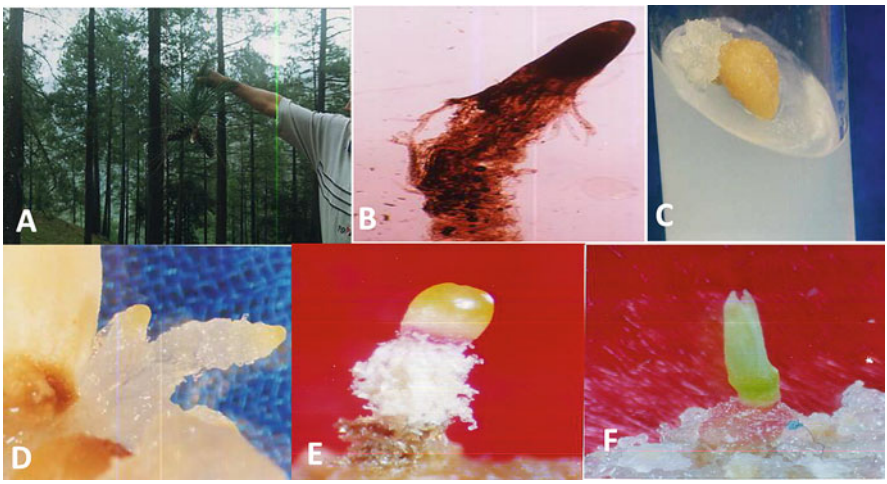

Fig. 2.4 Somatic embryogenesis in *Pinus roxburghii*: (**a**) immature cones, (**b**) zygote with compact head and suspensor, (**c**) callus from micropylar end, (**d**) somatic embryo with compact head and long suspensor, (**e**, **f**) stages of somatic embryos

2.8 International and National Status

At the International level, Muriithi et al. (1993) conducted plant regeneration in *P. roxburghii* using their seeds. The role of salicylic acid in the somatic embryogenesis of *P. roxburghii* was studied by Malabadi et al. (2008).

In India, the technique of somatic embryogenesis in *P. roxburghii* was studied by Arya et al. in 2000 as mentioned above. Besides this Mathur et al. (2000) also studied somatic embryogenesis in chir pine using immature zygotic embryos. Kalia et al. (Kalia et al. 2001, 2007) studied clonal propagation in *P. roxburghii* as mentioned in the above section. Bud break and plantlet regeneration was achieved successfully using terminal and axillary buds from the mature tree of *P. roxburghii* by Parasharami et al. (2003). Malabadi and Nataraja in 2006 studied cryopreservation and plant regeneration via apical domes in *P. roxburghii.*

2.9 Conclusion

Clonal propagation, better known as micropropagation is a commercially effective and practically oriented plant biotechnology technique applied to produce true-to-type plants using tissue and cell culture. Propagation of plants in tissue culture is used for the production of high-quality clones of standard plants and preserving germlines of commercially and economically valuable species. Micropropagation in various species of bamboo could be achieved using seeds, nodal segments, immature inflorescences, etc. as explants. In the case of *Eucalyptus*, clonal propagation was

found successful using the nodal shoots as explants, whereas in chir pines, it could be successfully done using seeds, shoot apices as well as the megagametophyte as explants. All these cultures were developed on Murashige and Skoog's medium supplemented with various concentrations of plant growth regulators like auxins (IAA, IBA, 2,4-D and NAA), cytokinins (BAP, Kinetin and BPA) and gibberellic acid (GA_3). The explants were surface sterilized with bactericidal and fungicidal agents before their inoculation under aseptic conditions. The technique of clonal propagation to raise the plantlets on a large scale was found useful as these trees are of high importance to mankind providing various resources of commercial and eco-sociological value. Where bamboos are utilized in agricultural, artistic, cultural, industrial, construction and household needs; *Eucalyptus* is used as a major source of biomass in paper pulp, fibre board, charcoal and fuelwood industries; and chirpines holds their values in ornamental purposes, to treat boils and gastric ailments, as biomass in manufacturing fireworks, disinfectants and insecticides and enter in some lubricating composition, hair fixing and nail polishing preparations, etc. Clonal propagation overcame the limitations of conventional agricultural practices and made it possible to conserve and propagate elite germlines all year round.

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3 In Vitro Production of Medicinal Compounds from Endangered and Commercially Important Medicinal Plants

Neha Sharma and Hemant Sood

Abstract

Medicinal plants are known for curing various disorders and ailments throughout the world. The therapeutic properties of these plants are attributed to the medicinal compounds produced by them. Growing demands of these medicinal compounds have urged a necessity to concentrate the research on enhancing their production by engaging different techniques. In this article, we have assessed different plant tissue culture techniques which could be applied for enhancing the production of commercially important secondary metabolites. Here, we have discussed the usage of cell/tissue culture techniques for mass propagation and bioactive metabolite production in endangered medicinal plants under in vitro conditions. Role of hairy root cultures, elicitation and precursor feeding, endophytes, and nanoparticles has also been studied for enhancement of commercially important bioactive compounds. Further, we have also highlighted the importance of metabolic engineering and CRISPR-based approaches for understanding the biology of biosynthesis of medicinal compounds.

Keywords

Secondary metabolites · Medicinal compounds · Micropropagation · Elicitors · Endophytes · Metabolic engineering

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3.1 Introduction

The importance of medicinal plants has been known to humans since ancient times and is also recommended for their medicinal properties by traditional medicinal systems like Unani, Ayurveda, and other ancient texts including Atharva Veda, Charaka Samhita, Rig Veda, Sushruta Samhita, etc. Conferring by World Health Organization (WHO), 65–80% people of developing nations cannot access the expensive modern pharmaceutical products and depend on medicinal plants to endure their prime healthcare needs (Palhares et al. 2015). The herbal medicines are less expensive and safer than synthetic or modern drugs (Karimi et al. 2015). Approximately 25% of modern medicines and 60% of anticancer medicines are obtained from natural resources (Palhares et al. 2015). Medicinal compounds are mainly secondary metabolites, which are the resultant of primary metabolites and involved in directly in the life of plant cells by playing role in their defense, signaling, and chemical adaptations to environmental stresses. These medicinal compounds are obtained from various plant organs like bark, flower, leaves, stem, root, seed, or whole medicinal plant and can vary in a tissue and organ specific way during different developmental stages (Wink 2003). Secondary metabolites are mainly categorized as terpenoids (derived from acetyl coenzyme A or glycolysis cycle intermediates), phenolics (aromatic rings 11 bearing a hydroxyl functional group), and alkaloids (nitrogen containing compounds).

Due to emergence of new products like health care, personal care, and immunity-boosting formulations, industrial demand for herbal products has increased and will further tend to rise in future. Estimates show that the international demand for plant derived drugs is likely to approach ~US$5000 billion by end of the year 2050 (Pan et al. 2014). Currently, majority of herbal collections are obtained from their natural habitat and only 10% are contributed by the cultivated medicinal plants. The escalating demand of herbal products is affecting the dwindling population of medicinal plants in wild. Slow growth rate, complex accumulation patterns, narrow geographic ranges along with unorganized and extensive harvesting of medicinal plants are making them more prone to extinction and loss of genetic diversity. Also, uneconomical chemical synthesis and inaccessibility of quality plant material in adequate amounts hinder in satisfying the increasing industrial demands. Therefore, biotechnological tools offer valuable alternatives for production of desirable medicinal compounds by enhancing their biosynthesis and accumulation. In this chapter, we have reviewed different techniques and approaches used for production as well as enhancement of commercially important compounds in medicinal plants.

3.2 Medicinal Compounds Production Using Cell/Tissue Culture Techniques

In medicinal plants, cell/tissue culture techniques are promising methods for controlled biosynthesis of numerous bioactive compounds. These are environment friendly and act as cost-effective alternative to chemical synthesis of medicinal

compounds. These techniques enable the in vitro grown plants to biosynthesize particular medicinal compounds analogous to the intact plants and also aid in producing secondary metabolites at large scale without any seasonal constraints. Utilizing cell/tissue culture techniques, various medicinal plants can be established under aseptic conditions using different explants including meristems, leaves, stems, and roots for mass propagation and production of bioactive metabolites. Secondary metabolite accumulation is genotype specific; therefore, high metabolite content producing genotypes or cell/organ lines needs to be selected for induction of in vitro cultures of medicinal plants. Various valuable medicinal compounds are biosynthesized in callus or suspension cultures, while some metabolites are produced in more organized structures like shoots, roots, glands, or somatic embryos. Callus or suspension cultures have been widely utilized for production of secondary metabolites and can be efficiently upscaled for production of important medicinal compounds under in vitro conditions (Kapoor et al. 2018; Kumar et al. 2020). Organ culture under aseptic conditions is required for biosynthesis of some medicinal compounds which need differentiated structure or organ for production of medicinal compound. For example, *Panax ginseng* roots for saponins, foliar glands of *Hypericum perforatum* for hypericins and hyperforins, *Nicotiana tabacum roots for* biosynthesis of lysine to anabasine and its leaves for conversion of anabasine to nicotine (Hussain et al. 2012). Numerous studies have been attempted on medicinal compounds production in medicinal plants using cell suspension or organ/tissue culture techniques and some of these studies have been summarized in Table 3.1. Biosynthesis of bioactive compounds under in vitro conditions is dependent on various factors including culture media composition (carbon source, macronutrients, micronutrients, other organic compounds and plant growth hormones), pH of media, explant or inoculum concentration, and other suitable environmental conditions like light, temperature, aeration, and agitation. Optimization of these factors aids in improvement of metabolite productivity. There are studies where higher metabolite content in cultured cells has been reported compared to native medicinal plants like camptothecin in *Ophiorrhiza mungos* (Deepthi and Satheeshkumar 2017), shikonin in *Onosma bulbotrichom* (Bagheri et al. 2018), and vinblastine and vincristine produced in *Catharanthus roseus* (Mekky et al. 2018). Cell/tissue culture techniques offer various advantages for medicinal compound production such as biosynthesis of secondary metabolites under in vitro conditions is more reliable, simpler, and predictable. Also, the process of extraction of valuable phytochemicals from in vitro grown cell suspensions or tissue culture plants is fast and effective in comparison to isolation from whole plants. Interfering secondary metabolites can also be avoided in cell cultures. However, cost-effective parameters need to be considered for their economic production at large scale.

Table 3.1 Production of bioactive medicinal compounds in plants via cell/tissue culture techniques

Plant	Medicinal compound	Properties	Type of culture	References
Adhatoda vasica	Vasicinone	Bronchodilatory, cardiac-stimulating, and anti-anaphylactic effects	Callus and stem	Panigrahi et al. (2017)
Ailanthus altissima	Alkaloids	Antibacterial, antioxidant, anti-progestogenic, antiviral, antidiarrheal, anti-inflammatory, antipyretic, analgesic, antihistaminic, antiparasitic, cytotoxic, insect repellent	Suspension	Anderson et al. (1987)
Ammi majus	Umbelliferones	Antihypertensive, anti-inflammatory, can also treat bronchitis, hepatitis, gastrointestinal infections, and asthma	Shootlet	Krolicka et al. (2006)
Artemisia annua	Artemisinin	Antimalarial	Callus	Baldi and Dixit (2008)
Aspidosperma ramiflorum	Ramiflorin	Antimicrobial	Callus	Olivira et al. (2001)
Azadirachta indica	Azadirachtin	Antioxidant, antibacterial	Suspension	Sujanya et al. (2008)
Brucea javanica	Cathine	Anticancer, antimalarial, and anti-inflammatory	Suspension	Wagiah et al. (2008)
Camellia chinensis	Flavones	Antimutagenic, antitumour, antioxidant, anticoagulant, antiviral	Callus	Nikolaeva et al. (2009)
Catharanthus roseus	Vincristine	Anticancer	Callus	Mekky et al. (2018)
Coptis japonica	Berberine	Anti-inflammatory, immunomodulatory, antioxidative, cardioprotective, hepatoprotective, and reno-protective	Suspension	Morimoto et al. (1988)
Fritillaria cirrhosa	Peimissine, verticine, and verticinone	Antitussive and expectorant	Bulblets and callus	Chang et al. (2020)
Fritillaria unibracteata	Alkaloids	Antitussive	Shoot	Gao et al. (2004)

(continued)

Table 3.1 (continued)

Plant	Medicinal compound	Properties	Type of culture	References
Hypericum perforatum	Hypericins	Wound healing and anti-depressive	Shoot	Kornfeld et al. (2007)
Hypericum perforatum	Hypericin	Wound healing and anti-depressive	Suspension	Hohtola et al. (2005)
Nicotiana tabacum	Nicotine	Angiogenic, antispasmodic, sedative, discutient, diuretic, emetic, expectorant, irritant, narcotic	Suspension	Mantell et al. (1983)
Ophiorrhiza rugosa	Camptothecin	Anticancer, antibacterial	Shoot	Vineesh et al. (2007)
Panax ginseng	Saponins	Homeostasis, anti-Alzheimer, anti-amnesia, and anticancer	Cell suspension	Wu and Li (2002)
Papaver somniferum	Morphine, codeine, noscapine, papaverine	Analgesic, narcotic, sedative, stimulant	Callus	Oluk (2006)
Picrorhiza kurroa	Picroside-I	Hepatoprotective, antioxidant, immunomodulatory, antimalarial, anti-inflammatory, anti-cancerous, neuroprotective, anti-asthmatic, and antidiabetic	Shoot	Sharma et al. (2015a, b)
Podophyllum hexandrum	Podophyllotoxin	Anticancer	Suspension	Chattopadhyay et al. (2001)
Rauvolfia serpentina	Serpentine	Antipsychotic	Callus	Salma et al. (2008)
Rauvolfia serpentina	Reserpine	Antihypertensive	Callus	Nurchgani et al. (2008)
Rhodiola rosea	Salidroside	Neuroprotective, anti-stroke, anti-depressive, anti-traumatic	Shoot	Tasheva and Kosturkova (2010)
Salvia officinalis	Flavonoid	Anti-inflammatory and antinociceptive	Shoot	Grzegorczyk and Wysokinska (2008)
Taxus baccata	Taxol	Anticancer	Suspension	Hirasuna et al. (1996)
Withania somnifera	Steroidal lactone	Aphrodisiac, sedative, rejuvenative	Callus	Mirjalili et al. (2009)

3.3 Hairy Root Culture

Hairy roots are comprised of differentiated transformed roots which are produced by infection caused by *Agrobacterium rhizogenes.* T-DNA located in root-inducing (Ri) plasmid of this pathogen is transferred and integrated into the genome of host plant, thereby leading to formation of hairy roots at the wounded site. These proliferating roots show lateral branching, self-regulating rapid growth, absence of geotropism and are genetically stable (Hussain et al. 2012). Unlike natural roots, which are accessible at particular period of the year, these proliferating roots could biosynthesize bioactive compounds throughout the year without the effect of seasonal variations. Attributed to high productivity of secondary metabolites, this technique has become popular tool to produce same medicinal compounds corresponding to the wild type roots. Therefore, hairy roots provide an imperative alternative to natural plant material for biosynthesizing many valuable medicinal compounds. Production of some important medicinal compounds using this technique has been listed in Table 3.2.

3.4 Role of Elicitors and Precursor Feeding for Enhancement of Medicinal Compounds Production

Elicitors act as the signal molecules which can be biotic or abiotic in nature and are capable of inducing or enhancing the production of specific secondary metabolites by initiating defense or stress related responses. Biotic elicitors are partially purified extracts of biological origin like fungus, bacteria, yeast, or the plant itself. Some examples of biotic elicitors include polysaccharides, glycoproteins, inactivated enzymes, alginate, xanthan, yeast extract, fungal homogenate, etc. Abiotic elicitors are substances which are of non-biological origin and are categorized in physical, chemical, and hormonal factors such as UV rays, light, temperature, heavy metal salts, osmotic stress, antibiotics, jasmonates, methyl jasmonic acid, salicylic acid, acetyl salicylic acid, abscisic acid, etc. (Halder et al. 2019). They have been extensively used in cell/organ and hairy root cultures of different plant species to enhance the production of medicinal compounds. Further, they can also trigger the outflow of intracellular products and ease the extraction and purification of the targeted compounds. Application of different biotic and abiotic elicitors for production of various medicinal compounds has been summarized in Table 3.3.

The approach of supplementing the extrinsic precursors to growth medium is also used to enhance the production of bioactive compounds. For production of desired metabolites, if the endogenous levels of certain precursors are low, then supplying the precursors of key biosynthetic steps enhances the production of those metabolites (Bourgaud et al. 2001). Several reports have shown the effect of feeding suitable precursor for increasing the productivity and accumulation of target metabolites in different medicinal plants under in vitro conditions. Exogenous feeding of tryptamine and loganin has enhanced the secologanin production in mutant cell lines of *Catharanthus roseus* (Whitmer et al. 1998). In another study, exogenous

Table 3.2 Medicinal compounds produced in some plants using hairy root culture techniques

Plant name	Medicinal Compound	Properties	References
Aconitum heterophyllum	Aconites	Anticancer, anti-inflammatory, antimicrobial, pesticidal	Giri et al. (1997)
Agastache rugosa	Rosmarinate	Antioxidant, anti-inflammatory, antimutagenic, antimicrobial, antiviral, astringent	Lee et al. (2007a, b)
Angelica gigas	Deoursin	Anticancer, antibacterial, antinematodal	Xu et al. (2008)
Artemisia annua	Artemisinins	Antimalarial	Liu et al. (2002)
Astragalus mongholicus	Cycloartane saponin	Antimicrobial, antifungal	Ionkova et al. (1997)
Beta vulgaris	Betalains	Antioxidant, anti-inflammatory	Pavlov et al. (2005)
Brugmansia candida	Tropane alkaloids, hyoscyamine, scopolamine Scopolamine and hyoscyamine Scopolamine and hyoscyamine	Anti-asthmatic, anticholinergic, narcotic, anesthetic	Marconi et al. (2008)
Catharanthus roseus	Alkaloids	Anticancer	Hanafy et al. (2016)
Centella asiatica	Asiaticoside	Wound healing, memory improvement, cognition, and mood modulation	Nguyen et al. (2019)
Coleus forskohlii	Forskolin and rosmarinic acid	Anti-inflammatory and antipyretic	Li et al. (2005)
Echinacea purpurea	Alkamides	Immunostimulatory, anti-inflammatory	Romero et al. (2009)
Fagopyrum esculentum	Rutin	Antioxidant, anti-inflammatory, anticancer, antimicrobial, cardioprotective, hypolipidemic, antidiabetic, reno-protective, wound healing, anti-stress	Lee et al. (2007a, b)
Gentiana macrophylla	Gentiopicroside	Antimicrobial	Zhang et al. (2010)
Gingko biloba	Terpenoids	Antioxidant, improve blood flow to brain	Ayadi et al. (2003)
Linum flavum	Lignans	Anticancer	Renouard et al. (2018)
Lithospermum erythrorhizon	Shikonin	Antimicrobial	Tatsumi et al. (2016)
Panax ginseng	Ginsenosides	Neuroprotection, anticancer, antidiabetic, hepatoprotective, immunomodulatory	Murthy et al. (2017)

(continued)

Table 3.2 (continued)

Plant name	Medicinal Compound	Properties	References
Papaver somniferum	Morphine, sanguinarine	Analgesic, narcotic, sedative, stimulant	Le Flem-Bonhomme et al. (2004)
Picrorhiza kurroa	Picroliv	Hepatoprotective, anti-inflammatory, anticholestatic, antiulcerogenic, anti-asthmatic, antidiabetic, immunomodulatory	Verma et al. (2015)
Rauvolfia micrantha	Ajmalicine and ajmaline	Neuroprotective	Sudha et al. (2003)
Rubia akane	Alizarin and purpurin	Anticancer, antimalarial, antimicrobial, antifungal, antioxidant	Lee et al. 2010
Silybum marianum	Flavonolignan	Hepatoprotective, anticancer, antihepatitis	Rahnama et al. (2008)
Swertia japonica	Amarogenetin	Antibacterial, antihepatitis, anticholinergic, chemopreventive, anti-lieshmanial	Ishimaru et al. (1990)
Withania somnifera	Withanoloid A	Aphrodisiac, liver tonic, anti-inflammatory	Murthy et al. (2008)

supplementation of geraniol, 10-hydroxygeraniol, or loganin significantly increased tabersonine levels in hairy roots of *Catharanthus roseus* (Morgan et al. 2000). Bemani et al. (2013) have observed that application of phenylalanine to suspension cell cultures of *Corylus avellana* L. has increased the production of taxol along with the antioxidant activity and cytotoxic effects. Lately, Kumar et al. (2016) have found that exogenous supply of precursors like cinnamic acid and catalpol influences the synthesis of picroside-I in shoot cultures of *Picrorhiza kurroa.* Recently, Thakur et al. (2019) have reported enhanced picrosides content via nutrition feeding method in aeroponic and hydroponic system of *Picrorhiza kurroa*. Hence, elicitation and precursor feeding under in vitro conditions can be used as a viable tool for enhancing the production of pharmaceutically important secondary metabolites which are otherwise produced in low quantities.

3.5 Role of Endophytes in In Vitro Production of Medicinal Compounds

Endophytes are microbes that exist inside the plant living tissues without causing any negative effect to them. These microbes have the capability to biosynthesize secondary metabolites similar to that produced by their host plants (Köberl et al. 2013). They produce secondary metabolites due to the horizontal gene transfer between host and endophyte. These secondary metabolites empower them to create supremacy over other invading pathogens or enhance plant defense and consequently securing themselves against various pathogens (Mousa et al. 2016; Naik

Table 3.3 Different elicitors utilized in production of secondary metabolites in plants

Plant name	Medicinal compound	Elicitor	Culture type	References
Andrographis paniculata	Andrographolide	Methyl jasmonate	Cell suspension	Sharma et al. (2015a, b)
Ammi majus	Xanthotoxin	Copper	Shoot	Purohit et al. (1995)
Ambrosia artemisiifolia	Thiarubrine A	*Protomyces gravidus*	Hairy root	Bhagwath et al. (2000)
Azadirachta indica	Azadirachtin	*Claviceps purpurea*	Hairy root	Satdive et al. (2007)
Bacopa monnieri	Bacosides	Jasmonic acid, methyl jasmonate, pH, sucrose, copper	Shoot	Naik et al. (2010), Sharma et al. (2013), Munish et al. (2015)
Catharanthus roseus	Vinblastine and vincristine	Sodium chloride	Embryogenic tissues	Fatima et al. (2015)
Corylus avellana	Paclitaxel	Sucrose	Cell suspension	Sara-Alsadat et al. (2015)
Digitalis purpurea	Digitoxin	Salicylic acid	Shoot	Patil et al. (2013)
Datura metel	Hyoscyamines, scopolamines	Salicylic acid	Root	Ajungla et al. (2009)
	Atropine	Silver, *Escherichia* coli, *Bacillus cereus, Staphylococcus aureus*	Hairy root	Zahra et al. (2015)
Echinacea pupurea	Derivatives of caffeic acid	Gibberellic acid	Hairy root	Abbasi et al. (2012)
Gymnema sylvestre	Gymnemic acid	Methyl jasmonate, salicylic acid, *Aspergillus niger, Escherichia coli, Bacillus subtilis, Saccharomyces cerevisiae*	Cell suspension	Chodisetti et al. (2013), Chodisetti et al. (2015)
Glycyrrhiza uralensis	Glycyrrhizic acid	Salicylic acid	Adventitious root	Li et al. (2015a)
Hypericum adenotrichum	Hypericin and pseudohypericin	Sucrose, polyethylene glycol	Seedling	Omer et al. (2013)
Hypericum hirsutum	Hypericin and pseudohypericin	Salicylic acid	Shoot	Coste et al. (2011)
Hypericum perforatum	Hypericin and pseudohypericin	Chitin, pectin, dextran	Shoot	Sonja et al. (2015)
	Phenylpropanoid and naphtodianthrone	Chitin, *Fusarium oxysporum, Phoma*	Cell suspension	Sonja et al. (2015)

(continued)

Table 3.3 (continued)

Plant name	Medicinal compound	Elicitor	Culture type	References
		exigua, Botrytis cinerea		
Melissa officinalis	Rosmarinate	Ozone	Shoot	Tonelli et al. (2015)
Perovskia abrotanoides	Cryptotanshinone and tanshinone IIA	Methyl jasmonate, sorbitol, silver, yeast extract	Adventitious roots	Arehzoo et al. (2015)
Picrorhiza kurroa	Picroside-I	Methyl jasmonate, sodium nitroprusside, abscisic acid, seaweed extract	Shoot	Sharma et al. (2016)
Plumbago indica	Plumbagin	Jasmonic acid	Hairy root	Gangopadhyay et al. (2011)
Plumbago rosea	Plumbagin	Jasmonic acid, yeast extract	Cell suspension	Silja et al. (2014)
Pueraria thomsnii	Puerarin	Ozone	Cell suspension	Sun et al. (2012)
Salvia castanea	Tanshinone	Methyl jasmonic acid, silver	Hairy root	Li et al. (2015b)
Salvia miltiorrhiza	Tanshinone	Methyl jasmonate, salicylic acid, gibberellic acid, *Trichoderma atroviride*	Hairy root	Yuan et al. (2008), Qianliang et al. (2013), Xiaolong et al. (2015)
Salvia officinalis	Diterpenoid	Methyl jasmonate, sodium salicylate	Shoot	Izabela et al. (2009), Kracun-Kolarevic et al. (2015)
Silybum marianum	Silymarin	Methyl jasmonate, yeast extract	Cell suspension	Firouzi et al. (2013)
Stevia rebaudiana	Steviol glycoside	Proline, polyethylene glycol	Callus and cell suspension	Pratibha et al. (2015)
Taverniera cuneifolia	Glycyrrhizic acid	Methyl jasmonate, *Mucor hiemalis, Rhizobium leguminosarum*	Root	Awad et al. (2014)
Vitis vinifera	Resveratrol, trans-resveratrol, stilbene, viniferins	UV radiations, methyl jasmonate, salicylic acid, chitin, silver, cadmium, cobalt	Cell suspension	Taurino et al. (2015), Xu et al. (2015)

(continued)

Table 3.3 (continued)

Plant name	Medicinal compound	Elicitor	Culture type	References
Withania somnifera	Withanolide A, withanone, and withaferin A	pH, methyl jasmonate, salicylic acid	Hairy root	Praveen et al. (2012), Sivanandhan et al. (2013)
	Withanolide A	pH, sucrose	Cell suspension	Praveen et al. (2010)

et al. 2019). Production of important medicinal compounds by endophytes has been summarized in Table 3.4. Therefore, endophytic microbes constitute an important source for bioactive medicinal compounds and in future these could be harnessed for production of important metabolites in large scale.

3.6 Metabolic Engineering

Metabolic engineering is optimization of cellular processes and metabolic routes in an organism to improve secondary metabolites production (García-Granados et al. 2019). This technique involves manipulation of metabolic fluxes and endogenous metabolic pathways for enhancing the biosynthesis of specific compounds. The carbon flux towards the targeted product can be increased by overexpression of selected genes or by knocking down/out the key genes of competitive pathways. Recombinant DNA tools such as RNA interference (RNAi), antisense RNA, co-suppression, dsRNA mediated gene silencing, etc. can be used to upregulate metabolic pathways of desired products and obscuring the key genes responsible for production of unwanted metabolites. Wagner et al. (2008) have reviewed that gene knockdown and overexpression approaches can be used to alter secondary metabolite production in plants and have mentioned various examples such as high amounts of pelargonidin and reduced amounts of flavonols in *Nicotiana tabacum,* decreased ginsenoside production in *Panax ginseng*, and high reticuline accumulation in *Papaver somniferum.* Targeting transcription factors can aid in overcoming flux bottlenecks as well as gene expressions in particular tissues and the presence of multiple enzymatic steps in a metabolic pathway (Grotewold 2008). In *Artemisia annua*, artemisinin and artemisinic acids were enhanced by overexpressing jasmonic acid responsive *AP2/ERF* transcription factors (Yu et al. 2012). Overexpression of transcription elements like *ORCA2* or *ORCA3* in cell suspension/hairy roots cultures of *C. roseus* has enhanced the biosynthesis of different medicinal compounds like ajmalicine, catharanthine, serpentine, and tryptamine (Sun and Peebles 2017). In plants, various pathways such as shikimate, polyketide, and terpenoid routes are involved in biosynthesis of secondary metabolites, which can be reconstructed in heterologous hosts for overproduction and mining of important medicinal compounds (Chandran et al. 2020).

In the recent times, clustered regularly interspaced short palindromic repeat (CRISPR) genome editing system has transformed the metabolic engineering and

Table 3.4 Some medicinal compounds biosynthesized by endophytes

Plant name	Medicinal compound	Producing microorganism	References
Azadirachta indica	Azadirachtins	*Eupenicillium parvum*	Kusari et al. (2012)
Artemisia annua	Artemisinin	*Colletotrichum* sp., *Pseudonocardia sp.*	Wang et al. (2001), Li et al. (2012)
Apodytes dimidiata	Camptothecin	*F. solani*	Shweta et al. (2010)
Camptotheca acuminate	Camptothecin	*Alternaria* sp., *Aspergillus* sp., *Fusarium solani, Trichoderma atroviride*	Kusari et al. (2009a), Pu et al. (2013), Su et al. (2014)
Catharanthus roseus	Vinblastine	*Talaromyces radicus*—CrP20	Palem et al. (2016)
	Vincristine	*Fusarium oxysporum, Talaromyces radicus*—CrP20	Zhang et al. (2000), Palem et al. (2016)
Dysoxylum binectariferum	Rohitukine	*Fusarium proliferatum*	Kumara et al. (2012)
Ginkgo biloba	Ginkgolide B	*Fusarium oxysporum*	Cui et al. (2012)
Juniperus communis	Podophyllotoxin	*Aspergillus fumigatus*	Kusari et al. (2009b)
Juniperus recurva	Podophyllotoxin	*F. oxysporum*	Kour et al. (2008)
Macleaya cordata	Sanguinarine	*F. proliferatum*	Min et al. (2014)
Nothapodytes foetida	Camptothecin	*Entrophospora infrequens*, *Neurospora* sp.	Puri et al. (2005), Amna et al. (2006), Rehman et al. (2008)
Podophyllum hexandrum	Podophyllotoxin	*Alternaria* sp., *A. fumigatus*, *F. solani*	Yang et al. (2003), Kusari et al. (2009b), Nadeem et al. (2012)
Podophyllum peltatum	Podophyllotoxin	*Phialocephala fortinii*	Eyberger et al. (2006)
Podocarpus sp.	Taxol	*A. fumigatus*	Sun et al. (2008)
Rhizophora annamalayana	Taxol	*F. oxysporum*	Elavarasi et al. (2012)
Taxus baccata L.	Taxol	*Fusarium redolens, Cladosporium* sp.	Garyali et al. (2013), Kasaei et al. (2017)
Taxus celebica	Taxol	*F. solani*	Chakravarthi et al. (2008)
Taxus chinensis	Taxol	*Botrytis* sp., *F. solani, Metarhizium anisopliae, Mucor rouxianus, Ozonium* sp.	Hu et al. (2006), Deng et al. (2009), Liu et al. (2009), Miao et al. (2009), Wei et al. (2010)
Taxus cuspidata	Taxol	*Botrytis* sp., *Pestalotiopsis versicolor*	Zhao et al. (2008), Kumaran et al. (2010)
Taxus globosa	Taxol	*Nigrospora* sp.	Ruiz-Sanchez et al. (2010)
Taxus x media	Taxol	*Cladosporium cladosporioides* MD2	Zhang et al. (2009)

playing role in manipulating metabolic pathways to biosynthesize desired medicinal compounds. CRISPR technology can be employed in different approaches for manipulating the biosynthetic pathways which include controlling transcription factors to trigger or block specific effectors of metabolic pathways, enzyme manipulation, enzyme inhibition, blocking branch pathways, switching path to alien metabolite, removing limited availability to precursor, translational regulation, protein modification, etc. (Sabzehzari et al. 2020a, b). Alagoz et al. (2016) have knocked out benzyl isoquinoline alkaloids pathway gene, viz. *4OMT2* in *Papaver somniferum* and observed reduced morphine, thebaine levels in edited plants. Similarly, in *Salvia miltiorrhizarosmarinic acid synthase* (*SmRAS*) gene of phenolic acid metabolic pathway has been edited using CRISPR/Cas9 technology (Zhou et al. 2018). They have found declined rosmarinate and lithospermic acid B contentsin edited hairy root lines, specifically in the homozygous mutants. Additionally, enhanced level of rosmarinic acid precursor 3,4-dihydroxy phenyl lactic acid was observed. Therefore, the study suggested that the rosmarinic acid synthase enzyme is important for biosynthesis of rosmarinic acid and can be targeted to increase the level of desired metabolites.

3.7 Nanoparticles Mediated Secondary Metabolite Production

Nanoparticles are 1–100 nm sized particles, which can interfere with various signaling pathways and can modulate secondary metabolite production in plants; however, the exact mode of action behind this modulation is yet to be uncovered (Marslin et al. 2017). Zhang et al. (2013) have reported 3.9-fold enhanced artemisinin content by silver nanoparticle treatment in *Artemisia annua* L. hairy roots. In *A. thaliana*, silver nanoparticles have upregulated anthocyanin and flavonoid biosynthetic genes (Garcia-Sanchez et al. 2015). Recently, Jasim et al. (2017) have studied the consequence of silver nanoparticles treatment in *Trigonella foenum graecum* L. and observed a substantial increase in diosgenin concentration. Nanoparticles have potential to increase secondary metabolite production in plants; however, deeper understanding is still required to fully exploit this technique to produce commercially important medicinal compounds.

3.8 National and International Status of Medicinal Compounds in Commercially Important Medicinal Plants

India harbors diverse potential for medicinal plants due to its different climatic zones, geographical variance, and rich biodiversity. Medicinal compounds of these plants are used in modern therapeutic drugs throughout the world, for example, use of berberine from *Coptis japonica* as cardioprotective, hepatoprotective, and renoprotective (Morimoto et al. 1988), reserpine, serpentine from *Rauwolfia serpentina* root in hypertension (Salma et al. 2008), vinblastine and vincristine, from *Catharanthus roseus* for cancer treatment (Mekky et al. 2018). Several types of

research have been conducted by national and international research community to enhance medicinal compounds production in commercially important medicinal plants. The demand of medicinal plants is constantly increasing around the globe. China, India, Nigeria, the United States of America (USA), and WHO are significantly investing on herbal medicinal research, so that it can contribute to global health (WHO 2002).

3.9 Conclusion

Medicinal plants have been used to cure various diseases since early times. Slow growth rate and threat of extinction of some medicinal plant species along with the inaccessibility of superior plant material in suitable amounts hamper in meeting the increasing industrial demands. Biotechnological approaches provide an additional production system to prevail over inadequate availability of bioactive and commercially important medicinal compounds. Cell/tissue culture techniques aid in multiplication of medicinal plants under in vitro conditions which provide an incessant, reliable source of natural products and thus relieve pressure from their natural habitat. Advances in hairy root cultures and endophytes can lay out new modes for cost efficient, viable production of low volume pharmaceutically important secondary metabolites. Elicitation with various biotic and abiotic elicitors has been broadly practiced for production of medicinal compounds in different species and will further aid in meeting growing demand of these natural metabolites. Metabolic engineering has opened new gateways to produce important metabolites by manipulating endogenous biochemical pathways. The emergence of CRISPR/Cas system has also opened new doors towards next-generation metabolic engineering which can assist in better elucidation of secondary metabolite production in plants.

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Double Haploid Production and Its Applications in Crop Improvement

4

Awadhesh Kumar Mishra, Rajesh Saini, and Kavindra Nath Tiwari

Abstract

Haploid plants are those having gametophytic chromosome number while doubled haploids are haploids that are produced after chromosome duplication. The production of haploids and doubled haploids (DHs) through gametic tissues allows a single-step development of complete homozygous lines from heterozygous parents. DHs shorten the time required to produce homozygous plants in comparison with the conventional breeding which requires several generations of selfing. The production of haploids and DHs provides a particularly attractive biotechnological tool, and the development of haploidy technology and protocols to produce homozygous plants had a significant impact on agricultural systems. Nowadays, these biotechnologies represent an integral part of the breeding programs of many agronomically important crops. There are several available methods to obtain haploids and DHs, of which in vitro anther or isolated microspore culture is the most effective and widely used. This chapter throws the light on the current status of knowledge on the production of haploids and DHs through anther or pollen cultures.

Keywords

Anther culture · Doubled haploids · Inbred lines · Embryogenesis

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Abbreviation

DH	Doubled haploid
MCS	Multi-cellular structure
PCD	Programmed cell death
PEG	Poly-ethylene glycol
SAM	Shoot apical meristem

4.1 Introduction

In recent years, microspore culture for doubled haploids (DH) production has become a routine biotechnological tool for value addition in crops. Doubled haploids (DHs) are plants derived from a single immature pollen grain and doubled artificially to form homozygous diploids. DH individual has two identical homologs, so that the amount of recombination information is equivalent to a backcross. DHs may be transferred between different laboratories and environments for assessing the effect of the environment on gene expression. Successful production of doubled haploids on a routine basis would reduce cultivar development time, and provide excellent recombinant inbred lines for molecular mapping applications. The first DH crop plant and its use in genetic analysis (e.g., establishing chromosomes maps) were reported by Forster et al. (2007). The development of DH plants also has a direct implication in breeding as it achieves homozygosity in segregating populations in a single generation (Dwivedi et al. 2015). The value of DHs for breeding and genetic studies has long been recognized and now widely used for the crop breeding program (Baenziger et al. 1984; Asif 2013; Li et al. 2013). This breeding tool not only eliminates the several generations needed to attain genetic stability and uniformity in breeding line but also significantly reduces the size of populations needed to find a desired genotype (Ren et al. 2017). DH breeding through microspore culture is well developed in several herbaceous plants (Prem et al. 2012). In last two decades, the DH technology has emerged as an efficient tool for inbred line development in breeding program (Chaikam et al. 2019). It is also used for in vitro screening for complex traits like herbicide-resistance and abiotic stress tolerance such as salinity tolerance, drought, and cold. It is useful for developing linkage maps by combining the molecular markers (Melchinger et al. 2011). The common methods used for DH production are microspore culture, anther culture (Doi et al. 2010), and ovary/ovule culture (Doi et al. 2013). A wide range of genetic variability has been induced after mutagenic treatments for use in plant breeding and crop improvement programs. The embryogenic microspores are the prime targets for mutagenic treatment with the chemicals such as ethyl methane sulfonate (EMS) (Beversdorf and Kott 1987), sodium azide (NaN_3) (Polsoni et al. 1988), *N*-methyl-*N*-nitrosourea (MNU) (Cegielska-Taras et al. 1999), and *N*-ethyl-*N*-nitrosourea (ENU) (Swanson et al. 1988). They have also been treated with physical mutagens such as gamma rays

(McDonald et al. 1991), X-rays (McDonald et al. 1991), and UV rays (Ahmad et al. 1991). DH has been developed in at least 200 plant species and is widely used in Brassicas and cereals, including wheat, barley, rice, and maize (Forster et al. 2007; Dunwell 2010; Germana 2011; Dwivedi et al. 2015). Haploids are generated by in vitro procedures based on the culture of immature male and female gametophytes and by in vivo procedures based on inter- and intraspecific hybridization causing uniparental chromosome elimination. Once haploid plants become available, their genome must be doubled to produce fertile DH lines. However, the efficiency of embryogenesis in anther culture was quite low and large genotypic variation was observed. Gynogenesis is an alternative process for haploid and/or DH induction. Gynogenic regeneration has been obtained through in vitro culture of unfertilized ovules, ovaries, and whole flowers in several economically important crops such as onion (Muren 1989; Alan et al. 2007), sugar beet (Van Geyt et al. 1987; Gurel et al. 2000), gerbera (Tosca et al. 1999), and cucumber (Gemes-Juhasz et al. 2002; Diao et al. 2009). The use of haploid has emerged as key strategies for agricultural crop improvement with desirable trait such as quality, crop yield, and resistance to environmental stresses. The haploids having single set of chromosome in sporophytic phase have become a valuable source to screen for desired traits or to introduce mutation in their genetic constitution. The DHs can be obtained by spontaneous or artificial doubling of chromosomes. The DHs are homogenous at all loci and achieve complete homozygosity. The in vitro production of haploid for crop improvement has been successfully achieved in many crops like barley, wheat, maize, rice, potato, tomato, brassicas, sunflower, grapes, and many more. The DHs are an excellent source for evolutionary studies, cytoplasmic research, and gene mapping. In this chapter, we will focus on recent advancement and elementary principles in haploid production along with their usefulness in crop improvement.

4.2 Normal Process of Embryogenesis in Plants and Initiation of Haploid Production

The plant life shows the haploid and diploid stages. The haploid spores are produced through diploid plant body. Generally, in higher plants, anther and ovary are important organs in which meiosis takes place. Sporogenous tissues after reduction division gave rise to pollen grains (n) in anther and egg/ovum (n) in ovary. After pollination, pollens grains germinate to give rise male gametophyte and haploid male gametes. These gametes through pollen tubes enter inside the ovary. Pollen tube through ovary when enters inside the embryo sac, male gamete gets fused with egg and zygote is produced. This zygote after mitotic divisions gave rise to embryo (2n). This embryo remains dormant or germinates to give rise to diploid stage that is the vegetative body of the plant (Fig. 4.1). Haploid production can be achieved through intact anther culture or microspore (pollen grain) culture. Ovary or ovule culture is also helpful in development of haploid production. In this process, suitable donor plant flower buds are selected. The stage of the developing microspore is determined using appropriate techniques and one should confirm that microspore is

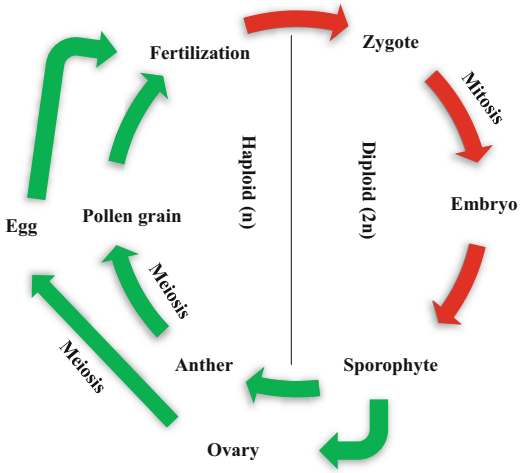

Fig. 4.1 Normal life cycle of angiosperm plant showing haploid and diploid stages

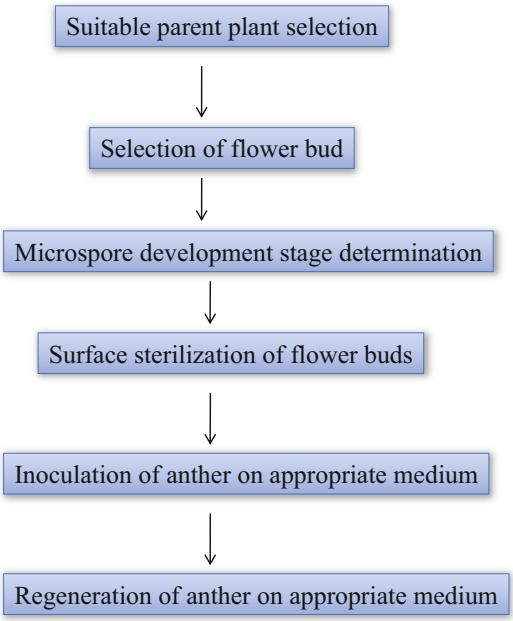

Fig. 4.2 Steps involved in haploid production via anther culture

in the tetrad stage. After that, surface sterilization protocol for flower buds should be applied for obtaining contamination free explants. Anthers/microspores are isolated with care and inoculated on appropriate regeneration medium. The regenerated shoots are haploid plants (Fig. 4.2). Similarly, haploid plant production through ovary or ovule culture can be achieved. In this process, from donor plant, ovary or ovule will be taken out and proper developmental stage of ovary/ovule will be determined by histology. After confirmation of developmental stage of ovary/

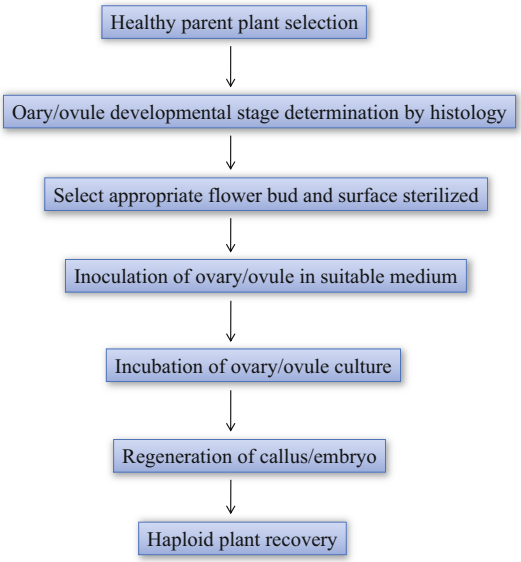

Fig. 4.3 Common steps for haploid production via ovary/ovule culture

ovule, the suitable flower buds are selected and surface sterilized. Ovary/ovule excised from surface sterilized flower buds is inoculated on suitable medium for regeneration/embryo which is derived from cultured ovule/ovary. This process of regeneration is given in Fig. 4.3.

4.3 General Fate of Pollen Grains

Embryogenesis in plants is a unique process and it can be initiated from a wide range of cells other than the zygote. Androgenesis refers to the development of embryos from anther or pollen grains (Touraev et al. 1997). Androgenesis represents an important tool for research in plant genetics and breeding, since androgenic embryos can germinate into homozygous double haploid plants. Androgenic development can be completed in characteristic phases: acquisition of embryogenic potential, initiation of cell division, and pattern formation. In flowering plants, the male reproductive processes occur in the stamens. During normal process of microsporogenesis, the diploid cells (pollen mother cell/microspore mother cell) undergo meiosis and produce haploid microspores. Normally, the microspores (n) divide mitotically and differentiate into 2-or 3-celled male gametophytes. The principle of androgenesis is to shift the fate of the microspores from gametophytic to sporophytic development (Fig. 4.4). Microspores are uninucleate (single nucleus) and contain large vacuole. Further these pollen grains can follow any one of the following developmental process: (1) it can undergo mitosis and produce haploid mass of the tissues; (2) microspores can undergo symmetric divisions which after

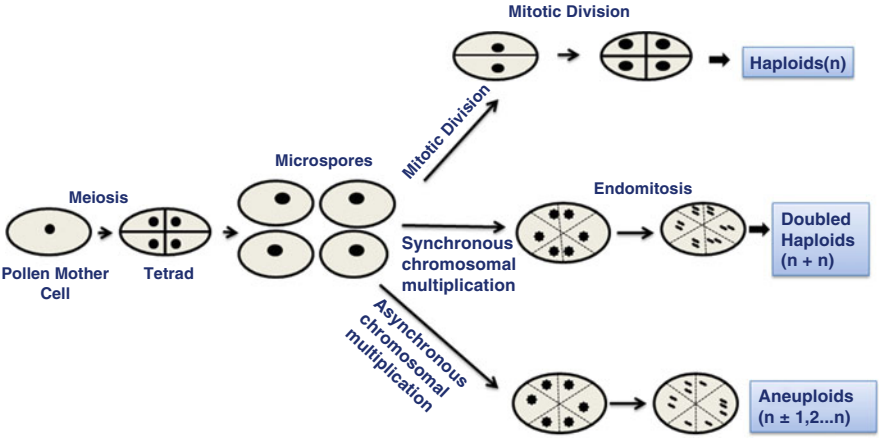

Fig. 4.4 Schematic representation of androgenesis, showing the origin of haploids, doubled haploids, and aneuploids

endomitosis produce double haploids; (3) these pollen grain after asymmetric division may produce aneuploids with varied number of chromosomes.

4.4 Cellular and Molecular Aspects of Androgenesis

The embryogenic development during androgenesis is divided into three main characteristic overlapping phases: Phase I, acquisition of embryogenic potential by stress involves repression of gametophytic development and leads to the dedifferentiation of the cells to acquire an androgenic capacity. Mainly in this phase, microspores enlarge and appear as star-like structure. The environmental and physiological factors also influence this developmental phase. Phase II, it involves initiation of cell division leading to the formation of multicellular structures (MCSs) confined within exine wall; Phase III, in this phase specific pattern development was observed so embryo-like structures (ELS) are developed, it is referred as androgenic embryoids, which is released out after breaking the exine wall, and further organization of shoot apical meristem (SAM), scutellum, and root meristem take place. A timeline of the three different phases during androgenic development in the model species barley is shown in Fig. 4.5. Rapeseed (*Brassica napus* L.), wheat (*Triticum aestivum* L.), tobacco (*Nicotiana* spp.), and barley (*Hordeum vulgare* L.) are considered as model species to study the mechanisms of stress-induced androgenesis (Touraev et al. 1997) due to their high regeneration potential. In addition to stress induced androgenesis, the hormone modulated various gene expression programs are associated with each phases. In response to stress like cold, salinity, osmotic and hypoxia, plant cells produce abscisic acid (ABA) (Zeevaart and Creelman 1988). Reynolds and Crawford (1996) reported a gene encoding an early cysteine-labeled

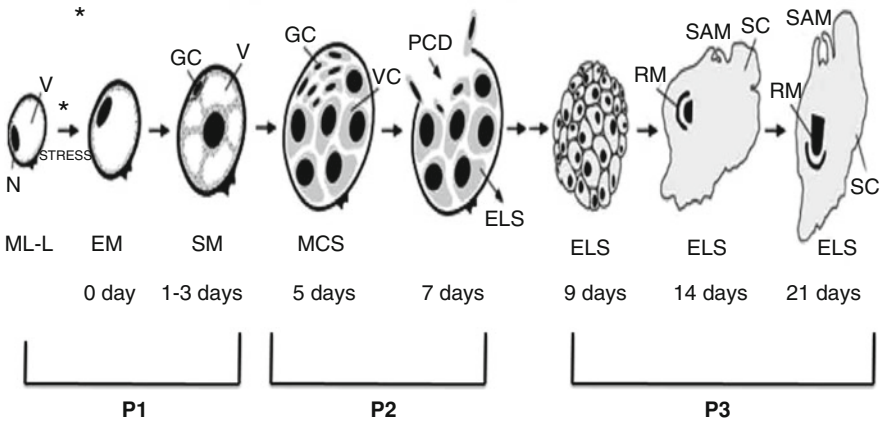

Fig. 4.5 Cellular and molecular aspects of androgenesis in the model species barley completed in the three different phases of embryogenic development. P_1: embryogenic potential acquisition; P_2: cell division initiation; and P_3: Formation of the pattern (ML-L: mid-late to late-uninucleate microspore; EM: enlarged microspore; SM: star-like microspore; V: vacuole; GC: generative cell; MCS: multicellular structure; PCD: programmed cell death; ELS: embryo-like structure; SAM: shoot apical meristem; SC: scutellum; RM: root meristem)

class II metallothionein protein (EcMt) from initiation of wheat androgenesis and it is closely related to the peak of ABA production. Another important gene from the family of Alcohol dehydrogenase (ADH3) during stress treatment to induce the barley androgenesis is correlated with high regeneration efficiency and these expressions is modulated by ABA level (Maraschin et al. 2005a, b). These relations to ABA suggest that it plays important role in the expression of specific gene during the initiation of androgenesis induced by stress. It indicates that besides ABA signaling, other hormonal signaling process is also involved in gene expression during androgenesis induction. The members of heat shock protein (HSP) family may be associated with the acquisition of embryogenic potential by heat and starvation (Barany et al. 2001). Another gene which is the member of GST family is likely to be associated with the cell against the harmful effect of ROS which are responsible during the acquisition of embryogenic potential (Pasternak et al. 2002). Based on observations, it was reported that proteolytic gene metalloprotease (*FtSH*) is correlated with androgenic response of barley microspore and is associated in degrading photosystem II reaction center D1 protein upon its irreversible photo-oxidative damage (Lindahl et al. 2000). The mutational approach might show that the gene *FtSH* is associated with the formation of normal green choloroplast during regeneration of green plants from the microspore (Yu et al. 2004). The functional studies of BABY BOOM (BBM), a member of the AP2/ERF family of transcription factors in rapeseed and *Arabidopsis*, represent the first androgenic-related gene identified which has a putative role in co-coordinating the phase of initiation of cell division and it triggers the embryogenic development during induction of androgenesis (Boutilier et al. 2002). AGAMOUS like 15 (AGL15) is an another

important regulatory protein which is translocated into the nucleus after initiation of cell division during androgenesis, apomixes as well as somatic and zygotic embryogenesis. AGL 15 is a member of MADS domain family that acts as a transcription factor and involved in the embryogenesis (Perry et al. 1999). Genes like *LEC1* and *LEC2* also play key regulatory role in coordinating the morphogenesis and maturation phase of embryogenesis, therefore, it is master regulator of embryogenesis (Harada 2001). Similarly, the gene *DcSERK* is transiently expressed and increased the efficiency of somatic and zygotic embryogenesis during the initiation of embryogenic development up to globular stage (Schmidt et al. 1997). However, endosperm specific gene *ZmAE1* and *ZmAE3* are transiently involved at early stage in endosperm development (Magnard et al. 2000). The expression of *ELS* gene helps in epidermal differentiation and layer specification in both androgenic as well zygotic embryos (Yeung et al. 1996; Maraschin et al. 2003). Programmed cell death (PCD) plays important roles that are associated with barley androgenesis induction, could possibly trigger the programmed removal of the "weakest" cells and has a role in sculpting the globular embryo (Lam 2004). The gene expression shows a comprehensive overview of microspore embryo formation by molecular mechanism.

4.5 Cytological Behavior of Microspore During Androgenesis

Microspore/pollen grain during normal process of embryogenesis undergoes unequal division producing bigger tube cell and smaller generative cell. Further generative cell differentiates into two male gametes. During the culture of pollen grain, this normal behavior of pollen grain during embryogenesis changes and it follows different pathways of differentiation (Fig. 4.6). Pathway I follows the symmetric division of microspore in which both generative and vegetative cells of equal size are produced. After second symmetric division, this binucleate cell gives rise to tetrad which ultimately gives mass of cell after several divisions. It means both generative and vegetative cell are involved in this multicellular mass induction. This kind of development is found in *Brassica napus*. Pathway II involves general mode of division at initial stage as found in majority of the plants. Uninucleated microspore divides asymmetrically to produce larger vegetative cell and smaller generative cell (Maraschin et al. 2005b). Generative cell degenerates either initially or after one/two divisions. Vegetative cell after second mitosis gives two cells and further divisions in these two cells result in multicellular haploid mass development. *Hordeum vulgare* and *Triticum aestivum* are the common examples of this pathway. Pathway III has been observed in *Hyoscyamus niger* and in this initial asymmetric division produces smaller generative and larger vegetative cell, but the further developmental process is performed by generative cell. Generative cell divides mitotically and symmetrically to produce two cells and further divisions in these two cells are responsible for the production of haploid tissues. Vegetative cell either does not divide or undergo division to a limited extent to form suspensor-like structure. Pathway IV shows initial development as like normal microspore development. After asymmetrical mitosis I, it forms larger vegetative and smaller

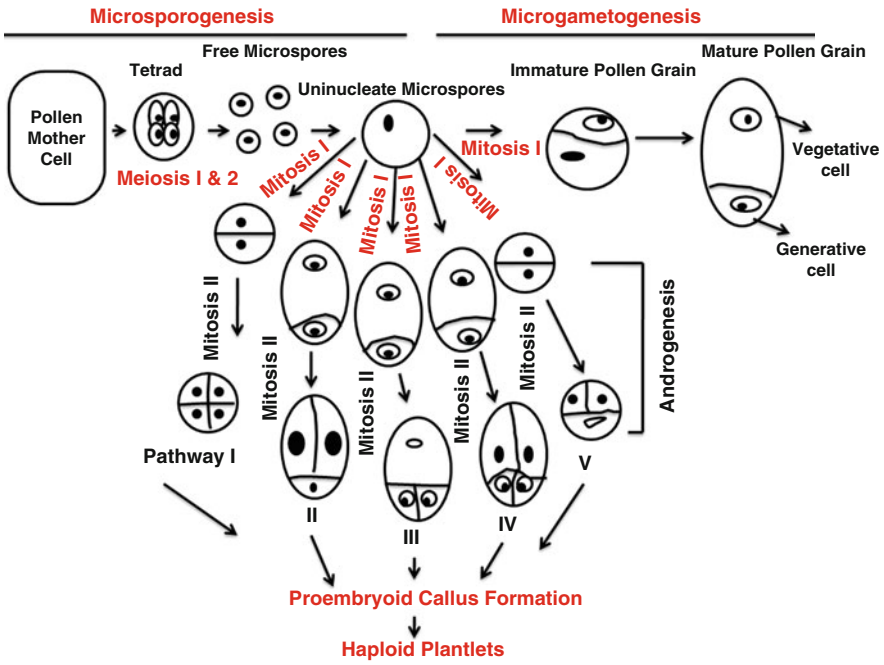

Fig. 4.6 Cytological behavior of microspores after the culture on nutrient media

generative cell. Further both the cells took part in mitosis II and form four celled microspore in which two larger and two smaller cells are derived from both generative and vegetative cell. Further, both vegetative and generative cells equally divide repeatedly and produce multicellular mass which helps in organogenesis. *Datura innoxia* is the common example of this pathway. In pathway V, microspore undergoes symmetrical mitotic division to produce two equal size cells, one is vegetative and another is generative. The vegetative cell later follows mitosis II to form two cells of equal size, which take active part in further cell division. Generative cell either ceases to divide or degenerates. *Brassica napus* is a well-known example of this pathway.

4.6 Isolated Microspore Culture

Inoculation of mechanically isolated microspores from anther on the culture media is known as isolated microspore culture. It is an efficient method for androgenesis and DH production. In this process, microspores, preferably at the uninucleated stage, are obtained from young anthers and then cultured in vitro where they develop into either callus tissue or embryoids, that is regenerated into plantlets (Dubas et al. 2014, Salas et al. 2012a). The disadvantage of anther culture is that plantlets may be originated from haploid microspores as well as from diploid anther wall tissue.

Microspore culture eliminates this problem. Haploid nature of regenerated plant is confirmed through this process. It is an ideal method for genetic manipulation as it starts from single cells that can be better regulated. Technological advances have now enabled microspore culture to achieve reasonably high yields in some crop genotypes, such as canola, barley, and wheat (Gil-Humanes and Barro 2009; Broughton et al. 2014).

There are many other advantages of microspore culture. In the study of genetic recombination in higher plants, study of mode of differentiation from single cell to whole organism, hybrid development, mutation study, transfer of disease and insect resistance, haploid are used in the molecular biology and genetic engineering, genome mapping. However, the limitations of microspore culture over anther culture are the high level of management and expertise is required. The doubling of haploid does not always result in the production of homozygosity, formation of albinos especially with the cereals that affect the loss of plant due to albinism. There is little chance of isolating the haploid from the mixture of various ploidy levels since higher ploidy levels are easily outgrown. Tissue or callus comprises a chimera of diploid, tetraploid, and haploid cells.

4.7 Anther Culture for Haploid and Double Haploid (DH) Production

Anther containing microspores, when cultured on the nutrient media is able to induce regeneration. It is a common approach for haploid production. The haploids are the individuals with gametic chromosome (n) in somatic cells. Predominantly haploid plants originate from meiotically divided tissue that develops into embryo without fertilization. In other words haploids are sporophytic plants containing chromosomes numbers as like gametes. On the contrary, the double haploid is a genotype formed when haploid cells that is egg or male gamete cells undergo chromosome doubling and the resulting individuals are completely homozygous. The simplest method of haploid induction through the androgenesis pathway is the in vitro culture of the whole immature anthers, having microspores. Guha and Maheshwari (1964) firstly discovered this method. There are number of factors that affect the efficiency of anther culture. These include the developmental stage of immature microspores/pollen grain, donor plant's growth condition, plant genotype, type of culture medium, different type of PGRs, and their concentration, type, intensity, and duration of stresses (Isah and Umar 2020; Hentour et al. 2020). The proper bud size, the thickness of anther wall, and many other important factors that should be considered when anther culture is initiated. Younger anthers should be preferred over older one because it has thin walls that facilitate the entry of effective factors leading to a better response and growth of its internal microspores. Thick wall of anther acts like a barrier in absorption of effective factors, which are responsible for inducing growth and multiplication of microspores (Rivas-Sendra et al. 2020). Flower bud position within the inflorescence can help to isolate younger anthers before maturation. The selection of suitable flower buds containing microspores at

suitable developmental stage is also very important step in anther culture studies. Flower bud size cannot predetermine for anther isolation because it varies from species to species (Gu et al. 2014). It has been observed in kiwifruit (*Actinidia arguta* Planch.) that callus induction with the late-uninucleate stage of microspores in anther was more effective than those anthers in which microspores were in tetrad, early-uninucleate, and binucleate stages (Wang et al. 2018). In spite of numerous studies on androgenesis in different plants, there is still low induction of this process and regeneration of green plants. The emergence of albino plants and low frequency of the doubled number of chromosomes in haploid plants are significant problems (Lantos et al. 2014). It is difficult to develop an efficient method of regeneration of plants from anther cultures because several factors are influencing the efficiency of androgenetic induction (Dagustu 2008). Prolonged exposures of cell cultures to high concentration of 2,4-D affect the frequency of regeneration of plants and cause chromosomal abnormalities (Deambrogio and Dale 1980; Ziauddin and Kasha 1990). The major controversies influencing the efficiency of DH production include species and genotype dependency (Ei-Hennawy et al. 2011; Murovec and Bohanec 2012), a high proportion of albinism (Kumari et al. 2009; Makowska and Oleszczuk 2014; Sriskandarajah et al. 2015), high frequencies of clones via androgenesis (Oleszczuk et al. 2014), and genome instability such as aneuploidy due to somaclonal variation (Oleszczuk et al. 2011; Wędzony et al. 2015).

4.8 Factors Affecting Androgenesis

The anther culture media composition varies with the genotype, age of anther as well as the condition under which the donor plants are grown (Burbulis et al. 2005; Buyukalaca et al. 2004). The genotype and physiological status of donor plant play a significant role in determining the frequency of pollen production (Salas et al. 2011; Basay and Ellialtıoglu 2013; Rivas-Sendra et al. 2017; Heberle-Bors 1985; Nowaczyk et al. 2016). The age of the donor plant also affects the response in microspore culture for androgenesis in several species of angiosperm plants (Shtereva et al. 1998). It is usually recommended that microspore should be isolated from young and vigorous stage of the plants (Chuong et al. 1988). Another important factor that affects the androgenesis is the anther wall factor. The anther wall provides nourishment in the development of isolated pollen in a number of species. The yield of microspore derived embryo is regulated by the induction and differentiation stages of microspore (Karakullukcu and Abak 1993; Salas et al. 2012a, b). Induction of haploid can be enhanced by maintaining the anther or flower buds at low temperature. The pretreatment of the anther such as cold treatment 3–5 °C (Ciner and Tipirdamaz 2002), or heat shock treatment at 30 °C for 24 h (Sangwan and Sangwan-Norreel 1990) stimulates the androgenic process. Anthers exposure to cold treatment causing a 90° shift in the division plane of a microspore has resulted in a symmetrical division that enhances the anther culture response (Obert et al. 2005). The culture media also influences the anther-derived callus induction (Na et al. 2019). The concentration of sucrose in the culture media also influences the callus

induction from anthers (Burbulis et al. 2005), although various researchers have suggested different optimal levels of sucrose to improve the response in microspore culture (Chen et al. 1998). Mathapati et al. (2019) observed that higher concentration of sucrose in the media influenced the haploid induction. N6 and MS media with high concentration of $FeSO_4.{}_7H_2O$ also affect the androgenesis (Goncharova et al. 2020). Media supplemented with $AgNO_3$, Fe-EDTA, and myoinositol supported anther-derived callus induction in Seolhyang strawberries (Na et al. 2019). It is reported that the media supplemented with the activated charcoal also influenced the embryo formation in *Capsicum annuum* L. anther culture (Ciner and Tipirdamaz 2002). The endogenous level of hormones along with nutritional status of the tissue of anthers (Sunderland and Dunwell 1977) directly affects the regeneration from the microspore. The optimum proportion of auxin and cytokinin in the medium has stimulated the induction of androgenesis (Ciner and Tipirdamaz 2002). Iron in the medium, chelated iron, culture density, and concentration of myoinositol, glutamine, pyrrolidone (PVP) and spermidine affects the androgenesis. There are few reports that glutamine alone (Akhtar 2013) or in combination with polyvinyl pyrrolidone (PVP) and spermidine (Górecka et al. 2014) and coconut water (Żur et al. 2008) affects the morphogenic response of induced callus on different callus regeneration media. Anther-derived embryogenesis and regeneration were influenced by the osmoticum like mannitol and polyethylene glycol (PEG) in various systems (Ayed et al. 2010). The oxidative stress such as change in superoxide dismutase and guaiacol peroxidase level also influenced the in vitro anther culture and hence affects the process of androgenesis by induced chromosomal breakage and rearrangement (Çakmak et al. 2019). The light intensity and photoperiod are also important factors that determine the androgenic response of the plant (Dunwell 1981). The microspore developmental stages are another important factor for embryogenesis or organogenesis and it depend upon the size of the floral bud (Adhikari and Kang 2017), and temperature during bud collection. The seasonal influence affects the androgenesis. It was observed that anther form the first flush of flower in the season was found to be more responsive (Sunderland 1971). However, at the level of microspore culture, different factors like basal medium (type, strength, and pH), shock treatment and culture condition (Dumas de Vaulx and Chambonnet 1982; Ellialtıoglu and Tipirdamaz 1999), the concentration of arabinogalactan protein (Makowska et al. 2017), glutathione and mannitol (Zielinski et al. 2020) play a key role in the developmental process in different plant species. The brassinosteroids (BRs) are the class of plant hormones controlling the plant developmental process (Hu et al. 2017) and influencing the cell division, cell elongation, cell expansion, and cell wall formation by exogenous application (Hacham et al. 2011). BRs control maize seedling elongation by regulating cell elongation, thus affecting shoot and root lengths, mesocotyl length and the root: shoot ratio (Hartwig et al. 2012; Pacifici et al. 2015). Addition of some additives can improve haploid induction efficiency. Plant growth regulators (PGRs) are one of such additives that are directly involved in haploid induction by involving in signaling and gene expression pathways. PGRs improve the acclimatization capability of the plants to the changing environment. The type of PGRs and auxin: cytokinin ratio play very important role in

androgenesis. However, its efficiency in haploid induction depends on type of species. 2, 4-D has a very efficient role in somatic embryo induction in number of species (Sakina et al. 2020; Mahato and Chaudhary 2019). Although it acts as efficient PGRs to induce calli but 2, 4-D is one of the PGRs that causes somaclonal variation in tissue culture studies and these variations are through DNA modifications which stimulate unorganized cell growth (Garcia et al. 2019). Haploid plants production was observed with combined action of NAA and kinetin (Maharani et al. 2020) isopentenyl adenine and IAA (Kumar et al. 2020a, b). Efficient callus induction and haploid plant production through anther culture was also influenced by using polyamines. Putrescine is a kind of polyamine that was found to be effective in haploid plant production (Heidari-Zefreh et al. 2019).

4.9 Production of Double Haploid Plants

The procedure for DH production includes two major steps: haploid induction and chromosome doubling. Consequently, the chromosome doubling of haploids derived from microspores is an important step in the practical application of microspore culture technology (Yuan et al. 2015). It is also possible to obtain haploids through a pathway known as gynogenesis from the unpollinated female gametophyte (Bohanec 2009). Chemical (usually colchicine) treatments for chromosome doubling may occur at either in vitro culture stage by using media containing the chemical to directly generate DH embryoids or callus or at a later stage on the regenerated haploid plantlets. The former is now the most often used methodology. Spontaneous or induced chromosome duplication of a haploid occurs, the resulting plant is called doubled haploid (DH). DH techniques have been well established in a range of economically important crop species, including major cereals and cabbages (Wedzony et al. 2009). DH technology offers the fastest and most efficient route to produce completely homozygous lines. Isozyme analyses (Bouvier et al. 2002), random amplified polymorphic DNA (RAPD) markers, and microsatellites can be utilized to assess homozygosity and to confirm the gametic origin of calluses and plantlets (Germana 2006). Optimization of chromosomal doubling protocols based on spontaneous chromosomal doubling and/or non-hazardous chemicals that could further increase the overall DH production efficiency. Colchicine is the most widely anti-microtubule agent used in vivo and in vitro (Castillo et al. 2009), but other doubling agents have also been used, such as oryzalin and trifluralin. The pretreatment of spike at low temperatures before isolating and inoculating anthers in a nutrient medium is the crucial step for the production of haploids in various other species of higher plants. This procedure not only increases the optimal stage duration of microspores development, but also induces their abnormal multiple division with callus and embryoids formation and also enhances the frequency of plant regeneration (Bilynska 2020). The process of hypomethylation in the genomic DNA by the compound 5-azacytidine (5-azaC) also influenced the production of DHs in wheat crop by anther culture. It increased the callus induction up to 38% and plant regeneration up to 50% (Belchev et al. 2004). An antimitotic compound like

colchicine extensively is used in microspore culture and has been shown to improve results in terms of green double haploid plant production (Ślusarkiewicz-Jarzina et al. 2017). Successful haploid and DHs production in selected plants is summarized in Table 4.1.

4.10 Applications of Double Haploid Plants

DH technology enables significant shortening of time during production of pure lines. Complete homozygosity of DH lines offers a higher phenotype to genotype correlation, thereby facilitating better estimation of quantitative trait loci (QTL) effects in marker trait association studies (Hyne et al. 1995). DH technology is an important tool for exploring the genetic diversity, for maintenance of genetic resources, and introducing novel variation to magnify the genetic base of elite germplasm (Brauner et al. 2019). Haploid Inducer Mediated Genome Editing (IMGE) is a recent application that enables direct genomic modification of commercial inbred lines and eliminates several costly and time-consuming steps when incorporating genome-edited traits into elite cultivars (Kelliher et al. 2019; Wang et al. 2019). Well-established DH plant production methods are applicable for breeding and research for several crop plants, e.g., rice, maize, barley, wheat, and triticale (Dunwell 2010; Germana 2011; Hensel et al. 2012; Niu et al. 2014). DHs have been widely used for cultivar development, genetic mapping, mutagenesis, and gene function studies (Ferrie and Mollers 2011; Hussain et al. 2012). DH production is well established in the range of economically important species belonging to the family *Solanaceae*, *Brassicaceae,* and *Poaceae* (Dunwell 1986; Hu and Yang 1986). In a few reports, it is useful in the improvement of some legumes and woody plants (Sangwan-Norreel et al. 1986; Bajaj 1990; Raghavan 1990; Wenzel et al. 1995; Germana 2006, 2009).

Advantage of double haploid technique is based on quick method to obtain 100% pure inbred lines (Venancio et al. 2019). Thus, the resulting inbred lines are highly homozygous. Rapid development of DH based inbred lines is useful in development of resistance against *Aspergillus flavus* (Pekar et al. 2019), aflatoxin (Ajithkumar et al. 2019), insect and disease (Ahman and Bengtsson 2019), drought and heat tolerance (Karkour et al. 2019). The double haploid technology needs two generations for development of homozygosity in inbred lines compared to six generation in conventional method. There are many applications of DH in plant breeding like mapping the qualitative trait loci (QTL) (Castro Aviles et al. 2020), back cross breeding (Brauner et al. 2019), bulk segregant analysis (BSA), hybrid sorting, fixation of heterosis, genetic distance, and phenotypic development analysis of cultivars (Hu et al. 2020).

Table 4.1 Haploid and Double Haploid (DH) production in some major plant species by anther, ovule, and embryo culture

No.	Plant name	Explant	Haploid/Diploid	References
1	*Musa balbisiana*	Anther	Haploid	Assani et al. (2003)
2	*Phlox drummondii*	Anther	Haploid	Razdan et al. (2008)
3	*Cocos nucifera* L.	Anther	Haploid	Perera et al. (2008)
4	*Medicago sativa* L.	Anther	Haploid	Zagorska and Dimitrov (1995)
5	*Capsicum annuum* L.	Anther	Haploid	Dolcet-Sanjuan et al. (1997)
6	*Cicer arietinum* L.	Anther	Haploid	Croser et al. (2011)
7	*Musa balbisiana*	Anther	Haploid	Bakry et al. (2008)
8	*Capsicum annuum* L.	Anther	Haploid	Luitel and Kang (2013)
9	*Hordeum vulgare* L.	Anther	Double haploid	Hoekstra et al. (1997)
10	*Oryza sativa L.*	Anther	Double haploid	Hooghvorst et al. (2020)
11	*Helianthus annuus*	Anther	Double haploid	Coumans and Zhong (1995)
12	*Solanum melongena*	Anther	Double haploid	Rivas-Sendra et al. (2017)
13	*Cucumis sativus* L.	Anther	Double haploid	Song et al. (2007)
14	*Citrus maxima* (Burm.)	Anther	Double haploid	Kawano et al. (2020)
15	*Citrus clementina*	Pistil	Haploid	Germanà and Chiancone (2001)
16	*Nigella sativa*	Seed	Haploid	El-Mahrouk et al. (2018)
17	*Gossypium barbadense*	Unpollinated ovule	Haploid	Kantartzi and Roupakias (2009)
18	*Guizotia abyssinica* (L. f.) Cass	Unpollinated ovule	Haploid	Bhat and Murthy (2007)
19	*Cucurbita pepo* L.	Unpollinated ovule	Haploid	Shalaby (2007)
20	*Gentiana triflora*	Unpollinated ovule	Haploid	Doi et al. (2011)
21	*Gentiana triflora*	Unpollinated ovule	Double haploid	Doi et al. (2011)
22	*Cucumis sativus* L.	Unpollinated ovary	Double haploid	Sorntip et al. (2017)
23	*Triticum durum* Desf.	Unpollinated ovary	Double haploid	Slama-Ayed and Slim-Amara (2007)
24	*Cucumis sativus* L.	Ovary	Haploid and double haploid	Deng et al. (2020)
25	*Allium cepa* L.	Flower bud and ovary	Haploid	Yarali and Yanmaz (2017)
26	*Larix decidua*	Zygotic embryo	Haploid	Van Aderkas and Bonga (1988)
27	*Larix decidua*	Zygotic embryo	Haploid	Nagmani and Bonga (1985)

(continued)

Table 4.1 (continued)

No.	Plant name	Explant	Haploid/Diploid	References
28	Wheat and Rice crosses	Zygotic embryo	Haploid	Bakos et al. (2005)
29	*Triticum aestivum* L.	Zygotic embryo	Haploid	Kumlehn et al. (1997)
30	*Hordeum vulgare* L.	Zygotic embryo	Haploid	Burun and Poyrazoglu (2002)
31	*Triticum aestivum* L.	Zygotic embryo	Haploid	Niroula et al. (2007)
32	*Zea mays* L.	Zygotic embryo	Haploid	Niroula et al. (2007)
33	*Linum usitatissimum* L.	Zygotic embryo	Haploid	Pretova and Williams (1986)
34	Sturt's desert pea	Pollen grains	Haploid	Sudhersan et al. (2008)
35	*Bupleurum falcatum*	Anther	Haploid	Shon et al. (2004)
36	Pepper	Anther	Haploid	Koleva-Gudeva et al. (2007)
37	*Manihot esculenta* Crantz	Flower	Haploids	Woodward et al. (2001)
38	*Beta vulgaris* L.	Seed	Haploid	Zhang et al. (2001)
39	*Primula forbesii* Franch.	Flower	Haploid	Jia et al. (2014)
40	*Cucurbita pepo*	Unpollinated ovule	Haploid	Metwally et al. (1998)
41	*Tagetes erecta* L.	Ovule	Haploid	Kumar et al. (2020)
42	Sweet orange cv. Tobias	Ovary	Double haploid	Cardoso et al. (2012)
43	Indica hybrid rice	Anther	Double haploid	Premvaranon et al. (2011)
44	*Brassica napus*	Immature zygotic embryo	Double haploid	Burbulis et al. (2007)
45	*Brassica napus*	In vitro cultured zygotic embryo	Double haploid	Burbulis and Kupriene (2005)

4.11 National and International Status of Haploid and Double Haploid (DH) Production

The production of haploid (H) and doubled haploid (DH) plants is one of the most important developments in the field of agriculture biotechnology. This technology is integrated with marker assisted selection which is emerged as a powerful tool for the development of the high quality plant materials. DH research successfully used in the development of several economically important cultivars of rape, wheat, rice, melon, pepper, tobacco, eggplant, triticale, mustard, etc., worldwide. The recent

discovery of a highly efficient centromere-mediated genome elimination technique of haploid induction in *Arabidopsis* (Ravi et al. 2014) has generated immense interest among researchers for the production of desired traits. Various research groups across globe are currently assessing its value in plant breeding. Multinational seed companies widely adopted DH technology in large-scale production of inbred lines for development of hybrid cultivars of herbs, spices, medicinal, and nutraceutical plants. This technology increased the efficiency of plant breeding. Recently, haploid inducer lines have also been created in *Arabidopsis thaliana*, by using targeted centromere manipulation of engineered centromeric histone protein 3 (CENH3) variants (Ravi and Chan 2010). However, this haploid induction method has not been reported in plant breeding programs so far. Thus, the possibility of the application of this approach in crops is highly promising. However, the establishment of viable CENH3-based haploidization methods is highly challenging for the scientists of the world. More recently, quantitative trait loci (QTL) have been genetically and physically mapped and used for haploid induction in novel species (Bazrkar-Khatibani et al. 2019). Based on the research it is emerged that the novel gene *MATRILINEAL* (MTL) associated with haploid induction will be available in the near future (Kelliher et al. 2017) in DH production.

Indian Council of Agricultural Research (ICAR) and the International Rice Research Institute (IRRI), Philippines are working on a haploid and DH production at the Central Rice Research Institute in Cuttack, India (http://www.crri.nic.in/crri_vision2030_2011.pdf) to cater the needs of rice breeding and to develop agronomical improved rice genotypes.

In our country, a private company "*In Vitro* International Bengaluru, India" (http://dbtncstcp.nic.in/html/Certified_TCUs/Vitro_International.html) had vast experience in the production of DH of field crops (mustard, canola, and maize) and offers the possibility of DH production in vegetable crops. Iowa State University, USA is offering expertise and services in DH technology for the development of maize inbred lines (www.plantbreeding.iastate.edu/DHF/DHF.htm). Due to the demand of DH, Heartland Plant Innovations (HPI)—a public-private partnership—has established a laboratory of double haploid at Manhattan, Kansas (USA) for use of public and private wheat breeders (Barkley and Chumley 2012). Thus, it is distinct that the worldwide efforts are going on in the field of DH production and its exploitation in the breeding process.

4.12 Future Prospects and Conclusion

Haploid plants and haploid-derived homozygous lines are useful in several domains of basic research in the realms of classical plant genetics and cytogenetics, modern molecular genetics including induced mutagenesis, site-directed mutagenesis, genetic transformation research, genome mapping and assessing distant genome relationships, gene dosage effects, analysis of linkages, mechanisms of the genetic control of chromosomal pairing, and in conventional plant breeding studies. The DH technology platform offers a rapid mode of truly homozygous line production that

helps to expedite crop breeding programs where homogeneity is an absolutely essential parameter for rapid crop development. Integration of the haploidy technology with other available biotechnological tools such as Marker Assisted Selection (MAS), induced mutagenesis, and transgene technologies could also effectively expedite the crop improvement programs running all across the globe. Thus direct incorporation of cloned genes at the haploid level following subsequent chromosome doubling may help accelerate stable integration of target gene(s) into several crops and/or higher plant species. To be useful, however, it is important to note that an efficient and reliable method of haploid and DH production will be essential. Recent research has very clearly demonstrated that induced chromosome elimination offers a very useful approach for rapid haploid plant production in cereals. However, further improvements in microspore culture could substantially bring in more changes in the not so distant future. It is interesting to note that the totipotent nature of the haploid cell is being efficiently and effectively explored in different facets of modern biological and agricultural research disciplines. By efficiently utilizing DH populations, QTLs associated with yield and yield components have been successfully identified allowing marker assisted breeding approaches to be employed in several crop improvement programs. The haploidy technique has played an important role in practical plant breeding as can be seen in widely grown DH cultivars in all the major continents where some of them have earned the recognition of dominant cultivars. Haploids and DHs are also useful in mutational studies (Da Silva et al. 2015). For the production of transgenic plants, co-culture of anther/pollen with *Agrobacterium* suspension resulted indirect gene transfer (Calayugan et al. 2020; Ohnoutková and Vlčko 2020) in haploid regenerants. Further, it may be used for the transgenic DHs production. Microspores can also be used for transformation purpose, but this method needs a precise estimation of the developmental stage of microspore. Thus, we can conclude that DHs can be widely used in several crop improvement program in different ways.

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Encapsulation Technology: An Assessment of Its Role in In Vitro Conservation of Medicinal and Threatened Plant Species

5

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Abstract

In the recent decades, many plant species are under severe threat mainly due to the destruction and shrinking of natural habitats caused by several anthropogenic activities, global climate change and overexploitation of plant resources. In the current scenario, the plant-based industry has expanded many folds due to an ever-increasing demand of plant-based medicines used in traditional medicinal system which has led many species to the brink of extinction. Conservation of such rare, threatened and commercially important plants has now become a global concern. Many biotechnological-based integrated programs are prerequisite to protect and preserve plant biodiversity. Plant tissue culture-based synthetic seed technology is one of the new emerging biotechnological tools which has now been widely used for the propagation, conservation and delivery of germplasm of many economically important and rare and endangered plant species. Synthetic seed technology coupled with slow growth culture and cryopreservation technique has emerged as a promising approach for conservation of many threatened plant species for short to medium and long-term storage. The application of syn-seed technology, i.e. encapsulation of embryogenic and non-embryogenic

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propagules for conserving rare and threatened medicinal plant species particularly those used in herbal pharmaceutical industry has been extensively studied in India and across the world in recent years. In this chapter, we provide new insights, recent and comprehensive information on the syn-seed technology, with the main focus on its role in in vitro conservation of medicinal and threatened plants of India and worldwide both.

Keywords

Conservation · Encapsulation · In vitro propagation · Synthetic seeds · Threatened plant

5.1 Introduction

Plants are being domesticated and cultivated by human beings since times immemorial for food, health and economic security and human still rely mostly on those crops that were domesticated thousands of years ago (Ross-Ibarra et al. 2007; Moshelion and Altman 2015). As a result of domestication, several cereals, pulses, oil yielding, vegetables and fruit crops occupy the main composition of human diet. With the advancement of human civilization and urbanization, and the ever-evolving of agricultural practices and their harmonious association with human, a number of agricultural, medicinal, horticultural and many more economically important plants became the integral part of our diet, medicines, cultures and religions (Schaal 2019). Since time immemorial and as per data available in ancient scriptures and literature, several plants have been used in traditional and folk practices for healing and therapeutic purposes. Interestingly, this traditional system of herbal medicine is still under practice in rural and tribal communities of many poor and developing countries across the world and a majority of the population is thus dependent on these economically important and potent medicinal plants. In addition, in the current scenario, due to high cost and multiple side effects posed by synthetic or allopathic drugs and absence of therapeutic treatment for numerous chronic diseases, a large population from developing and developed countries are shifting towards herbal medicines and phytonutrients for their primary healthcare (Patwardhan et al. 2005; Ekor 2014). Therefore, the demand of raw materials for preparation of plant-based medicines by pharmaceutical industries is increasing in recent years which has posed an adverse pressure on the natural population of many medicinally important plants, particularly those growing in wild and used in herbal medicine (Singh et al. 2009a). As per the Kew's Medicinal Plant Names Services (MPNS) ver. 9 database released in January 2020, the plant parts of more than 27,734 plants from 365 families of angiosperm and gymnosperm have been reported as being used as for treating various ailments (MPNS 2020).

In the last three to four decades, the global climate change and several and severe biotic and abiotic stresses, shrinking and destruction of natural habitats and overexploitation of many economically important plants due to rapid human population

growth and high demand of plant-based products have significantly influenced the biodiversity loss. This in turn has posed a danger to many economically important plant species and as per the literature available, has led to the extinction of many important plant species (Nandini and Giridhar 2019). According to recent data of International Union for Conservation of Nature (IUCN) Red List, out of 3,69,000 flowering plant species and 41,516 species described and evaluated by IUCN red list ver. 2020–2022, 16,667 species are listed as threatened (IUCN 2020). The declining wild populations of threatened and many more medicinally important species at an alarming rate have become a cause of serious concern and these species require an urgent attention for conservation, management and their further commercial utilization in the near future (Rajasekharan and Wani 2020).

Conservation and monitoring of all threatened species are practically impossible particularly in their natural habitat due to several reasons ranging from vast natural habitation, biotic and abiotic stresses and other factors such as pollination regime, seed dormancy, shift in the seed set, etc. responsible for the growth and revival of these species in their natural forms. Thus, in situ conservation method is insufficient to meet the challenges of conserving these threatened plant species as the wild populations have declined at critical levels (Rohini 2020). In addition, many medicinal plants have some distinct characteristics that make them difficult to conserve using conventional seed bank or field gene bank methods. Conventionally, conservation of medicinal and threatened plant species is constrained mainly due to poor viability and germination of seeds after storage, physiological dormancy, recalcitrance to seed/vegetative propagation, scanty information available on the reproductive biology of many wild medicinal plants, high risk of disease transfer during germplasm distribution and erosion of genetic resources (Sharma et al. 2019). Interestingly, in the recent years, with the advent of many biotechnological tools and techniques, ex situ conservation method is gaining popularity and momentum for the conservation and management of many threatened plants. This is due to the fact that it facilitates to conserve a species with value, i.e. potential economic use, conservation priority and biological and cultural importance and that too in controlled conditions and in less space requirements (Maxted 2013; Rohini 2020). Recent advances in plant tissue culture-based biotechnological tools and techniques have paved a new way for the rapid propagation, conservation and management of rare, endangered and other commercially important plant species (Phulwaria et al. 2013; Shasmita et al. 2018; Rajasekharan and Wani 2020). In all, micropropagation coupled with encapsulation-based synthetic seed technology has emerged as a promising approach for ex situ conservation and germplasm distribution of elite threatened plant species (Ara et al. 2000; Rai et al. 2009; Sharma et al. 2013; Faisal and Alatar 2019). The application of encapsulation technology in conserving threatened as well as medicinal plants particularly those used in herbal pharmaceutical industry has been extensively studied in recent years. Synthetic seed-based slow growth culture and cryopreservation technique has now been widely used to conserve many threatened plant species for short to medium and long-term storage. In this chapter, we will discuss the recent advances in in vitro conservation of medicinal and threatened plant species using encapsulation-based synthetic seed technology.

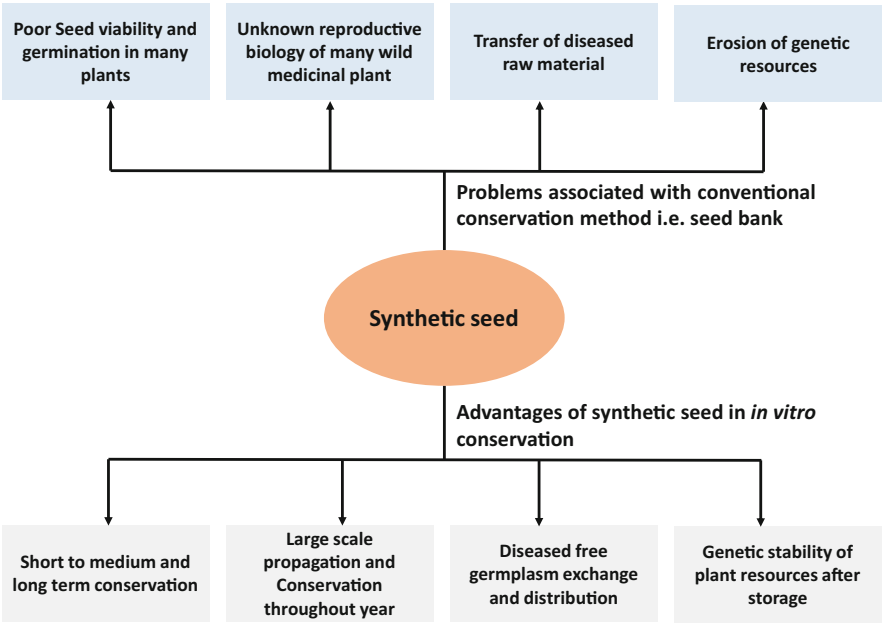

Fig. 5.1 Schematic illustration of advantages of synthetic seeds over conventional seed bank

The need of synthetic seed production and its advantages in medicinal plant is presented in Fig. 5.1.

5.2 Encapsulation Technology: A Resourceful Biotechnological Tool for Propagation, Conservation, and Germplasm Distribution

Encapsulation technology-based production of synthetic seed or artificial seed involves artificially encapsulation of somatic embryos or other vegetative tissues such as shoot tips, nodal segments, axillary buds, protocorms, etc. for plant propagation, conservation and germplasm exchange or distribution (Rai et al. 2009; Sharma et al. 2013). Since its discovery in 1980s (Kitto and Janick 1982; Redenbaugh et al. 1984), encapsulation technology has been successfully employed for propagation and conservation of a number of commercially important plant species including agricultural crops, forest trees, fruits, medicinal, ornamental and orchids, etc. (reviewed by Gray et al. 1991; Attree and Fowke 1993; Standardi and Piccioni 1998; Ara et al. 2000; Rai et al. 2009; Reddy et al. 2012; Sharma et al. 2013; Gantait et al. 2015; Faisal and Alatar 2019). Due to bipolar nature and its ability to forms root and shoot in one step, somatic embryo is a choice of explant for encapsulation and synthetic seed production in most of the plant species, however development of synthetic seed using somatic embryos is restricted only in those

crops for which a well-established somatic embryogenic system exists. In addition, asynchronous development, precocious germination during conservation and formation of low-quality somatic embryos are some other important hurdles in employing somatic embryos for synthetic seed development (Ara et al. 2000; Saiprasad 2001; Rai et al. 2008a). During recent decades, encapsulation of non-embryonic vegetative propagules like shoot tips, axillary buds, nodal segments, microbulbs, rhizomes, protocorm or protocorm-like bodies, etc. has received substantial attention as an appropriate explant and suitable alternative to somatic embryos for synthetic seed production in a numerous plant species studied (Bapat et al. 1987; Standardi and Piccioni 1998; Naik and Chand 2006; Rai et al. 2008b, c, 2009; Sharma et al. 2013; Gantait et al. 2015; Faisal and Alatar 2019). High shoot proliferation rate due to mitotic activity in apical and axillary meristems, low risk of genetic instability owing to somaclonal variations and better storage potential at low temperature are some potential advantages of unipolar explants being in use for propagation, germplasm conservation and exchange via synthetic seed (Sharma et al. 2013; Gantait et al. 2015). Different encapsulating or coating agents like sodium alginate, potassium alginate, sodium pectate, agar have been tested as hydrogel for synthetic seed production. However, the most of the successful studies carried out have pointed towards the usage of sodium alginate as encapsulating agent and calcium chloride as a polymerizing agent for the development of synthetic seed. Moreover, hardening and softening of alginate capsule are significantly influenced by sodium alginate and calcium chloride at different concentrations and also variable plant propagules used for encapsulation in different plant species (Rai et al. 2009; Sharma et al. 2013).

Moreover, an important application of synthetic seed is the propagation, conservation and delivery of rare hybrids, genetically modified plants, elite genotypes, rare, endangered and threatened (RET) plants (Rai et al. 2009). Encapsulation-based syn-seed technology facilitates the propagation of plants in both in vitro and ex vitro on standardized plant regeneration medium and under planting substrates such as soil, sand, soilrite, perlite or vermiculite, etc., respectively. Interestingly, encapsulation of explants taken from field grown plant followed by direct sowing of synthetic seed on planting substrates makes it a cost-effective technique and economically viable strategy for plant propagation at the commercial level (Sharma et al. 2013). Owing to better storage potential at low or ultra-low temperature (−196 °C) and its ability to recover plantlets after storage as well as easy handling due to small capsule size, synthetic seed approach can act as a potent tool in exchange of disease-free germplasm of elite genotypes between laboratories. In addition, production of synthetic seed is season independent and can be prepared throughout the year (Rai et al. 2009).

5.3 In Vitro Conservation of Medicinal and Threatened Plant Species

Encapsulation technology-based in vitro conservation of medicinal and threatened plants mainly includes slow growth culture techniques for short- or medium-term storage and cryopreservation for long-term storage. These in vitro conservation techniques offer storage of germplasm of important plant species in a comparatively small space under aseptic conditions. In recent decades, encapsulation technology has been successfully applied for propagation and conservation of several medicinally important and threatened plant species. Some recent reports of synthetic production in medicinal and threatened plants during the last two decades (2000–2020) are listed in Table 5.1.

5.3.1 Short- and Medium-Term Conservation

The main strategy to conserve plant germplasm for short- and medium-term using syn-seed technology is to store encapsulated propagules under growth-limiting conditions. This strategy is based on slow growth culture technique which allows encapsulated propagules to be held for few weeks to several months. The approaches adopted for the storage of synthetic seed for short- and medium-term include the incubation of synthetic seed at different temperatures mostly at 4 °C and/or dark condition and use of minimal growth medium by minimizing nutrient components in the medium or applying growth retardants or osmoticum for limiting the growth of cultures (Sharma et al. 2019). Majority of medicinal plants exhibited regrowth of synthetic seeds after storage at 4 °C. However, temperature requirement for optimal storage of synthetic seed may vary from species to species. In some medicinal and threatened plant species, storage of synthetic seed at 15 or 25 °C and/or in dark has also been reported advantageous for short-term conservation, for instances *Clitoria ternatea* (Kumar and Thomas 2012), *Mandevilla moricandiana* (Cordeiro et al. 2014), *Picrorhiza kurrooa* (Mishra et al. 2011), *Rhinacanthus nasutus* (Cheruvathur et al. 2013a), *Tylophora indica* (Gantait et al. 2017b), *Zingiber officinale* (Sundararaj et al. 2010). Plantlet conversion rate after storage at low temperature for different duration is an important characteristic feature of syn-seed technology-based in vitro conservation. Generally, regrowth ability of synthetic seed decreases with increase in storage period. Although a number of medicinal plants also exhibited high percent regrowth after storage for a longer duration when transferred to an appropriate medium. For example, synthetic seed of *Clitoria ternatea* stored at 4 °C exhibited 86% regrowth after 5 months of storage. Other than low temperature storage, use of growth retardant or minimal growth media has also been applied for storage of in vitro cultures in many plant species, however, very less work has been carried out on the application of growth retardant and minimal growth media in the preservation of medicinal plants in the form of synthetic seed. Mohanty et al. (2013a) established a short-term storage method of *Dendrobium nobile* using encapsulation technique and they reported that the incorporation of two osmotica sucrose and mannitol in

Table 5.1 Synthetic seed production in some medicinal and threatened plants (included only selected reports from 2000 to 2021)

Plant species	Explants used for encapsulation	References
Aconitum violaceum	Micro shoots	Rawat et al. (2013a)
Adhatoda vasica	Shoot tips	Anand and Bansal (2002)
Althaea officinalis	Nodal segments	Naz et al. (2018)
Anethum graveolens	Somatic embryos	Dhir et al. (2014)
Angelica glauca	Somatic embryos	Bisht et al. (2015)
Ansellia africana	Protocorm-like bodies	Bhattacharyya et al. (2018)
Aristolochia tagala	Nodal segments	Remya et al. (2013)
Arnebia euchroma	Somatic embryos	Manjkhola et al. (2005)
Azadirachta indica	Nodal segments	Padilla et al. (2021)
Bacopa monnieri	Shoot tips, nodal explant, somatic embryos	Ramesh et al. (2011), Khilwani et al. (2016), Rency et al. (2017), Sharma et al. (2020)
Balanites aegyptiaca	Nodal segment	Varshney and Anis (2014)
Cannabis sativa	Axillary buds	Lata et al. (2009)
Capparis decidua	Nodal segment	Siddique and Bukhari (2018)
Cassia angustifolia	Nodal segments	Bukhari et al. (2014), Parveen and Shahzad (2014)
Catharanthus roseus	Somatic embryos	Maqsood et al. (2012)
Centella asiatica	Somatic embryos, axillary buds/nodal explants	Joshee et al. (2007), Prasad et al. (2014)
Celastrus paniculatus	Nodal segments	Fonseka et al. (2019)
Ceropegia bulbosa	Nodal segments	Dhir and Shekhawat (2013)
Ceropegia barnesii	Nodes	Ananthan et al. (2018)
Ceropegia spiralis and *C. pusilla*	Shoot tips, nodal segments	Murthy et al. (2013)
Chonemorpha grandiflora	Shoot tips	Nishitha et al. (2006)
Chlorophytum borivilianum	Shoot buds	Dave et al. (2004)
Cineraria maritima	Micro shoots	Srivastava et al. (2009)
Clitoria ternatea	Somatic embryos	Kumar and Thomas (2012)
Crinum malabaricum	Somatic embryos	Priyadharshini et al. (2020)
Curcuma amada	Somatic embryos	Raju et al. (2016)

(continued)

Table 5.1 (continued)

Plant species	Explants used for encapsulation	References
Curculigo orchioides	Shoot buds	Nagesh et al. (2009), Dutta Gupta et al. (2019)
Cymbidium aloifolium	Protocorm	Pradhan et al. (2014)
Decalepis hamiltonii	Nodal segments	Sharma and Shahzad (2012)
Decalepis salicifolia	Shoot tips and nodal segments	Rodrigues et al. (2020)
Dendrobium nobile	Protocorm-like bodies	Mohanty et al. (2013a)
Digitalis davisiana	Shoot tips	Verma et al. (2016)
Eclipta alba	Shoot tips, nodal segments, somatic embryos	Ray and Bhattacharya (2010), Singh et al. (2010), Salma et al. (2019)
Eleutherococcus senticosus	Somatic embryos	Choi and Jeong (2002)
Glycyrrhiza glabra	Micro shoots	Mehrotra et al. (2012a)
Gymnema sylvestre	Nodal segment	Saeed et al. (2018)
Hedychium coronarium	Shoot segments	Behera et al. (2020)
Hemidesmus indicus	Somatic embryos, nodal segments	Cheruvathur et al. (2013b), Yadav et al. (2019)
Ipsea malabarica	Bulbs	Martin (2003)
Khaya senegalensis	Shoot tips and nodal segments	Hung and Trueman (2011)
Ledebouria revoluta	Somatic embryos	Haque and Ghosh (2016)
Ludwigia palustris	Nodal shoot segment	Fontanili et al. (2015)
Mandevilla moricandiana	Nodal segment	Cordeiro et al. (2014)
Mondia whitei	Somatic embryos	Baskaran et al. (2015)
Nerium oleander	Shoot tip and nodal segment	Hatzilazarou et al. (2019)
Ocimum basilicum	Nodal segment	Siddique and Anis (2009)
Ocimum gratissimum	Micro shoots	Saha et al. (2014)
Ocimum kilimandscharicum	Micro shoots	Saha et al. (2015)
Ocimum spp.	Shoot tips	Mandal et al. (2000)
Phyllanthus amarus	Shoot tips	Singh et al. (2006a)
Phyllanthus fraternus	Nodal segments	Upadhyay et al. (2014)

(continued)

Table 5.1 (continued)

Plant species	Explants used for encapsulation	References
Picrorhiza kurrooa	Micro shoots	Mishra et al. (2011)
Plectranthus amboinicus	Shoot apices	Arumugam et al. (2019)
Plumbago rosea	Axillary buds	Prakash et al. (2018)
Plumbago zeylanica	Embryoid callus	Jain et al. (2018)
Podophyllum peltatum	Axillary buds	Lata et al. (2010)
Pogostemon cablin	Nodal segments	Kumara Swamy et al. (2009)
Rauvolfia serpentina	Nodal segments, shoot tips	Ray and Bhattacharya (2008), Faisal et al. (2012), Gantait and Kundu (2017), Gantait et al. 2017a)
Rauvolfia tetraphylla	Shoots	Alatar and Faisal (2012), Faisal et al. (2013)
Rauvolfia vomitoria	Micro shoots	Mehrotra et al. (2012b)
Rhinacanthus nasutus	Somatic embryos	Cheruvathur et al. (2013a)
Ruta graveolens	Nodal segments	Ahmad et al. (2012)
Salix tetrasperma	Nodal segments	Khan et al. (2018)
Selinum tenuifolium	Somatic embryos	Joshi et al. (2006)
Serapias vomeracea	Protocorm-like bodies	Bektaş and Sökmen (2016)
Simmondsia chinensis	Nodal explants	Kumar et al. (2010)
Solanum nigrum	Shoot tips	Verma et al. (2010)
Sphagneticola calendulacea	Nodal segments	Kundu et al. (2018)
Splachnum ampullaceum	Moss buds	Mallón et al. (2007)
Spilanthes acmella	Shoot tips	Singh et al. (2009b)
Spilanthes mauritiana	Nodal segments	Sharma et al. (2009)
Sterculia urens	Nodal segments	Devi et al. (2014)
Stevia rebaudiana	Shoot tips	Nower (2014)
Swertia chirayita	Somatic embryos	Kumar and Chandra (2014)
Taraxacum pieninicum	Shoot tips	Kamińska et al. (2018, 2020)
Tecomella undulata	Nodal segments	Shaheen and Shahzad (2015)
Terminalia arjuna	Shoot tips	Gupta et al. (2014)
Tylophora indica	Nodal segments	Faisal and Anis (2007), Gantait et al. (2017b)
Urginea altissima	Shoot tips	Baskaran et al. (2018)

(continued)

Table 5.1 (continued)

Plant species	Explants used for encapsulation	References
Vanda coerulea	Protocorm-like bodies	Sarmah et al. (2010)
Vanda tessellata	Somatic embryos	Manokari et al. (2021)
Vitex negundo	Nodal segments	Ahmed and Anis (2010)
Vitex trifolia	Nodal segments	Ahmed et al. (2015), Alatar et al. (2017)
Withania coagulans	Micro-cuttings	Rathore and Kheni (2017)
Withania somnifera	Shoot tips, nodal segments	Singh et al. (2006b), Fatima et al. (2013)
Zingiber officinale	Micro shoots	Sundararaj et al. (2010)

encapsulation matrix affects significantly on storage duration. More recently, Kamińska et al. (2020) demonstrated the role of abscisic acid (ABA) in storage of synthetic seed of *Taraxacum pieninicum* under low temperature and found that shoot tip explant treated with ABA before encapsulation limits the growth and helped in extension of storage time up to 9 months. ABA regulates the physiological responses against the cold stress and its applicability in slow growth conservation has already been reported (Rai et al. 2011). In another study, Priyadharshini et al. (2020) attempted encapsulation technology for the conservation of an endemic and IUCN red listed critically endangered plant *Crinum malabaricum*.

5.3.2 Long-Term Conservation

Cryopreservation, storage of germplasm at ultra-low temperature in liquid nitrogen (−196 °C), is an ideal and most viable technique for long-term conservation of rare germplasm or genetic variants with economic values. Under such condition, cell division and thermally driven metabolic activities of cells are suspended, thus assuring the viability of germplasm for longer duration with less possibility of genetic alteration. One of the main advantages of cryopreservation is that germplasm can be stored in cryogenic bank in little space and cost of storage is much lower than that of other traditional ex situ conservation methods (Engelmann 2011; Sharma et al. 2019). Different types of plant parts like seed, pollen, flower buds, roots, tubers, zygotic embryos or in vitro derived explants like shoot tips, micro cuttings, somatic embryos, protoplasts, etc. can be used for conservation purposes using cryopreservation technique (Benson 2008). Among in vitro derived explants, shoot tip is one of the best choices for the cryopreservation of many medicinal plants because of assurance of direct regeneration and high multiplication rate due to presence of meristematic cells as well as regeneration of virus free and genetic stable plant after cryo-storage (Normah et al. 2019). Many in vitro derived explants have high water content; thus, these plant materials are supposed to be more sensitive to cellular dehydration under cold stress. However, use of cryoprotectants and encapsulation of such explants protect the tissues against the dehydration injury in cold

stress environment (Reed 2018). Although several cryopreservation methods have been used for conservation in recent years, however cryopreservation techniques based on encapsulation technology include encapsulation-dehydration and encapsulation-vitrification. In both synthetic seed-based techniques, explants are encapsulated before dehydration and freezing in liquid nitrogen, but both differ mainly in dehydration process. In encapsulation-dehydration technique, encapsulated explants initially undergo a physical dehydration process either by air drying in laminar air flow or with silica gel before freezing. Encapsulation-vitrification technique is based on osmotic dehydration in which encapsulated explants are first treated with a high concentration of vitrifying solution of cryoprotectants and then placed in liquid nitrogen for freezing (Engelmann 2011; Sharma et al. 2019). In comparison to the encapsulation-dehydration, encapsulation-vitrification is a better technique because explants are sufficiently dehydrated in vitrification solution without causing injury resulting in enhanced/fast and high post-freezing recovery growth (Sakai and Engelmann 2007). Moreover, cryoprotectants prevent the formation of ice crystals and eliminate the freezable waters from cryopreserved cells or tissues (Cordeiro et al. 2020). Direct exposure of explants to cryoprotectants in non-encapsulated vitrification technique sometimes causes cell or tissue damage due to chemical toxicity of vitrification solution. Encapsulation not only provides physical support for easy handling of small sized and fragile explants but also protects the plant materials from toxic effects of cryoprotectants (Ciringer et al. 2018). Both encapsulation-based cryopreservation techniques are easy to operate and have high post-storage recovery in most cases. During recent decades, both techniques have successfully been applied in a wide range of plant species including medicinal and threatened plants (Table 5.2). Encapsulation-dehydration technique has been used to conserve *Pteris adscensionis*, an endemic and critically endangered fern of Ascension Island (Barnicoat et al. 2011). More recently, Cordeiro et al. (2020) reported the successful cryopreservation of in vitro derived root cultures of *Tarenaya rosea,* an endemic species belonging to coastal plains of Brazil using encapsulation-vitrification technique. In another study, both encapsulation-dehydration and encapsulation-vitrification techniques have been applied for cryopreservation of *Hladnikia pastinacifolia*, a monotypic endemic and endangered species of Slovenia (Ciringer et al. 2018).

5.4 Some Medicinal and Threatened Plant Species Used for Synthetic Seed Production: Case Studies

5.4.1 *Bacopa monnieri*

Bacopa monnieri L., belongs to the family *Scrophulariaceae*, is an important medicinal herb and well recognized in Indian traditional ayurvedic system for its memory boosting effect and pharmacological activities (Ramesh et al. 2011). Due to diverse medicinal importance and overexploitation from its natural habitat for commercial purpose, *B. monnieri* was listed as threatened plant species during

Table 5.2 Synthetic seed-based cryopreservation of some medicinal and threatened plant species (included only selected recent reports from 2010 to 2020)

Plant species	Explants used for encapsulation	Cryopreservation technique	References
Ajania pacifica	Shoot tip	Encapsulation-dehydration	Kulus and Abratowska (2017)
Anemarrhena asphodeloides	Embryogenic callus	Encapsulation-vitrification	Hong and Yin (2012)
Artemisia herba-alba	Shoot tip	Encapsulation-dehydration, encapsulation-vitrification	Sharaf et al. (2012)
Asparagus officinalis	Roots	Encapsulation-dehydration	Carmona-Martin et al. (2018)
Astragalus membranaceus	Shoot tip	Encapsulation-dehydration, encapsulation-vitrification	Ming-Hua and Sen-Rong (2015)
Cymbidium eburneum and C. hookerianum	Protocorm-like bodies	Encapsulation-dehydration	Gogoi et al. (2013)
Dendrobium chrysanthum	Protocorm-like bodies	Encapsulation-vitrification	Mohanty et al. (2013b)
Dendrobium nobile	Protocorm-like bodies	Encapsulation-dehydration Encapsulation-vitrification	Mohanty et al. (2012)
Dioscorea bulbifera	Embryogenic callus	Encapsulation-vitrification	Ming-Hua and Sen-Rong (2010)
Hladnikia pastinacifolia	Shoot tip	Encapsulation-dehydration, encapsulation-vitrification	Ciringer et al. (2018)
Ipomoea batatas	Shoot apices	Encapsulation-dehydration	Agbidinoukoun et al. (2018)
Lamprocapnos spectabilis	Shoot tip	Encapsulation-vitrification	Kulus (2020)
Mandevilla moricandiana	Nodal segments	Encapsulation-dehydration	Cordeiro et al. (2014)
Osmunda regalis	Gametophytes	Encapsulation-vitrification	Makowski et al. (2016)
Petiveria alliacea	Somatic embryos	Encapsulation-dehydration	de Almeida Pettinelli et al. (2017)
Plantago algarbiensis	Nodal segments	Encapsulation-dehydration	Coelho et al. (2014a)
Pteris adscensionis	Gametophytes	Encapsulation-dehydration	Barnicoat et al. (2011)
Rabdosia rubescens	Shoot tip	Encapsulation-dehydration	Ai et al. (2012)
Tarenaya rosea	Root culture	Encapsulation-vitrification	Cordeiro et al. (2020)
Teucrium polium	Shoot tip	Encapsulation-dehydration	Rabba'a et al. (2012)
Tuberaria major	Shoot tip	Encapsulation-dehydration	Coelho et al. (2014b)
Ziziphora tenuior	Shoot tip	Encapsulation-dehydration	Al-Baba et al. (2015)

1990s (Khilwani et al. 2016). However, according to recent data base of IUCN red list of threatened species (IUCN 2020), this plant is now categorized as least concern (LC). In vitro propagation of this plant is well established using different explants (reviewed by Saha et al. 2020). In vitro conservation of this plant using synthetic seed approach was performed by many researchers. Both embryogenic, i.e. somatic embryo and non-embryogenic, i.e. shoot tips and nodal explants have been used for production of synthetic seed in *B. monnieri* (Ramesh et al. 2009, 2011; Muthiah et al. 2013; Khilwani et al. 2016; Rency et al. 2017; Sharma et al. 2020). Khilwani et al. (2016) developed synthetic seed by encapsulation of somatic embryos for short-term storage at 4 °C and 25 °C and found that they remained viable after storage at both temperatures after 140 days; however, the percent regrowth of synthetic seeds was higher when it was stored at 25 °C. Rency et al. (2017) demonstrated the effect of seaweed liquid extracts (SLEs) isolated from red algae *Gracilaria salicornia* on shoot and root induction from encapsulated shoot tips of *B. monnieri*. Recently, Sharma et al. (2020) established a method of mid-term conservation of *B. monnieri* applying slow growth of encapsulated shoot tips and nodal segments through reduction of oxygen with mineral oil overlay. Using this approach, they were able to conserve genetic resource of *B. monnieri* for 12 months. Applying different molecular markers, genetic stability of plants regenerated from encapsulated shoot tips or nodal segments as well as plants recovered after storage of synthetic seeds for different storage period have also been demonstrated in a few studies (Ramesh et al. 2011; Muthiah et al. 2013; Rency et al. 2017).

5.4.2 *Eclipta alba*

Eclipta alba (L.) Hassk syn. *E. prostrata* L., belonging to the sunflower family *Asteraceae*, is another medicinally important plant of tropical and subtropical regions of the world. It is an important component of many ayurvedic formulations particularly that are used in the treatment of liver diseases and hairs, skin and memory related disorders (Singh et al. 2012). In *E. alba*, synthetic seed has been developed using nodal segment and somatic embryo explants (Ray and Bhattacharya 2010; Singh et al. 2010; Salma et al. 2019). Singh et al. (2010) demonstrated the synthetic seed production in *E. alba* using in vitro derived nodal segments encapsulated in 3% sodium alginate and 100 mM calcium chloride with maximum percent plantlet conversion on MS (Murashige and Skoog 1962) medium containing 0.88μM BAP (6-benzylaminopurine). Encapsulated nodal segments could have also been stored at low temperature, but survival of synthetic seeds declined markedly following storage for 60 days. About 50% synthetic seeds were recovered and converted into plantlets after storage for 60 days. In another study, Ray and Bhattacharya (2010) optimized different storage conditions of encapsulated shoot tips and nodal segments and found highest regrowth in syn-seeds stored at 4 °C for 8 weeks. Using RAPD (Randomly Amplified Polymorphic DNA) analysis, they also validated the genetic stability of plantlets derived from synthetic seeds. Recently, Salma et al. (2019) succeeded to induce somatic embryos from nodal explants and

further synthetic seed production from encapsulation of torpedo stage somatic embryos in 2.5% sodium alginate and 75 mM calcium chloride with highest 93.33% germination rate.

5.4.3 *Hedychium coronarium*

Hedychium coronarium (family *Zingiberaceae*), an aromatic rhizomatous plant, is well-known for its medicinal values in Ayurveda and traditional system of medicine and almost all parts of this plant are used for the treatment of various diseases (Behera et al. 2018). According to recent data base of red list of threatened species (IUCN 2020), this plant is categorized as Data deficient (DD), but in many literatures, this species is enlisted as threatened species of some states of India and is one among the important plant species of conservation concern in India (Behera et al. 2018, 2020). More recently, Behera et al. (2020) reported synthetic seed development in *H. coronarium* by alginate encapsulation of in vitro shoot segments derived from multiple shoot cultures. An ideal synthetic seed of *H. coronarium* was prepared in 3% sodium alginate and 100 mM $CaCl_2.2H_2O$ (Fig. 5.2a). Maximum conversion of synthetic seed into plantlets (shoot and root both in one step) was

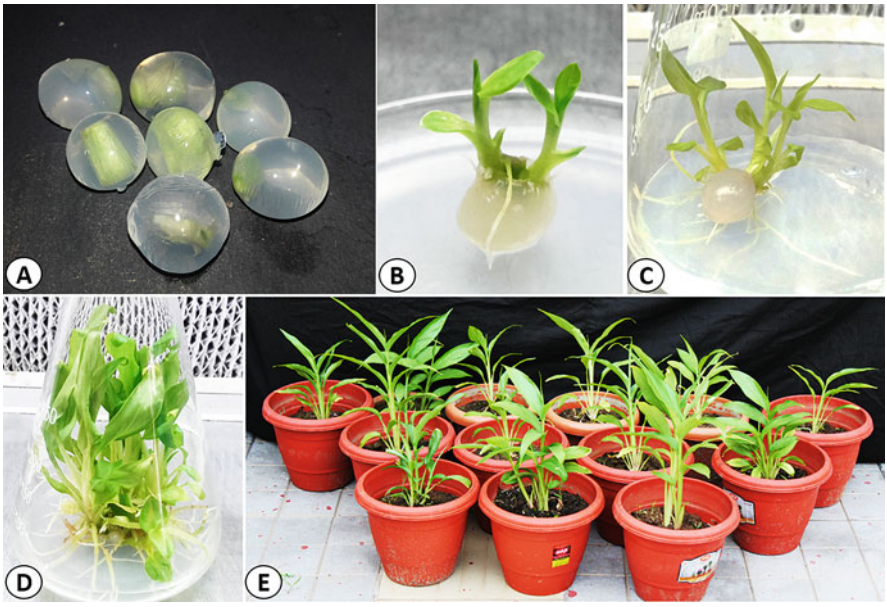

Fig. 5.2 Production of synthetic seed in *Hedychium coronarium.* (**a**) Synthetic seeds prepared by alginate encapsulation of axenic shoot segments. (**b**, **c**) Root and shoot emergence from synthetic seed on plant growth regulator free MS medium. (**d**) Plantlet conversion from synthetic seed exhibiting multiple shoots and roots. (**e**) Acclimatization of synthetic seed derived plants in pots containing garden soil

achieved on full-strength MS medium (Fig. 5.2b–d). Plants developed through synthetic seed were acclimatized successfully under ex vitro condition with 95% survival (Fig. 5.2e). Behera et al. (2020) also demonstrated short-term storage of synthetic seed at 4 °C and 25 °C for 8 weeks and showed 44.4% and 42.2% revival of plantlets on optimized regeneration medium, respectively. They also confirmed the genetic fidelity of plants regenerated from synthetic seeds and plants revived after storage at low temperature using two molecular marker systems.

5.4.4 *Picrorhiza kurrooa*

Picrorhiza kurroa (Family *Scrophulariaceae*) is an endemic plant species of alpine Himalayan region from Kashmir to Sikkim, India. Due to enormous medicinal properties, it is widely used in manufacture of many ayurvedic drugs and the world annual demand of this plant is more than that of its total annual production (Debnath et al. 2020). Due to overexploitation, it is also listed in one of the important medicinal plants of conservation concern. Some literatures also report its conservation status as threatened plant species (Rawat et al. 2013b; Debnath et al. 2020). In vitro conservation of *P. kurroa* through syn-seed technology has been reported by Mishra et al. (2011). Encapsulated microshoots were stored in a moist-environment for 3 months at 25 °C. Following 3 months of storage, about 90% synthetic seeds were converted into plantlets when cultured on half strength MS basal medium for regrowth. The genetic fidelity of plants developed after the storage was also evaluated through RAPD analysis.

5.4.5 *Rauvolfia* spp.

Rauvolfia spp., belong to the family *Apocynaceae*, are a group of plants with high secondary metabolite and medicinal values in Indian tradition medicine. *R. serpentina* is a representative and most important species. Other important species include *R. tetraphylla*, *R. micrantha* and *R. macrophylla*. Due to overutilization and extensive extraction of reserpine alkaloids, some *Rauvolfia* species have now been pushed to endangered categories (Mukherjee et al. 2019). In *Rauvolfia* spp., only unipolar shoot tips and nodal segments have been used for synthetic seed development (Ray and Bhattacharya 2008; Alatar and Faisal 2012; Faisal et al. 2012, 2013; Gantait and Kundu 2017; Gantait et al. 2017a). In vitro conservation of two *Rauvolfia* species, i.e. *R. serpentina* and *R. tetraphylla* through storage of synthetic seeds at different temperature has been reported by many workers, however in most of these studies storage of encapsulated propagules at 4 °C was most effective (Ray and Bhattacharya 2008; Alatar and Faisal 2012; Faisal et al. 2012, 2013; Gantait and Kundu 2017). Ray and Bhattacharya (2008) demonstrated the storage of encapsulated shoot tips of *R. serpentina* on three different temperatures (4, 12 and 20 °C) and found synthetic seeds incubated on 12 and 20 °C were not able to survive after 4 weeks, while synthetic seed stored at 4 °C showed 68.5% regrowth even after

14 weeks of storage. In another study, Gantait and Kundu (2017) observed higher post-storage survival of encapsulated shoot tips of *R. serpentina* stored at 25 °C as compared to the one stored at comparatively low temperature (8 °C) for 30 days.

5.4.6 *Withania* spp.

Genus *Withania*, comprising 23 species, is an important member of family *Solanaceae* (Bharti et al. 2016). Within genus *Withania*, *W. somnifera* and *W. coagulans* are two most economically and medicinally important plant species and both species are well-known for their several medicinal properties (Rathore and Kheni 2017; Shasmita et al. 2018). Medicinal attributes of both species are mainly due to the presence of steroidal lactones, i.e. withanolides in different parts of plant. Reproductive obstacles and overexploitation forced *W. coagulans* towards the extinction and now categorized as critically endangered species (Rathore and Kheni 2017). There are some published reports on synthetic seed production and their short-term storage in *W. somnifera* (Singh et al. 2006b; Fatima et al. 2013) and *W. coagulans* (Rathore and Kheni 2017). In all these studies, in vitro derived vegetative propagules shoot tips or nodal segments have been used for synthetic seed production. All these three studies also demonstrated the storage of synthetic seeds for short-term durations at low temperature. Moreover, Singh et al. (2006b) succeeded to obtain plantlet of *W. somnifera* from encapsulated shoot tips directly sown in sterile soilrite moistened with ¼ MS salts.

5.5 National and International Relevance

Owing to high demands in traditional medicines, overexploitation for other valuable plant based products, habitat loss, plant biodiversity particularly wild plant species used in herbal medicines are threatened globally and many of them are at high risk of extinction. Synthetic seed technology, an excellent method of ex situ conservation, is an internationally accepted strategy and is now frequently employed for germplasm conservation. In addition, transport of germplasm of threatened, medicinal or other commercially important plant species from one laboratory or extension center to other national or international laboratories without spread of disease and in a convenient way is an enviable property of this technique which proves its worldwide relevance.

5.6 Concluding Remarks and Future Prospects

Encapsulation technology is recommended as a potential tool for the propagation and conservation of germplasm of rare and endangered plant species or plants which have recalcitrant seeds or produce non-viable seeds. Due to its ability to retain their viability after cryo-storage or storage at low temperature for weeks to years, this

technique is gaining popularity among conservation biologists. Since first report in 1980s, practical significance of synthetic seed technique is still alive and it has been applied in a wide range of plant species for the propagation and conservation purpose. Despite advantages and progress achieved in last 2–3 decades, encapsulation technology has some limitations which need to be resolved. Due to bipolar nature, synthetic seed via encapsulation of somatic embryos has great significance but production of somatic embryos is restricted to some certain medicinal plant species. Alternatively, non-embryogenic vegetative propagules like shoot tips, nodal segments and other tissues have been used for synthetic seed production in majority of plant species. However, additional rooting steps, low conversion frequency and problems in acclimatization of synthetic seed derived plantlets limit its utility in a number of plant species. In addition, very little success after direct sowing of synthetic seed in soil is a major drawback of the practical applicability of this technique. Therefore, refinements in technique are necessary to resolve such limitations particularly, an improvement in the plantlet conversion rate during direct sowing in soil and after long-term conservation. Ultimately, improved synthetic seed technique will help in the restoration of medicinal and threatened plants to its natural habitat and make it more feasible for agriculture, horticulture, forestry and the plant-based industry.

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Somaclonal Variation in Improvement of Agricultural Crops: Recent Progress

6

Manoj K. Rai

Abstract

Genetic variability induced by cell and tissue culture provides a new source of variation within a species that can be utilized for crop improvement. The selection of novel variants through in vitro induced somaclonal variation helps in the generation of new cultivars with many important agronomic traits, which may be exploited in agriculture for breeding purpose. Some desirable characteristics can also be achieved by in vitro selection pressure technique. During the last 3–4 decades, several useful somaclonal variants particularly linked to the agronomic and agriculturally useful traits like yield, nutrient quality, disease resistance, abiotic stress tolerance, etc. have been selected and few of them have been released as cultivars for commercial production. However, selection of somaclonal variants for crop improvement has some major limitations like selection of undesirable traits, genetic instability and loss of regeneration after selection. Therefore, many efforts are needed to achieve desired results. In this chapter, the source and genetic basis of somaclonal variation, its detection methods and advantages of somaclonal variation in agriculture with main emphasis on some useful somaclonal variants released as a cultivar are discussed.

Keywords

Crop improvement · Genetic and epigenetic change · In vitro selection · Molecular marker · Plant tissue culture · Somaclonal variants

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6.1 Introduction

6.1.1 Somaclonal Variation: Causes, Sources and Detection Methods

The maintenance of genetic diversity/variability within a population is an important aspect of plant breeding. Genetic diversity, which enables the changes in the genetic composition of a species, is the basis of selection of superior varieties as well as crop improvement by offering a choice to the breeders for the development of new hybrids (Mohammadi and Prasanna 2003). Different factors such as mutation, genetic recombination and natural selection influence genetic variability in a species (Bhandari et al. 2017). In the last 3–4 decades, plant tissue culture technique has been widely exploited as an alternative means of plant propagation and crop improvement by generating genetic alterations in regenerated plants from cell and tissue culture and used to develop a new breeding line (Bhojwani and Dantu 2013). Although, tissue culture-induced genetic variation in in vitro micropropagated plantlets, commonly referred as 'somaclonal variation' (Larkin and Scowcroft 1981), is one of the main drawbacks in commercial micropropagation in which the regenerant is expected to be true to type (Neelakandan and Wang 2012). However, these tissue culture-induced variations have been efficiently used to develop new varieties with agriculturally important traits in a number of species (Karp 1995; Jain 2001; Rai et al. 2011; Krishna et al. 2016).

Theoretically, the regeneration of whole plant from a cell or tissue using in vitro culture technique is an asexual process which involves mitotic division in cells and there is no involvement of genetic recombination resulting in development of genetically uniform plants (Bairu et al. 2011). However, many stress and other factors during tissue culture processes like explants sources, genotypes, mode of regeneration particularly cell suspension and callus culture, length of culture period, culture environment and number of subculture cycles, use of imbalance concentrations of plant growth regulators, sugar, nutrients or other additives in culture media, etc. trigger spontaneous genetic, epigenetic or phenetic variations in tissue culture raised plants (Rani and Raina 2000; Bairu et al. 2011; Rai et al. 2012; Smulders and de Klerk 2011; Krishna et al. 2016). Other than spontaneous variation induced due to growth of cell, tissue or callus for several subcultures and further regeneration of plants from long-term cultures, somaclonal variation can be induced using in vitro selection technique for the development of useful somac1onal variants (Brar and Jain 1998; Jain 2001; Rai et al. 2011). Undesired phenotypic anomalies and variations occur in plants regenerated by in vitro culture technique may differ from the mother plant permanently or temporarily. Usually, somaclonal variants also referred to permanent variants, are genetic, heritable and non-reversible. Other temporary variations in tissue culture raised plants could also arise due to epigenetic changes and physiological factors which are non-heritable and reversible (Kaeppler et al. 2000; Bairu et al. 2011). Many studies also suggested that somaclonal variation is the result of pre-existing genetic variations in cultured cells and tissues which are

induced by different in vitro factors during tissue culture processes (Brar and Jain 1998; Bairu et al. 2011; Bhojwani and Dantu 2013).

Molecular basis of somaclonal variation has now been well explored in recent years. Due to several intrinsic and extrinsic factors, there is a high probability of genetic and epigenetic changes in regenerants, such as single base pair changes, changes in chromosome number or ploidy level, chromosomal aberrations and rearrangements, activation of transposable elements, etc. which may lead to mutations in plant cells and cause somaclonal variation (Bairu et al. 2011; Springer and Schmitz 2017). Hyper- or hypomethylation are two major phenomena which contribute to epigenetic changes in regenerants. Stressful situations due to artificial culture environment and requirement of high concentrations of PGRs and nutrients also linked to the generation of reactive oxygen species (ROS) and free radicals in in vitro regenerated plants which may also involve in DNA methylation changes (Krishna et al. 2016). Usually, in vitro regeneration methods involving undifferentiated cells like calli or protoplasts is considered to be most unreliable for clonal propagation and likely to be more sensitive to somaclonal variation in comparison to plantlets regenerated through apical and axillary meristems (Rani and Raina 2000; Bairu et al. 2011; Rai et al. 2012; Phulwaria et al. 2013; Krishna et al. 2016).

Somaclonal variation is detected not only by the changes in morphological characters of regenerants but also it is characterized by the multifaceted changes at cytological, biochemical and genetic or epigenetic levels (Bairu et al. 2011; Krishna et al. 2016). Earlier, detection of somaclonal variants was based on the alterations in morphological characters and cytological studies primarily analysis of numerical and structural changes in the chromosomes (Bhojwani and Dantu 2013). However, nowadays a wide range of most advanced tools and techniques are available for the detection of somaclonal variants particularly DNA based molecular markers (Bairu et al. 2011; Krishna et al. 2016), detection of DNA methylation using the methylation-sensitive amplification polymorphism (MSAP) (Gonzalez et al. 2013) and next generation sequencing (Park et al. 2020). Due to simple and easy to detection as well as ability to detect variation at early growth phase of regenerants, a number of PCR based molecular markers like amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), inter simple sequence repeat (ISSR), simple sequence repeat (SSR), start codon targeted (ScoT) polymorphism are preferred for the detection of somaclonal variation (Espinosa-Leal et al. 2018). In addition, many molecular markers are able to identify a specific genomic region that is linked with a useful agronomic and agriculturally important traits (Kalia et al. 2011). These DNA based molecular markers have been utilized not only in detection of somaclonal variations (Table 6.1) but also analysis of the genetic stability of tissue culture raised plants (Rani and Raina 2000; Bairu et al. 2011; Rathore et al. 2014; Krishna et al. 2016). Some recent reports published during the last decade (2011–2021) on the sources and causes of somaclonal variations and its detection methods are listed in Table 6.1.

Table 6.1 Some selected recent reports on somaclonal variation, possible causes and detection methods of somaclonal variants

Plant name	Mode of regeneration/ explants	Source/causes of somaclonal variation	Detection method	Reference
Ananas comosus	Leaf base explants	Epigenetic changes by hormonal induction, NaCl, and ABA	HDAC enzyme activity	Halim et al. (2018)
Asparagus officinalis	Callus induction from rhizome bud explants	Polyploidization	Flow cytometry	Regalado et al. (2015)
Brassica napus	Callus induction from hypocotyl explants	Change in DNA methylation level	DNA methylation estimation by HPLC	Gao et al. (2014)
Caladium hortulanum	Leaf explants	Type of leaf explants and auxin/ chromosome number change	SSR markers	Cao et al. (2016)
Chrysanthemum morifolium	Leaf protoplasts	Genome size aberration	Flow cytometry	Eeckhaut et al. (2020)
Coffea arabica	Embryogenic calli induced from leaf explants	Long-term cell cultures/ methylation and transposable elements changes	AFLP, MSAP, SSAP molecular markers	Landey et al. (2015)
Elaeis guineensis	Embryogenic suspension culture	Changes in genomic DNA methylation levels	HPLC estimation of global methylation rates	Rival et al. (2013)
Hordeum vulgare	Callus induction from spikes	Genome changes	IRAP and ISSR marker	Campbell et al. (2011)
Linum usitatissimum and *L. album*	Plump and smooth flax seeds	Number of subcultures	SSR, EST-SSR, IRAP and REMAP analyses	Noormohammadi et al. (2020)
Miscanthus giganteus	Callus induction	Long-term shoot culture/ DNA methylation	ISSR, RAPD and MS-ISSR markers	Cichorz et al. (2018)
Olea europaea	Somatic embryogenesis	Genotype and culture age	Biometric analysis	Bradai et al. (2016)

(continued)

Table 6.1 (continued)

Plant name	Mode of regeneration/ explants	Source/causes of somaclonal variation	Detection method	Reference
Saccharum spp.	TIBs	In vitro establishment and the number of subcultures	ISSR molecular markers	Martínez-Estrada et al. (2017)
Secale cereale	Somatic embryo	Genetic and epigenetic changes	Methylation-sensitive ISSR marker	Linacero et al. (2011)
Solanum tuberosum	Protoplasts and stem explants	Aneuploidy or structural chromosomal changes	Flow cytometry	Fossi et al. (2019)
Triticosecale (triticale)	Doubled haploids regenerants derived from isolated microspores	Culture technique and genetic background of donor plants induced genetic and epigenetic variation	MetAFLP and RP-HPLC	Machczynska et al. (2015)
Vanilla planifolia	Nodal segments/ shoot multiplied by ten subculture cycles	Number of subculture cycles	ISSR molecular markers	Pastelin-Solano et al. (2019)
Viola uliginosa	Callus induction from leaf and petiole fragments	Change in ploidy level	Flow cytometry	Slazak et al. (2015)

ABA abscisic acid, *AFLP* amplified fragment length polymorphism, *EST-SSR* expressed sequence tag SSR, *HPLC* high performance liquid chromatography, *HDAC* histone deacetylase, *IRAP* inter-retrotransposon amplified polymorphism, *ISSR* inter simple sequence repeat, *MetAFLP* methylation-sensitive amplified fragment length polymorphism, *MSAP* methylation-sensitive amplified polymorphism, *NaCl* sodium chloride, *RAPD* random amplified polymorphic DNA, *REMAP* retrotransposon-microsatellite amplified polymorphism, *RP-HPLC* reversed-phase HPLC, *SSAP* sequence-specific amplification polymorphism, *SSR* simple sequence repeat, *TIBs* temporary immersion bioreactors

6.1.2 Advantages and Disadvantages of Somaclonal Variation

In the last 2–3 decades, somaclonal variation and its advantages and disadvantages have been studied extensively in many commercially and agriculturally important crops. The main advantage of somaclonal variation is the creation of new genotypes without the use of sophisticated tools and technologies. Unlike the traditional plant breeding and transgenic technique, somaclonal variation allows us to generate a new trait without disturbing the useful traits that are already present (Bhojwani and Dantu

2013; Sahijram 2015). The selection of novel variants through induced somaclonal variation helps in the generation of new cultivars with many important agronomic traits which may be exploited in agriculture for breeding purpose, particularly in those crops having a narrow genetic base. Using somaclonal variant selection technique, few new cultivars with abiotic stress tolerance, disease resistance and other useful traits have been released in a number of crops including sugarcane, barley, wheat, rice, tomato, potato, mustard, banana, etc. (reviewed by Bhojwani and Dantu 2013).

Despite several advantages and some successful stories, somaclonal variation has also some serious limitations. Many studies reported that the results of somaclonal variation are uncontrollable and unpredictable which have no practical use in crop improvement. In addition, many epigenetic changes induced under in vitro conditions were non-heritable and not stable after selfing or crossing (Krishna et al. 2016). In some cases, somaclonal variants also showed undesirable features like reduced growth rate, aneuploidy, sterility, etc. (Brar and Jain 1998). Moreover, selection of variants is restricted only to those plants which have a well-established in vitro regeneration system. Usually, primary objective of micropropagation technology is to produce clones of elite genotypes but unwanted somaclonal variations reduce the commercial value of tissue culture raised plants (Bhojwani and Dantu 2013).

6.2 Somaclonal Variation Induced by In Vitro Selection Method and Crop Improvement

Induced mutation has contributed significantly to crop improvement worldwide. Tissue culture technique also offers a feasible method to induce novel variations in plants by culturing cell, tissues or organs on medium containing selective agents. Exposure of cells and tissues on a specific selective agent provides an opportunity to manipulate totipotent cells and select useful mutations at cellular level and permits the regeneration of plants with desirable characteristics (Suprasanna et al. 2009; Rai et al. 2011; Penna et al. 2012). A variety of selective agents are used to obtain a specific trait such as sodium chloride (NaCl) for salt tolerance, polyethylene glycol (PEG), D-mannitol or sorbitol for drought or water stress tolerance and culture filtrates or phytotoxin for disease resistance and only cell lines capable of tolerating such environment and survive after many cycles are selected (Van den Bulk 1991; Remotti 1998; Jain 2001; Rai et al. 2011). Induction of mutation and creation of genetic variation, i.e., somaclonal variation in cells, tissues and/or organs is the basis of in vitro selection technique. In most cases of in vitro selection experiments, one step selection method has been applied. In this selection process, cell, callus or other explants is exposed to maximum inhibitory level of selective agents and finally selected after 3–4 cycle of subculturing on tolerating level of selective agent. After stability test, selected cell/callus lines are finally subjected for the plant regeneration with desirable characteristics. A schematic illustration of in vitro selection and plant regeneration for salt stress tolerance is presented in Fig. 6.1. Another in vitro selection

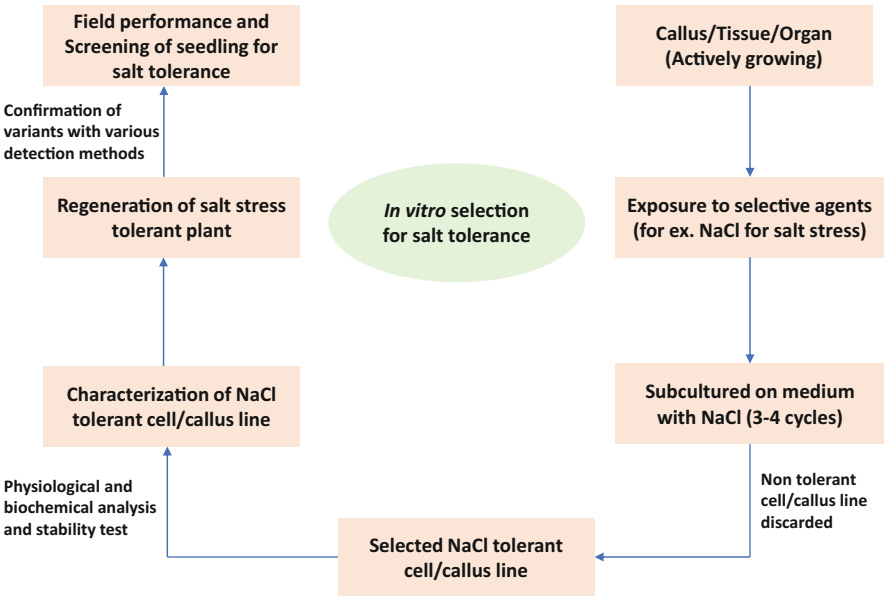

Fig. 6.1 A schematic illustration of in vitro selection and plant regeneration for salt stress tolerance

method, commonly known as stepwise selection, involves the exposure of culture to a selective agent with a gradual increase from sublethal to lethal concentration. During the past 2–3 decades, some traits of agronomic and agriculturally importance like abiotic stress tolerance particularly salt and drought, disease resistance, herbicide resistance, etc. have been selected using in vitro selection technique. Some recent reports (published during the last decade) on in vitro screening and selection of plants for abiotic stress tolerance and disease resistance are listed in Table 6.2.

6.2.1 In Vitro Selection for Abiotic Stress Tolerance

In the era of climate change, several abiotic stresses like salinity, drought, heat, metal toxicity, heat, frost, etc. adversely influence the productivity of many commercially important plants (Sreenivasulu et al. 2007; van Zelm et al. 2020). Therefore, selection of abiotic stress tolerant cultivars/varieties is a prerequisite to increase crop productivity. As compared to technologies involving transfer of foreign gene in a sexually incompatible crop, i.e. genetic transformation which has limited practical application due to silencing of transgene, use of antibiotic resistant gene as a selectable marker, consequent reduction of gene expression and low transformation frequency, tissue culture based in vitro selection technique provides a wider choice of selection of stress tolerant variants developed after mutagenic treatment under control and in vitro condition (Jain 2001; Rai et al. 2011; Penna et al. 2012; Rai and Shekhawat 2014). Using in vitro selection technique, many studies have been carried

Table 6.2 Some selected recent reports on screening and in vitro selection for biotic and abiotic stress

Plant species	Selecting agents	Abiotic stress/resistant against pathogen (biotic stress)	References
Beta vulgaris	Culture filtrate	*Fusarium oxysporum*	Yerzhebayeva et al. (2019)
Cenchrus ciliaris	Mannitol	Water stress	Carloni et al. (2017)
Citrus sinensis	EMS	*Xanthomonas citri* subsp. *citri* (citrus canker)	Ge et al. (2015)
Daphne jasminea and *Daphne tangutica*	Pb $(NO_3)_2$	Lead stress	Wiszniewska et al. (2015)
Eucalyptus tereticornis	D-mannitol	Drought stress	Singh et al. (2020)
Guizotia abyssinica	NaCl	Salt stress	Ghane et al. (2014)
Mangifera indica	PEG	Moisture stress (drought)	Pradhan et al. (2021)
Malus sp.	Culture filtrate	*Phytophthora cactorum* (collar rot disease)	Verma et al. (2021)
Oryza sativa	Gamma radiation + NaCl or Sorbitol	Salinity and drought stress	Abdelnour-Esquivel et al. (2020)
	PEG	Drought stress	Verma et al. (2013)
Passiflora edulis	Fungal culture filtrate and purified toxin fusaric acid	*Fusarium oxysporum* f. sp. *passiflorae*	Flores et al. (2012)
Punica granatum	Culture filtrate	*Xanthomonas axonopodis* pv. *Punicae* (bacterial blight)	Kumari et al. (2017)
Saccharum officinarum	PEG	Drought stress	Rao and Jabeen (2013)
	Gamma radiation + NaCl	Salt stress	Nikam et al. (2014)
Saccharum sp.	EMS + PEG	Drought stress	Masoabi et al. (2018)
Solanum tuberosum	Cd	Cadmium	Ashrafzadeh and Leung (2017)
Vanilla planifolia	Culture filtrate	*Fusarium oxysporum* f. sp. *vanillae*	Ramirez-Mosqueda et al. (2019)
Vigna radiata	NaCl	Salt stress	Rao and Patil (2012)
Withania somnifera	Phathotoxin	*Alternaria alternata* (leaf blight disease)	Chakraborty et al. (2020)

EMS ethyl methane sulphonate, *NaCl* sodium chloride, *PEG* polyethylene glycol

out to develop abiotic stress tolerant plants, especially for salt, drought and metal toxicity stress (reviewed by Jain 2001; Rai et al. 2011; Penna et al. 2012). In vitro selection technique has been used to produce abiotic stress tolerant cell line and plants in many species like *Cenchrus ciliaris* (Carloni et al. 2017), *Eucalyptus tereticornis* (Singh et al. 2020), *Mangifera indica* (Pradhan et al. 2021), *Oryza sativa* (Abdelnour-Esquivel et al. 2020), *Saccharum officinarum* (Rao and Jabeen 2013), *Solanum tuberosum* (Ashrafzadeh and Leung 2017), etc. For this purpose, a number of culture systems, i.e. callus, protoplast, cell suspension cultures, somatic embryos, shoot cultures, etc. have been applied, but in most of cases callus, protoplast or cell suspension cultures were preferred (Penna et al. 2012). In majority of salinity and drought tolerance studies, NaCl and PEG are used as a selective agent, respectively. However, other selective agents like KCl, Na_2SO_4 and $MgSO_4$ (for salt stress) and mannitol, sorbitol, sucrose (for drought tolerance) have also been used in many other studies (Rai et al. 2011). Usually, aluminium chloride and $Pb(NO_3)_2$ have been applied for the selection for aluminium and lead stress tolerance, respectively. In some cases, explants initially treated with chemical mutagens like ethyl methane sulphonate (EMS) or irradiated with gamma radiation, then grown on media containing selective agents (Nikam et al. 2014; Masoabi et al. 2018; Abdelnour-Esquivel et al. 2020). Most of works done on the selection and characterization of cell lines tolerant to abiotic stress are based on adaptive role of compatible solutes primarily proline and glycine betaine, activities of antioxidative enzymes and ion homeostasis (Rai et al. 2011). More recently, callus cultures of mango selected for drought tolerance using PEG as selective agent, Pradhan et al. (2021) have found that osmolytes like proline play a main contributor in adaptation against stress. They also observed that the activity of different antioxidative enzymes, i.e. catalase, super oxide dismutase (SOD), peroxidase and glutathione reductase (GR) was increased in the calli tolerant to PEG.

6.2.2 In Vitro Selection for Disease Resistance

Owing to susceptibility to various pests and pathogens, the yields of many important crops are significantly reduced and damages caused by several diseases highly impacted on massive economic loss and food security. In nature, plants have evolved many defence mechanisms to confer resistance against the pathogens such as production of pathogenesis-related proteins (PR proteins), antimicrobial peptides, antimicrobial metabolites or defence signaling pathways (Li et al. 2020; van Esse et al. 2020). However, sometimes these mechanisms apply only on single or selective pathogen. Therefore, there is a need to develop a broad-spectrum resistance mechanism because it confers resistance against more than one pathogen (Li et al. 2020). As a result of tissue culture-induced somaclonal variation, a wide range of plant characteristics such as disease sensitivity and resistance traits can be altered. A more realistic approach for the regeneration of disease resistant plant is the exposure of cultures to culture filtrate (CF), phytotoxin and sometimes pathogen itself and generating resistant cell/callus line. This in vitro approach has been proven effective

to obtain disease resistant somatic variants in a number of agricultural crops (Van den Bulk 1991; Remotti 1998; Jain 2001; Rai et al. 2011; Anil et al. 2018). Initially, in vitro selection technique was studied mostly for *Fusarium* wilt resistance with some important solanaceous plants including tomato, potato and tobacco, but nowadays this technique for disease resistance has been extended to cereals, legumes, oil yielding plants, medicinally important plants, etc. (Remotti 1998; Rai et al. 2011; Anil et al. 2018), some recent studies are also listed in Table 6.2.

Other than biotic and abiotic stress tolerance, in vitro selection technique has also been used to develop crops with high nutritional value, secondary metabolites or other important useful traits (Suprasanna et al. 2009; Penna et al. 2012).

6.3 Somaclonal Variants of Agricultural Crops Released as New Cultivars

The somaclonal variant selection has led to develop several new cultivars with improved agronomic traits including high yields, improved nutrient and fruit quality, disease resistant or abiotic stress tolerance, etc. and after evaluation at laboratory and field trial, some new cultivars are also released for commercial production (Table 6.3).

6.3.1 Banana

Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *cubense*, is a most destructive fungal disease affecting banana production across worldwide. In Taiwan, Taiwan Banana Institute generated few somaclonal variants from local cultivar Giant Cavendish. One variant, namely GCTCV-215-1 acquired through somaclonal variation showed only 4.8% average wilt incidence compared with 39.1% on local cultivar Giant Cavendish (Hwang and Ko 2004). Finally, this variant was released as cv. Tai Chiao No. 1 for commercial planting in 1992. Later, Taiwan Banana Institute also selected another prominent variant GCTCV-218 against *Fusarium* wilt, which was released in 2002 as cv. Formosana (Hwang and Ko 2004). This cultivar was superior in many important traits like robust pseudo stem, thicker leaves, high yield and heavier fruit bunches, etc.

6.3.2 Barley

In the 1980s, a spring barley cultivar ‘Leger’ was cultivated extensively in Eastern Canada due to its high yield, but this cultivar was very susceptible to powdery mildew caused by *Erysiphe graminis* f. sp. *hordei*. Li et al. (2001) generated three somaclonal variants of barley from embryo-induced callus of cultivar Leger, which were found resistant to *Erysiphe graminis* f. sp. *hordei* after field trial of 2 years. Out of three, one variant, namely ‘AC Malone’ was registered as a new cultivar in

Table 6.3 Some selected commercial cultivars of agriculturally important crops developed from somaclonal variations

Crop/plant name	Cultivar name	Characteristics of somaclonal variant	Cultivar registered in country	Reference
Banana (*Musa acuminata*)	Tai Chiao No. 1 (GCTCV215–1)	*Fusarium* wilt resistant	Taiwan	Hwang and Ko (2004)
Barley (*Hordeum vulgare*)	AC Malone	Resistance to powdery mildew	Canada	Li et al. (2001)
Mustard (*Brassica juncea*)	Pusa Jaikisan (Bio-902)	High yield	India	Katiyar and Chopra (1995)
Potato (*Solanum tuberosum*)	White Baron	Non-browning tuber	Japan	Arihara et al. (1995)
Rice (*Oryza sativa*)	Dama	Resistant to *Piricularia*	Hungary	Heszky et al. (1996)
Sugarcane (*Saccharum officinarum*)	Ono	Resistance to Fiji disease	Fiji	Daub (1986)
	Phule Savitri (Co 94012)	Better sucrose content and moderately resistant to red rot and smut diseases	India	Jalaja et al. (2006)
	VSI 434	High cane and sugar yield, moderately red rot resistant	India	Tawar et al. (2016)
Sweet orange (*Citrus sinensis*)	EV1 EV2 N7-3 N13-32 OLL-4 OLL-8 Valquarius SF14W-62 UF 111-24	Better yield and fruit quality	USA	http://www.ffsp.net/varieties/citrus/ Germana et al. (2020)
Tomato (*Lycopersicum esculentum*)	DNAP-17	Resistant to *Fusarium*	USA	Evans (1989)
Wheat (*Triticum aestivum*)	Hezu 8	High yield, early maturity, disease resistance, tolerance to moisture as well as good grain quality	China	Gao (1992)

Canada. They also found the performance of regenerated lines in respect to high yield and disease resistance was stable over generations.

6.3.3 Mustard

Although somaclonal variation has been observed in many mustard species, but only one somaclonal variant of *Brassica juncea* has been released for commercial cultivation. In India, Pusa Jai Kisan (Bio-902), a somaclone of *B. juncea* generated from seedling callus of parent cultivar 'Varuna' was released as a new cultivar in 1994 (Katiyar and Chopra 1995). In comparison to parent variety 'Varuna' and other traditional varieties, Pusa Jai Kisan performed better in terms of seed yield and other important traits like maturity, seed size and oil content. Pusa Jai Kisan is one of the high demanding mustard cultivars of India and is very popular among farmers.

6.3.4 Potato

Many articles on somaclonal variation of potato have been published and few also claimed to generate improved variants in terms of many agronomic traits like size, shape and number of tubers and starch content, disease resistance, etc. (Bhojwani and Dantu 2013). Due to high yield and good cooking characters, Danshakuimo was a popular and dominant variety of potato in the Japanese market in 1980s. However, discolouring of tuber flesh from white to brown after peeling was a major disadvantage of this variety. Arihara et al. (1995) developed a non-browning somaclonal variant 'White Baron' from the protoplast culture of Danshakuimo. Other than non-browning character, tuber of this variety was oval in shape with relatively shallow eyes. Later, this variety was selected for commercial use in Japan.

6.3.5 Sugarcane

Sugarcane is considered as a model crop for the study of somaclonal variation and crop improvement. Sugarcane was the first crop in which Heinz (1973) reported phenotypic and genotypic variation in tissue culture raised plantlets. He obtained first somaclone resistant to Fiji disease and demonstrated the application of somaclonal variation in crop improvement. Later, Larkin and Scowcroft (1983) obtained a toxin-tolerant variant in sugarcane from cultures exposed to eyespot toxin. Due to narrow genetic base, sugarcane is a potential candidate for the somaclonal variation. Till date, significant progress has been made on generation of somaclonal variants in sugarcane linked with various traits like yield, sugar content, disease resistance, plant height, etc. (Manchanda et al. 2018). However, only three cultivars of sugarcane have been released applying somaclonal variation technique. Krishnamurthi and Tlaskal (1974) isolated a subclone of sugarcane 'Pindar 70-31' from the susceptible cultivar 'Pindar', which showed high resistance

to Fiji disease. Later, this subclone was released as new cultivar with the name of 'Ono' (Daub 1986). In India, 'Phule Savitri' (Co 94012), a somaclone of sugarcane generated from a popular cultivar 'CoC671' was released as a new cultivar for the cultivation in Maharashtra state. The performance of 'Phule Savitri' was better than parent cultivar in terms of maturity, high sucrose content and resistance to red rot and smut (Jalaja et al. 2006). Second cultivar of sugarcane 'VSI 434' from India was released by Vasantdada Sugar Institute, Pune, which was obtained from variety CoC671 and it has high cane yield and sugar content than parent variety (Tawar et al. 2016).

6.3.6 Sweet Orange

Citrus improvement programme of University of Florida evaluated more than 2000 sweet orange somaclones regenerated from protoplasts, nucellar seedling or callus of two most popular 'Hamlin' and 'Valencia' sweet oranges cultivar (Grosser et al. 1997) and one non-commercial selection 'Orie Lee Late'. After evaluation of field trial, a total nine cultivars were released for the commercial production (Table 6.3; Germanà et al. 2020).

6.4 National and International Relevance

Using induced somaclonal variation practices, many cultivars of agriculturally important crops have been released at national and international level and some are highly popular among farmers. In Indian perspective, one variety of mustard, e.g., Pusa Jaikisan (Bio-902) and two cultivars of sugarcane, e.g., Phule Savitri and VSI 434, developed by the use of somaclonal variation, have been released for commercial exploitation. Some useful somaclones have also been selected for the development of improved varieties of globally important crops like rice, wheat, banana, barley, maize, potato, tomato, sugarcane, citrus, etc. which shows its worldwide acceptance.

6.5 Concluding Remarks and Future Prospects

Tissue culture-induced variation, i.e., somaclonal variation is considered as a resourceful tool for plant biologists and crop breeders to create new plant varieties. Unlike genetically modified (GM) crops, this technique does not have any socio-ethical hurdles and new variety can be developed without use of sophisticated tools. In addition, it has the potential of creating novel variants particularly linked to the agronomic and agriculturally useful traits like yield, nutrient quality, disease resistance, abiotic stress tolerance, etc. that can be further exploited in crop improvement programmes. During past years, many somaclones have been selected in a number of agricultural crops, however, only a few have been released for commercial purposes.

Despite some successful stories, selection of somaclonal variants for crop improvement has some major limitations like epigenetic adaptation, selection of undesirable traits, genetic instability and loss of regeneration after selection, field trial for several generations. The problems of genetic instability and loss of regeneration can be solved by the selection of suitable explants, which have high regeneration potential and produce stable somaclonal variants. Furthermore, priority should be given to those cultivars or varieties, which propagated vegetatively and having narrow genetic base. The success of somaclonal variation in a plant species is primarily determined by the frequency of stable variants. An understanding of the fundamental biochemical and molecular pathways involves in selection of stable somaclonal variants needs to be explored. More molecular research will help in the detection of variants, assessment of genetic and epigenetic stability or instability and identification of candidate genes linked with a specific trait.

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7 Genetic Fidelity Studies for Testing True-to-Type Plants in Some Horticultural and Medicinal Crops Using Molecular Markers

Sapna Tyagi, Deepak Rajpurohit, and Amit Sharma

Abstract

Micropropagation of commercial crops represents a major success of in vitro culture technology profitably. However, a major problem often encountered with the use of tissue culture techniques is the occurrence of somaclonal variation, which is often inheritable as it represents induced genetic changes. Genetic variation could ruin valuable genetic stocks maintained under in vitro conditions and make them useless in plant improvement. Thus, genetic fidelity of the in vitro derived plantlets should be tested as early as possible. Currently, different molecular analytic techniques have been used to assess genetic fidelity of regenerated plants, of which DNA-based molecular markers have gained paramount importance. This review describes the use of various molecular markers such as RAPD, RFLP, AFLP, SSR, ISSR, and SRAP in micropropagated plants of some commercial horticultural (apple, banana, guava, and strawberry) and medicinal (aloe, ashwagandha, chirayita, and stevia) crops for the assessment of genetic fidelity and trueness-to-type plant.

Keywords

Genetic fidelity · Micropropagation · Molecular markers · True-to-type plants

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7.1 Introduction

In vitro culture techniques are practiced for micropropagation of superior genotypes of commercially important species. This technique is being successfully employed in plant species especially those having longer growth cycles. In vitro propagation of horticultural trees is quite promising for obtaining the mass multiplication of elite genotypes. Cloning of mature trees is generally preferred over seedling because it is often not possible to determine whether these seedlings have the genetic potential to develop the desired qualities later in their life cycle (Nanda et al. 2004). The commercial multiplication of fruit tree is one of the major successes of tissue culture technology profitably (Rani and Raina 2000). As more and more farmers are showing interest in raising in vitro grown plants, the quality of the planting material needs to be tested for consistent characters to ensure same produce, ensuring farmer's income. Clonal fidelity is one of the most important aspects of study in tissue culture raised plants. The main aim of in vitro micropropagation is to obtain true-to-type plants to maintain the characters of mother plant, but, during in vitro culture, there is a chance of genetic changes which are commonly known as "somaclonal variations" (Larkin and Scowcroft 1981). The occurrence of genetic defects as a result of somaclonal variation in the micropropagated plantlets severely restricts the scope of micropropagation for commercial practice; thus, the somaclonal variation is not a desired event. These variations are induced due to stress caused by unusual in vitro conditions, frequent sub-culturing, and influence of culture conditions like alterations in the supply of nutrients, different hormone concentrations and their ratios. These factors induce heritable DNA damages, thus hinder the exact clonal nature of the progenies. So there is a need to assess the genetic fidelity of micropropagation system before exploiting it at commercial level (Goswami et al. 2013). Several strategies have been employed to ascertain the genetic stability, each of them having merits and limitations (Alizadeh et al. 2015). Techniques based on morphophysiological, biochemical, and cytological approaches are mainly based on characters, which can be affected by the in vitro manipulation, environment, and types of explants hence, somaclonal variation is difficult to resolve. DNA-based molecular markers are a versatile tool to monitor somaclonal variation, test the genetic fidelity of micropropagated plants and verify genotypes with the desired response under in vitro culture conditions. The major advantage of DNA molecular markers is its objective analysis and its results are easy to share between laboratories.

Molecular markers are useful in screening regenerated plants with accuracy, as these are unaffected by environmental factors (which can alter phenotypes), hence overcome the limitations of morphological and biochemical markers and produce reliable and reproducible results. To assess somaclonal variations in tissue culture raised plants, suitable molecular marker system is required to be identified. Molecular markers offer plant geneticists and breeders a set of genetic tools that are abundant, non-deleterious and reliable. Marker systems have been successfully used over the last several decades to construct genetic maps, assess genetic diversity, and locate genes of interest in a number of agriculturally important crops for the

desired traits (Garcia et al. 2005). For instance, restriction fragment length polymorphisms (RFLPs), randomly amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs), SSRs and ISSR markers have proved their utility in crop improvement programs as well as studies on genetic diversity (Gupta et al. 2010). The use of one type molecular marker to assess the fidelity of in vitro propagated plants can be inadequate. Recently, various authors used multiple molecular marker types to study somaclonal variation in regenerants of several plant species. Perusal of literature revealed that variability occurs in tissue culture raised plants; hence, for successful use of in vitro technology, assessment of genetic fidelity for true-to-type nature of regenerated plants by molecular markers is prerequisite for their commercial exploitation. This review provides a literature on the use of molecular markers for the assessment of genetic fidelity and trueness-to-type among mother plant and micropropagated progeny which is useful in studies for developing marker-assisted selection strategies.

7.2 Molecular Markers for the Analyses of In Vitro Cultured Plants

Molecular markers have widespread applications in characterization of germplasms, validation of genetic relationships, assessment of genetic diversity, varietal identification, and clonal fidelity testing (Anand 2000). Molecular markers served as important tool for characterization of in vitro horticultural fruit crops. Several medicinal plants were also evaluated by molecular markers. Various molecular techniques designed to check genetic fidelity in tissue derived plants are random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR) and inter simple sequence repeat (ISSR). RAPD, ISSR and SSR have been immensely explored by various workers for establishing the genetic stability in many horticultural and medicinal plants.

7.2.1 RFLP Markers

Restriction fragment length polymorphism (RFLP) is the only marker system representing hybridization based marker. This involves the use of restriction enzymes and hybridization of the target fragment by labeled probe (Thoungamba and Potshangbam 2017). RFLP exhibits high reproducibility, codominant inheritance, easy data transferability between laboratories and provides locus-specific markers. Genetic fidelity of micropropagated plants with respect to their nuclear, mitochondrial and chloroplast genomes can be determined with appropriate selected probes by RFLP technology (Abe et al. 2002; Devarumath et al. 2002). Among various DNA-based markers, though restriction fragment length polymorphism (RFLP) can be used for screening genetic stability of tissue-cultured plants, but the technique is time consuming, requires high quality and quantity of DNA and prior

sequence information for developing radio labeled probe. The method involves use of expensive enzymes, radioactive probes, tedious southern blotting method and extensive care. The RFLP markers have been employed for genetic diversity and population genetic study in fruit and herbal plants (Bhat et al. 1995).

7.2.2 Random Amplified Polymorphic DNA (RAPD)

To trace the genetic or epigenetic changes at the genome level, RAPD marker has been used intensively (Arnholdt-Schmitt and Schaffer 2001; Leelambika and Sathyanarayana 2011). RAPD is a convenient, economic, simple, cost-effective and rapid method (Bornet et al. 2002). It needs very small amount of DNA to give rapid and accurate identification. It requires no probes and prior sequence information and small number of primers can generate a very large number of fragments from different regions of the genome and hence multiple loci can be examined very quickly (Kumari et al. 2009).

7.2.3 AFLP Marker

AFLP is designed by combining the reliability of RFLP and efficiency of PCR-based techniques (Vos et al. 1995). It has now become a preferable technique for assessment of genetic fidelity in tissue cultures. The basis of this technique is to identify genomic restriction fragments by PCR amplification and applicable on any complex DNA. AFLP is an efficient, dominant, and high genomic abundance marker. AFLP can be generated easily without initial development of any primer or probe and does not require sequence analysis. It provides a high degree resolution and has proven to be a useful technique to assess genetic stability of the in vitro propagated clones, but it is labor-intensive and technically challenging as it involves many time-demanding steps. The main drawback of the technique is the lack of reproducibility when different sequencing platforms are used; hence, it is difficult to transport data between laboratories (Peleg et al. 2008). AFLP is very sensitive for detecting genetic polymorphism, but requires relatively large amounts of high-quality DNA; however, it is technically more demanding and used relatively frequent (Teixeira da Silva et al. 2007).

7.2.4 Simple Sequence Repeat (SSR) Marker

Simple sequence repeats (SSR) also known as microsatellites (Litt and Luty 1989) markers are short tandem repeats of two to eight base pair motifs, which can be easily detected by polymerase chain reaction (Kalia et al. 2011). SSRs are extensively used in analyzing genetic stability of in vitro raised plants as it is highly abundant throughout the genome and has many desirable characteristics like codominant inheritance, locus-specific, ability to detect heterozygous and homozygous nature

(McCouch et al. 1997), high level of polymorphism, informative, highly reproducible, strong discriminatory power (Kumar et al. 2011; Nookaraju and Agrawal 2012; Ashwini et al. 2015). It is frequently used in many studies because of cheaper cost, but requires previous information about region containing repeated sequences. The efficiency of microsatellite marker development depends on the abundance of repeats in the target species and the ease with which these repeats can be developed into informative markers which can monitor genetic variations occurred due to deleterious mutations during in vitro culture, because they reflect a relatively high rate of mutation and genetic variability (Lopes et al. 2006).

7.2.5 Inter Simple Sequence Repeats (ISSR) Marker

The inter simple sequence repeats (ISSR) were first developed by Zietkiewicz et al. (1994) to rapidly differentiate between closely related individuals. This technique is also very simple, fast, cost-effective, highly discriminative and reliable (Marri et al. 2002). It permits detection of polymorphisms in microsatellites and intermicrosatellites loci, which are abundant throughout the eukaryotic genome without previous knowledge of the DNA sequence (Ishii et al. 1987). This technique is also a very simple, fast, cost-effective and is suitable to discriminate closely related genotype variants (Kumar et al. 2016) and reliable. In this, very small amount of template DNA is required. These markers are more reproducible than RAPD markers and applied successfully in studying the genetic diversity in plants (Bornet et al. 2002). These markers enjoy advantages of random markers and do not require flanking sequences for development of primer (Israeli et al. 1991). This technique combines the benefits of AFLP and SSR markers with random amplified polymorphic DNA (RAPD) marker's universality (Eshraghi et al. 2005). These are useful where genome sequences are not available (Jain 1993). Their major drawback is that they are dominant in nature and follow Mendelism (Jarret and Gawel 1995). Although more repeatable results are produced by ISSRs, but they have shown less productive to detect polymorphisms for some primer combinations (Nguyen and Wu 2005).

7.2.6 Genetic Fidelity of Horticultural Crops

7.2.6.1 Apple

Apple (*Malus* × *domestica* Borkh.) is an important fruit crop of family *Rosaceae*. Its few varieties are cultivated which are favored by the market and can ensure early and profitable production. As a result, local cultivars are severely threatened with the risk of extinction, so germplasm conservation and multiplication protocols are needed. Almost none of the available apple cultivars entirely meets the high demands of propagators, growers and the rapidly changing apple market (Noormohammadi et al. 2015). Propagation of apple through tissue culture technology has been reported by many workers all over the world. But there may be chances of genetic variation in

plants derived from in vitro cultures (Dobránszki and Teixeira da Silva 2010). Little attention has been given to assess the genetic stability of micropropagated plants in apple. Virscek-Marn et al. (1998) studied somaclonal variation in different apple cultivars and rootstocks by RAPD in the regenerated plants of Golden Delicious Bovey and Goldspur cultivars, which were obtained by adventitious shoot regeneration from apple leaves. Modgil et al. (2005) used RAPD and ISSR markers to assess the genetic fidelity of micropropagated plantlets of apple raised from adventitious buds and also reported that micropropagated plantlets of apple cultivar MM 106 developed from axillary buds exhibited 23% polymorphism showing somaclonal variations. Gupta et al. (2009) observed no phenotypic variability in in vitro propagated plantlets regenerated through axillary bud and shoot apices of apple rootstock EMLA111. Analysis of RAPD profiles revealed that out of the ten RAPD primers, eight primers exhibited similarity between micropropagated and mother plants, but two primers, OPA-04 and OPE-14 showed difference between micropropagated progeny and mother plants.

No variation was observed in leaf-derived plantlets of rootstock of apple Golden Delicious Bovey and Goldspur using RAPD markers (Viršcek-Marn et al. 1998). Contrary to this, Caboni et al. (2000) found variations in these plantlets, but they did not observe any polymorphism in in vitro plants developed from apical meristems of rootstock of apple Jork 9. Negri and Tosti (2000) have found genetic similarity in micropropagated apple cultivars using RAPD, but little variations were observed in preserved (slow growth) micropropagated plants. Similarly, little somaclonal variations were observed in plantlets developed through axillary buds in Gala' family apple (McMeans et al. 1998). This showed that the type of explants used and the way of regeneration like direct axillary branching, organogenesis via callusing and somatic embryogenesis plays important role in determining the extent of genetic variation (Salvi et al. 2001). It has been observed that in vitro plants obtained from organized meristem are not always true to type (Devarumath et al. 2002) and even well-organized axillary bud tissues generated somaclonal variations (Rahman and Rajora 2001). Plants developed from axillary buds of rootstock MM106 showed genetic variation (Modgil et al. 2005), but plantlets from rootstock M7 showed no variation in RAPD analysis (Modgil et al. 2017). In contrast, Noormohammadi et al. (2015) observed 53% or 46% polymorphism using ISSR markers, which showed significant difference between mother plants and *in vitro* cultured plantlets in rootstocks M7 and M9. Pathak and Dhawan (2010) recorded no variation in micropropagated plants regenerated through axillary buds of clonal apple rootstock MM111 and "Merton 793" (Pathak and Dhawan 2012).

No genetic variation was observed in plantlets of "Gala" derived from leaf segments (Montecelli et al. 2000). Regenerated adventitious shoots from leaves of apple rootstock Pingyitiancha showed monomorphic banding profiles of SSR markers and found same as mother plant (Jin et al. 2014). SSR markers CH03g07, CH05c02, CH05d11, CH05e03, GD96, GD147 and GD162 exhibited low levels of polymorphism among plantlets of six apple cultivars (Goldrush, Rebra, Romus3, Romus4, Idared, and Florina) developed in vivo, in vitro and from cryocollection, which confirms the uniformity among them (Butiuc et al. 2019). Such data suggested

that genotype, explant type and shoot multiplication method, or the number of subcultures affect the genetic stability among plantlets (Dobránszki and Teixeira da Silva 2010). The selection of suitable molecular marker also plays an important role in detection of genetic polymorphism.

7.2.6.2 Banana

Banana is one of the most common and important fruit crops. It is a large monocotyledonous herb. Banana is one of the most widely used fruits, belongs to the genus *Musa*. Propagation of banana through micropropagation has gained importance because of its potential to produce genetically uniform, pest- and disease-free planting materials (Venkatachalam et al. 2007). It is a first fruit crop that uses tissue culture for mass propagation and till then it is continued to be multiplied through tissue culture. Work on propagation of banana from different explant sources and regeneration pathways through in vitro techniques has been done by several researchers (Novak et al. 1989). Up to 50% variations were observed in micropropagated Cavendish bananas (Israeli et al. 1996). Maria and Garcia (2000) and Chaudhary et al. (2015) ascertained genetic stability in banana using RAPD technique. Martin et al. (2004) confirmed significant genetic variation in in vitro corm-derived plants of banana cultivar Grande Naine on testing with RAPD. Lakshmanan et al. (2007) screened 50 RAPD and 25 ISSR primers to establish genetic fidelity in in vitro plantlets developed from axillary shoot of banana variety and observed no variation. Sheidai et al. (2008) recorded (51.40%) polymorphism using RAPD primers and primer OPI-07 produced highest 24 bands. Shiddalingeswara et al. (2014) screened 200 random RAPD primers to establish variation between micropropagated Robusta banana and off type orchard grown variants.

RAPD and SRAP analysis of banana cultivar Williams revealed genetic similarity in micropropagated plants and mother plant. All the RAPD primers generated monomorphic bands only. SRAP primer me1 + em2 produced one polymorphic band (Ismael and Mohamed 2018). Muslim et al. (2019) observed that 4 primers (OPA15, OPA19, OPC03 and OPH09) showed unique banding pattern. Rout et al. (2009) marked the potential of ISSR marker for identification of different varieties of banana and detection of genetic uniformity of micropropagated plantlets and ascertained 35% to highest 66% dissimilarity. No variation was observed by Nandhakumar et al. (2017) in plantlets developed from somatic embryos obtained from immature male flowers of banana cultivar Rasthali (AAB) and very less variation (3.6% polymorphism) in Grand Naine (AAA) through ISSR markers. When tested for genetic fidelity by ISSR primers, no discernable variation was seen among mother plant and micropropagated plantlets of cultivar Rasthali, while in cv. Grand Naine, very low loci were found to be polymorphic. Ray et al. (2006) reported that in *Musa,* the extent of instability caused in in vitro culture was associated with cultivar instead of the culture conditions. Whereas Krikorian et al. (1993) reported that frequency of variants in a particular cultivar was restricted to individual primary explant instead of clones.

7.2.6.3 Guava

Psidium guajava L., a highly nutraceutical fruit, belongs to the family *Myrtaceae*, is commonly known as "guava" (Chandra et al. 2010). Guava (*Psidium guajava* L.) is an important fruit crop possessing adequate nutritional quality and its availability throughout the year makes it economically valuable. Being rich in vitamin C, it is competent with that of oranges (Conway 2001). Guava is also capable of producing value added products (Karanjalker et al. 2013), viz. juice, jelly, jam, nectar, blended products etc. Various marker systems have been employed to assess the genetic fidelity of regenerated plants, each with their own benefits and drawbacks. Sharma et al. (2007) investigated diversity in 22 *Psidium guajava* L. cultivars and suggested the use of RAPD markers in genetic diversity analysis. Alizadeh and Singh (2009) suggested the use of a combination of markers for assessment of genetic stability that can amplify different regions of the genome. Youssef et al. (2010) screened five RAPD primers and found 71.4% of amplified DNA bands were conserved and an average of 10.6% showed polymorphism among each parent and their micropropagated plantlets, which was less than that found in donor parents developed from seeds of the same mother plant. Liu and Yang (2012) have reported ISSR markers for assessment of genetic fidelity in guava and observed 11 primers producing 93 scorable distinct amplicons and very low variability of 1.65% indicated genetic stability in micropropagated guava. Monomorphic banding pattern of SSR and ISSR primers revealed genetic stability in micropropagated plants and the mother plant and confirms the reliability of in vitro propagation system for guava (Rai et al. 2012). Plant regenerated from somatic embryos developed from immature zygotic embryos of some commercial cultivars (Allahabad Safeda, Lalit, Sardar and Shweta) assessed through RAPD, ISSR and SSR markers showed no polymorphism and genetic fidelity was maintained in regeneration protocol (Kamle et al. 2013). In spite of phenotypic variations, genetic fidelity in regenerated plants from leaf of guava cultivar Lucknow 49 was observed when tested with simple sequence repeat (SSR) markers (Rawls et al. 2015) and found only three primers showed heterozygosity which were in agreement with previous findings (Sitther 2014) of assessment in guava germplasm in the USA. Kumar et al. (2020) developed three novel g-SSR loci $(CT)_{14}$, $(GT)_{12}$ and $(AAC)_8$ for microsatellite-enriched libraries.

7.2.6.4 Strawberry

Strawberry (*Fragaria ananassa* Duch.), a perennial herb belongs to family *Rosaceae*, is well known for its fruit throughout the world. The berry is low-calorific and possesses high fiber contents. Their processed products like jams, jellies, juice extracts or flavorings, frozen whole berries, and many other products made them most exploited berry (Childers 1980). They are reservoir of many antioxidants and dietary glutathionine (Larson 1988). The fruit has anticancerous (Kresty et al. 2001), antiageing properties and can revert the effect of memory loss (Bickford et al. 2000). The conventional way of production of strawberry is not adequate to meet the commercial demand. To multiply elite selections, conserve germplasm, and develop suitable cultivars in the minimum time, in vitro techniques are used (Debnath 2013). Boxus (1974) developed

techniques to produce micropropagated plants of strawberry, but problem of variability in microshoots was still prevailing, so it is important to ensure the quality of materials before introduction to productive fields. Boxus et al. (2000) observed somaclonal variations in some cultivars of strawberry, after a high number of subcultures and suggested 5–10 subculturings only. Studies showed that the genotype of plant influences the level of variability occurred during subculturings of tissues in vitro (Hammerschlag et al. 2006). Polymorphism at DNA level among phenotypically similar somaclonal variants was reported in strawberry (Damiano 1997). Arnau et al. (2003) demonstrated that to obtain the correct fingerprinting pattern of strawberry cultivars, only few markers are required in the ISSR technique. The use of ISSR markers in the assessment of strawberry genotypes provides great information on genetic polymorphisms.

Debnath et al. (2008) also confirmed the feasibility of ISSR markers in evaluation of genetic similarity and genetic diversity in strawberry cultivars and developed RAPD and ISSR marker based assessment protocol to identify genetic diversity in strawberry and recorded trueness-to-type of micropropagated plantlets. Despite morphological variations observed in in vitro plants obtained from bioreactor derived tissue culture, tissue culture on gelled medium, and conventional runner cuttings of strawberry cultivar Bounty Debnath (2009) found homogenous ISSR profile confirming the clonal fidelity of micropropagated plants and donor control plants. Vandana et al. (2012) screened 15 EST-SSR markers to assess the genetic fidelity in micropropagated plants of strawberry genotypes Festival and Sweet Charlie and obtained clear and monomorphic bands from 12 primers confirming true-to-type character of the tissue-cultured plants. Ùuuan et al. (2012) evaluated the suitability of RAPD techniques to assess genetic stability and uniformity of in vitro ornamental strawberry plantlets of "Pink Panda" and "Serenata" varieties, originated from meristems through axillary buds proliferation. RAPD profile showed similar banding pattern suggested no variations when compared with that of control plant.

7.2.7 Genetic Fidelity in Medicinal Plants

Owing to uses in herbal medicines, plants of pharmaceutical importance are exhaustively exploited and their demand is increasing day by day. There is a constant demand of appropriate planting material, which could be accomplished by the plant tissue culture techniques. Clonal propagation of desired uniform, healthy plants through in vitro culture is the only way for production of large numbers of plantlets in a short time. Analysis of genetic fidelity in medicinal plants with the help of molecular markers ensures no variation in the formulation of drugs among the batches (Ipek et al. 2003).

7.2.7.1 Aloe

Aloe barbadens is an important medicinal plant of family *Liliaceae* and widely used herb in various Ayurvedic formulations. It is useful in multipurpose applications in pharmaceutics to cosmetics with promising economic return (Gantait et al. 2014). Its

number of formulations is widely available in the market for the treatment of skin disorders, general skin care cosmetics, and health food. The leaf gel is known to possess important biological properties such as antiinflammatory, anticancerous, anti-viral, anti-bacterial, immune-enhancing, and parasite-killing activities (Reynolds and Dweck 1999). It is also used as laxative, antihelminthic, stomachic, astringent, hemorrhoid remedy, uterine stimulant, disinfectant and used for wound healing (Choi and Chung 2003). It effectively cures UV-damaged and sun-damaged skin (Bunyapraphjatsara et al. 1996).

Work on mass propagation of aloe has been conducted by various workers as its natural propagation is rather slow for commercial purposes (Groenewald et al. 1975). Molecular marker studies on *Aloe vera* have been published by using AFLP (amplified fragment length polymorphism), RAPD (random amplified polymorphic DNA) and ISSR (inter simple sequence repeats) techniques (Alagukannan and Ganesh 2016; Kumar et al. 2016). The first report on analysis of variations generated in tissue culture in aloe was published by Cavallini et al. (1993). Although in vitro plantlets showed phenotypic similarity with wild *Aloe vera* plants, Zarreen et al. (2017) found 33% polymorphism through RAPD and 25% polymorphism by ISSR primers and reported genetic variation among them. Gantait et al. (2010, 2011) successfully employed ISSR markers to assess the genetic fidelity of the aloe clones developed from direct organogenesis, which confirms the true-to-type status of *Aloe vera*. Rathore et al. (2011) observed no variations in plantlets developed directly from axillary buds; however, plantlets established indirectly from callus produced at the base of the inflorescence axis showed 80% polymorphism through RAPD primer and 73.3% using ISSR primer, which confirmed that callusing is not suitable for generating true-to-type plants. Haque and Ghosh (2013) observed genetic fidelity in regenerates of *A. vera* established through shoot bud culture using RAPD primer.

Sahoo and Rout (2014) detected no genetic variations using RAPD and ISSR primers among plants regenerated from leaf explants. Similarly, Khatuna et al. (2018) also observed genetic fidelity in micropropagated plants developed from shoot tips tested through RAPD and ISSR primers and found it suitable for producing true-to-type plants of *Aloe vera*. RAPD profiles of the donor and somaclones of shoot apical meristems revealed polymorphic bands, which confirmed genetic difference among them and ITS-1 sequences also showed little variation (Das et al. 2015).

7.2.7.2 Ashwagandha

Ashwagandha or *Withania somnifera* (L.) Dunal also known as "Indian ginseng" (Deshpande 2005) is an important medicinal plant of family *Solanaceae*. Roots, leaves, and seeds of the plant are used in Ayurvedic and Unani medicines. Its root possesses number of alkaloids and generally used for general and sexual weakness, female disorder, leucorrhea and neuromuscular system, prevent old age symptoms, remove acidity, and prevent osteoarthritis. The entire plant of *W. somnifera* is used for the treatment of tuberculosis, rheumatism, inflammatory conditions, and cardiac diseases (Asthana and Raina 1989). In addition, it is used as an antitumor, antibiotic, anticonvulsant and CNS-depressant agent (Sharma and Dandiya 1992). It reverses

the symptoms of Alzheimer's disease. *W. somnifera* displays an appreciable spectrum of morphological and phytochemical variability (Kumar et al. 2007). So, there is a need of uniform planting material to maintain uniformity and efficacy of herbal drug preparations.

Mallubhotla et al. (2010) assessed the RAPD profile of micropropagated plants of *W. somnifera* WSR cultivar and confirmed the clonal fidelity among them. First time, Nayak et al. (2013) demonstrated in vitro clonal propagation of *W. somnifera* from cotyledonary nodes and assessed the genetic stability in them through PCR-based marker systems, RAPD and ISSR markers. Both RAPD and ISSR primers revealed 100% monomorphic and similar banding pattern, which confirmed the genetic fidelity among regenerated plants and mother plants. Fatima et al. (2015) investigated PCR-based single primer amplification reaction (SPAR) methods, which involved direct amplification of minisatellite DNA (DAMD) markers and reported no variation in DNA fingerprinting patterns among the micropropagated and the donor plants. Similar results have been observed when genetic fidelity among micropropagated plants and donor plant was tested using RAPD and ISSR markers (Fatima et al. 2016). Shetty and Nareshchandra (2012) observed the uniformity in RAPD profile of plantlets developed through tissue culture protocols in *Withania somnifera.* Contrary to this, Trivedi et al. (2017) ascertained somaclonal variations up to 50% (OPA19 RAPD primer) in regenerated plantlets; however, RAPD primer OPA05, OPA10, OPB01 and OPC12 showed no polymorphism.

7.2.7.3 Chirayita

Swertia chirayita (Roxb. ex Fleming) H. Karst. is an important medicinal herb belonging to family *Gentianaceae* (Kshirsagar 2015). The plant is used in treatment of fever, malaria, skin diseases, liver disorder and diabetes (Selvam 2012). All the plant parts are used as herbal drugs and reported in Indian British and the American pharmacopeias and in traditional systems of medicines such as the Ayurveda, Unani, and Siddha. Increasing pharmaceutical demand has led to overexploitation and unsustained destructive harvesting forced it into critically endangered category (Joshi and Dhawan 2007). Poor seed set, low viability, low seed germination percentage and delicate field handling of the seedlings hinder agro-technology development in *Swertia chirayita.* Hence, alternative ways for propagation of this plant are highly recommended. Tissue culture technology offers the opportunity to develop new better-adapted germplasm.

Joshi and Dhawan (2007) found that axillary multiplication results in true-to-type plants and confirmed the genetic fidelity in plantlets of *S. chirayita* developed in vitro through axillary multiplication of shoots by ISSR markers even after 42 cycles. Similarly, Chaudhuri et al. (2007) also ascertained genetic fidelity in plantlets developed through nodal explants. Sharma et al. (2016) assessed genetic fidelity in *Swertia chirayita* and found 25 RAPD primers produced monomorphic bands which showed 97% similarity and 10 ISSR markers produced monomorphic bands with 95–98% similarity among in vitro raised shoots and parental plants. Kapoor et al. (2019) screened 23 RAPD and 20 ISSR markers and observed 82% and

87% similarity, respectively, among in vitro raised plants and mother plant of *Swertia chirayita* with little polymorphism.

7.2.7.4 Stevia

Stevia rebaudiana (Bertoni), commonly known as sugar leaf is one of the important medicinal plants belonging to family *Asteraceae*. It is used as a substitute for sugar in most of the regions of the world. The leaves possess sweet-tasting chemicals known as steviol glycosides (stevioside and rebaudiosides) which is 300 times sweetener than sugar (Madan et al. 2010; Yadav et al. 2011). The leaves are commercially used as non-caloric sweetener that does not affect blood glucose levels (Gantait et al. 2015), thus safe for diabetic patients. It helps in weight management and blood pressure (Gantait et al. 2015). It also possesses antimicrobial, antiinflammatory, antihypertensive, and antioxidant properties (Das et al. 2010). In addition to its non-caloric sweetening properties, it has many therapeutic values as antihyperglycemic, anticancerous agent (Jeppensen et al. 2002, 2003), and antihypersensitive (Chan et al. 1998; Jeppensen et al. 2002). It prevents dental caries (Fujita and Edahira 1979). It also possesses antimicrobial and contraceptive properties (Melis 1999). Stevia product has been given GRAS status by FDA of the USA. Production of Stevia through seed germination is poor and in some cases does not produce viable seeds (Yadav et al. 2011).

Stevia is conventionally propagated through seed and cutting, but showed poor seed germination due to infertile seeds (Carneiro et al. 1997; Goettemoeller and Ching 1999). Self-incompatibility, insufficient pollinator activity and poor seed set resulted in heterozygosity among plants which affected the concentration of glucosides in leaves and have low multiplication rate (Gantait et al. 2018). Clonal propagation via cuttings is also inadequate (Sakaguchi and Kan 1982). The first genetic linkage map for *S. rebaudiana* was constructed by Yao et al. (2011) based on RAPD markers. Das et al. (2011a, b) gave first report of genetic variation in stevia tissue culture using biochemical and molecular markers. RAPD markers were analyzed and detected 35.5% polymorphic loci and 62.5% different positions in the karyotypes. Thiyagarajan and Venkatachalam (2012) screened 42 RAPD markers and found 29 markers producing distinct bands with unique amplification products. No genetic variability was detected among the tissue culture raised plantlets and reported that nodal explants can be successfully exploited for the commercial purpose without risk of genetic instability. Modi et al. (2012) established true-to-type nature of tissue-cultured derived plants and observed no somaclonal variations using RAPD and ISSR markers. Study conducted by Hassanen and Khalil (2013) to assess the genetic fidelity among in vitro raised plants and mother plant through SDS-PAGE and isoenzyme analysis revealed 100% similarity, whereas molecular analyses using RAPD, ISSR and AFLP showed 44.70%, 22.03%, and 24.00% polymorphism, respectively. Lata et al. (2013) found monomorphic and comparable bands in ISSR profiles of micropropagated plants and mother plants, which confirmed the genetic stability among them.

Soliman et al. (2014) observed similarity among in vitro plants and mother plants up to five subcultures and then low to high genetic variations with increase in time

using ISSR markers. Singh et al. (2014) reported that in vitro plants of Stevia obtained from callus showed genetic variation, whereas those from nodal segments showed no variation. Singh et al. (2017) tested genetic fidelity by RAPD and ISSR markers and observed that in vitro raised plantlets without any callusing were true-to-type compared to mother plants. Similar to Lata et al. (2013), Rania et al. (2017) also found identical DNA profile of plantlets obtained from axillaries bud explants and mother plants using ISSR markers, which revealed no genetic variability.

7.3 Global Status of the Study

At Institute of Forestry and Pomology, Beijing, China, Jin et al. (2014) developed adventitious shoots from leaves of apple rootstock "Pingyitiancha" and assessed genetic fidelity using SSR markers. In vitro clonal propagation protocols for different horticultural crops and different varieties of fruits have been developed in all over the world and to assess the clonal fidelity in these micropropagated crops, various molecular markers have been developed. Studies conducted on ex vitro and in vitro apple cultivars—Florina, Idared, Rebra, Goldrush, Romus 3, Romus 4, and the rootstocks M9 and M26 by Vălimăreanu et al. (2010) at Institute of Biological Research, Romania to assess clonal fidelity by RAPD markers revealed no genetic variations. Saifullah Khan et al. (2011) in Pakistan screened 13 varieties of the cultivated banana, procured from INIBAP, Belgium, using RAPD-DNA markers and also assessed the genetic fidelity among tissue culture raised plantlets of Cavendish Basrai (CB), cultivar of banana growing in the Sindh province of Pakistan. They found no variation up to eight passages, after that variation in leaves was observed. At Genetic Engineering and Biotechnology Research Institute (GEBRI), Minoufiya University, Egypt, 40,000 in vitro cultured banana plants of cultivar "Grand Naine" were analyzed at different growth stages to study differences in its 23 variants using RAPD markers by Abdellatif et al. (2012). Similarly, Sheidai et al. (2008) at Shahid Beheshti University, Tehran, Iran, investigated 30 decamer RAPD primers to study somaclonal variation at different stages of subculturings in banana cultivar Valery and observed that occurrence of somaclonal variation increased with increase in time period of subculturings. First of all, Risterucci et al. (2005) constructed a library of microsatellite-enriched (GA)n and (GT)n and characterized 23 nuclear simple sequence repeat (SSR) loci in the guava species *Psidium guajava.* These new SSR markers proved to be a powerful tool for genetic studies in guava. Liu and Yang (2012) studied genetic fidelity in guava cultivar grown in a greenhouse using 21 ISSR primers at Department of Natural Resources and Environmental Design in support with Center of Excellence for Post-Harvest Technology and the Agricultural Research Program, North Carolina, USA. In the USA, work on assessment of clonal fidelity in 31 plants of guava cultivar "Lucknow 49" regenerated by micropropagation was conducted at Morgan State University, Baltimore and Fort Valley State by Rawls in 2015, in which 16 SSRs detected the same allele, only slight variation in locus mPgCIR07 was detected. Boxus (1974) introduced micropropagation of strawberry. Sutan et al. (2010) in University of

Pitesti evaluated the genetic stability and uniformity of micropropagated ornamental strawberries "intergeneric hybrids Pink Panda" and "Serenata" plants by using RAPD markers. Studies conducted on development of RAPD and ISSR markers to identify genetic diversity in strawberry were carried out at Atlantic Cool Climate Crop Research Centre, Canada which were also used to test genetic fidelity in bioreactor micropropagated plants of strawberries (Debnath 2013). The very first report of assessment of tissue culture generated variation in aloe was published by Cavallini et al. (1993). Gantait et al. (2014) at Serdang, Selangor, Malaysia wrote a review update on advancement of in vitro culture in *Aloe vera,* where he discussed technique to assess the clonal fidelity of micropropagated plantlets. Several approaches were taken for aloe such as molecular approaches, cytological assessment, and biochemical assays. Khatun et al. (2018) at National Institute of Biotechnology, Ganakbari, Dhaka, Bangladesh assessed the suitability of 20 RAPD and 12 ISSR primers to assess clonal fidelity among in vitro *A. vera* plants. First molecular RAPD marker in *S. rebaudiana* was studied by Yao et al. (2011). Soliman et al. (2014), Matariya, Cairo, Egypt attempted in vitro propagation of Stevia through multiple shoot regeneration from nodal segments and analyzed the genetic stability of micropropagated and mother plants using ISSR primers which confirmed success of micropropagation protocol. Lata et al. (2013) at University of Mississippi, USA micropropagated in vitro plants from nodal segments containing axillary buds and screened hardened plants of *Stevia rebaudiana* to assess clonal fidelity using inter-simple sequence repeat (ISSR) markers.

7.4 National Status

In India, Pathak and Dhawan (2010) conducted first study on application of ISSR markers in evaluating genetic fidelity in apple in in vitro plantlets of apple. Gupta et al. (2009) at Solan, HP micropropagated EMLA 111, a clonal rootstock of apple through axillary bud and shoot apices and assessed the genetic fidelity among micropropagated plants through RAPD markers. Deepthi et al. (2007) characterized the 11 tissue-cultured variants of Grand Naine banana growing in farmers orchard in Bangalore using RAPD markers and observed significant variations. At JNU, New Delhi, Rustagi et al. (2016) micropropagated Indian Banana variety Matti and tested the genetic fidelity using RAPD marker. At Division of Crop Improvement and Biotechnology, Central Institute for Subtropical Horticulture, Lucknow, India, Kamle et al. (2013) developed somatic embryogenesis protocol for plantlet regeneration of four commercial cultivars, Allahabad Safeda, Lalit, Sardar, and Shweta of guava (*Psidium guajava* L.) and assessed genetic fidelity among regenerates using RAPD, ISSR, and SSR molecular markers. From their studies, the RAPD, ISSR, and SSR markers were proved to be useful in confirming the stability among somatic embryogenesis regenerated plants. At Agricultural University, Pusa, Bihar, Sharma et al. (2012) tested the clonal uniformity in micropropagated plants of two commercially important genotypes Festival and Sweet Charlie of strawberry using EST-SSR markers. At National Research Centre for Medicinal and Aromatic Plants, Anand,

Gujarat, India, a superior line of *A. barbadensis* for aloin-A from NBPGR, New Delhi was micropropagated using shoot meristems by Samantaray and Maiti (2008) and 40 decamers were used to determine the genetic integrity of in vitro raised plants. Haque and Ghosh (2013) at University of Calcutta worked on high frequency microcloning of *Aloe vera* and their true-to-type conformity by molecular cytogenetic assessment of 2 years old field growing regenerated plants through RAPD analysis which revealed no genetic variation. Khan et al. (2016) at IARI, New Delhi used a rapid and efficient protocol for in vitro multiplication of genetic modified *Stevia rebaudiana* (Bertoni) used RAPD primers to check the genetic fidelity of in vitro produced plants and found that all tissue culture plants were genetically same and true to type. Singh et al. (2017) in Udaipur, Rajasthan, India established high frequency mass multiplication of stevia using in vivo and in vitro nodal explants. Assessment of clonal fidelity of in vitro raised plantlets was performed by RAPD and ISSR molecular analysis, which revealed that all the regenerated plants of stevia through tissue culture were true-to-type compared to mother plants. Joshi and Dhawan (2007) at TERI, New Delhi assessed the genetic fidelity of *Swertia chirayita* plantlets multiplied in vitro by axillary multiplication up to 42 passages through ISSR marker assay and confirmed the fact that axillary multiplication is the safest mode of micropropagation to produce true-to-type progeny. Sharma et al. (2016) at Arni University, Kathgarh, Indora, HP, India developed in vitro culture protocol for *Swertia chirayita* and clonal fidelity has been checked by two marker systems ISSR and RAPD and regenerated plants showed high clonal fidelity. An efficient in vitro regeneration for medicinally important herb was developed and the genetic fidelity was assessed using RAPD and ISSR markers. Kapoor et al. (2019) at Solan developed protocol for micropropagation of endangered medicinal plant *Swertia chirayita* and tested its trueness type through RAPD and ISSR markers. Shetty and Chandra (2012) at Kalyan West, Thane, Maharashtra used *Withania somnifera* (L) Dunal variety JA 20 (Jawahar Asgund 20), JA 34 (Jawahar Asgund 34) GLV (Gujarat local variety) and wild plants were regenerated using callus culture. DNA fingerprinting showed no variation between regenerates and mother plants of wild plants which were selected for the present investigation. Fatima et al. (2013) at Aligarh Muslim University, Aligarh regenerated the plantlets from non-embrogenic, synthetic seeds containing axillary buds of *Withania somnifera* L. and assessed the genetic stability among the clones through RAPD and ISSR markers and confirmed true-to-type regenerants. Nayak et al. (2013) at Cuttack, Odisha first time demonstrated the candidature of cotyledonary nodes derived from axenic seedlings for in vitro clonal propagation of *W. somnifera* and assessed the clonal fidelity in in vitro regenerated plants using RAPD and ISSR markers.

7.5 Conclusion

As the demand in horticultural and herbal pharmaceutical sector is increasing day by day, so to maintain constant supply of efficient and superior quality material, there is a need to explore the advances in biotechnological techniques. Plant tissue culture

offers important technique for rapid multiplication of such commercially important plants. Propagation of plants in in vitro conditions followed by acclimatization induces some changes at morphological and cytogenetic level, which cannot be scored phenotypically. In order to check such variations, standardization of planting material for homogenous supply in market is necessary. Detection of these variations through molecular tools at early stage can help in refining the micropropagation protocol and eliminating the genetic instable plants. Molecular markers assured the genetic fidelity in regenerated plantlets and confirmed the reliability of the developed protocol for commercial exploitation.

Among the various molecular markers explored, combination of two or more marker systems which act on different loci of genome is more suitable then single marker for accurate evaluation of genetic fidelity in micropropagated plants.

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Callus Culture Approach Towards Production of Plant Secondary Metabolites

8

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Abstract

Plant cell culture is a budding biotechnological tool for the production of valuable medicinal products, flavours, colourant, and essence. However, only a few plants containing these compounds have been used in commercial scale production. Productivity constraints are mainly linked to the lack of detailed scientific knowledge and less understanding of the genetics and biochemistry process. The plant cell culture provides broader applications in the field of pharmacology, pharmacy, medicine, agriculture, and horticulture. Since the natural supply of medicinal herbs is limited and overexploitation often destroyed the natural habitat and led to the extinction of the species. But nowadays various in vitro technological interventions and plant cell culture platform provides a significant role in the production of bioactive ingredients and further enrichment and enhancement of the secondary metabolites. This book chapter emphasizes on highlights and significant scientific knowledge based on selected medicinal plants along with

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their pharmaceutical potential and year-round in-house production of key metabolites via plant cell culture method.

Keywords

Medicinal plants · Phytochemistry · Secondary metabolites · In vitro · Plant cell culture

8.1 Introduction

Plants with medicinal properties have been used since ancient times. Several attributes of plant products, such as pharmaceuticals, agrochemicals, and nutrition have been utilized extensively. Nowadays, almost all the world's inhabitants rely on plant-based goods. Also, in the present scenario, numerous biotechnological tools give rise to promising prospective for the development of plant-derived natural metabolites under in vitro systems as plant cell culture. Due to the rapid destruction of natural habitats of valuable plant species, in vitro propagation techniques can aid in the conservation of such species. Being sessile, plant exerts many stressful and extreme environmental conditions, so to cope up with such situations, i.e. biotic and abiotic stresses, plant produce secondary metabolites for their adaptive defence mechanism (Jeandroz and Lamotte 2017; Zhai et al. 2017; Kumar et al. 2020). The cohort of biochemical complexes in plants can be encouraged by various external stress stimuli, i.e. pathogens, oxidative stress, drought, elicitors, wounding, and allelopathy. Furthermore, these are internally arbitrated by signal transducers such as jasmonate, salicylic acid, and their derivatives (Isah 2019). In comparison to biotic elicitors, physical agents (cold, heat, osmotic pressure, and UV light) and chemical agents (ethylene, antibiotics, salts, heavy metals, and fungicides) also participate to enhance the metabolite content (Efferth 2019). These elicitors curb the gene expression in response to chemical and physiological stimuli and encourage enzyme amalgamation, thus facilitating the development of several secondary metabolites. Subsequently, certain secondary metabolites have not only defensive roles but also therapeutic potential for the benefit of human beings. Plant cell cultures consequently serve as a fascinating platform for the efficient and sustainable production of valuable secondary metabolites. Plant cell culture is becoming a well-established method for the processing of natural plant products in the nutrition, medicine, and pharmaceutical industries. Natural products produced in in vitro system have started to gain substantial consideration due to their advantages over the production of natural condition (Ochoa-Villarreal et al. 2016). It proved as an efficient system for the production of metabolites, regardless of the limitations arising from geographical, seasonal and deteriorating wild plant populations. This platform will also help to create a powerful supply chain and help to mitigate environmental issues and conserve plant biodiversity (Nossov 2012; Wilson and Roberts 2012; Ochoa-Villarreal et al. 2016). Notably, the in vitro processing by cell

culture methods of phytochemicals limits the need for water usage, as associated with existing regimes of agricultural production.

Commercialization of natural products produced by plant cell culture has also gained significant market approval, as they are labelled as non-GMOs (Murthy et al. 2015). Plant cell culture is proving to be the most effective and reliable way of processing natural products, such as the production of geranium in the flavouring and fragrance industries (Chen and Viljoen 2010). Moreover, by using an appropriate culture medium and adequate phytohormone ratios, in vitro cultures of most of the plant species can be established. Subsequently, cell suspension cultures can be established by adding such cells to the liquid medium after the efficient formation of friable callus. These cultures have a substantial scale-up capability for industrial level bioreactors system for bioactive molecules production. Several methods have been established in recent years to improve cultivation conditions and yield by using in vitro plant cell culture approaches. In this chapter, we specifically highlight the callus and suspension cultures procedure and approaches used for the accumulation and production of valuable secondary metabolites in *Rhodiola imbricata*, *Picrorhiza kurroa*, *Fritillaria roylei*, *Stevia rebaudiana,* and *Dactylorhiza hatagirea* (Figs. 8.1 and 8.2).

8.2 International and National Importance/Status

Plant cell culture has a long history in the manufacturing of medicines, cosmetics and nutraceuticals. Globally, approximately more than 50 plant cell culture-based products have been launched in the cosmetics industry (Eibl et al. 2018). The overall market demand of *Rhodiola* raw extract is approximately 20–30 tonnes raw material/year (Tao et al. 2018; Rattan et al. 2020) (Table 8.1). Companies like Nature's way; Chansha Organic Herb Inc.; iHerb; Amax Nutra Source; Swanson health products are actively involved in producing, promoting, and marketing of high-value *Rhodiola* enriched formulations and products (Rattan et al. 2020). Whereas in case of *P. kurroa*, the price of picrosides, i.e. P-I is Rs. 2590/mg, P-II is Rs. 2590/mg, and P-III is Rs. 21,758/mg (Table 8.1). Major firms in *Picrorhiza* extract and formulations are Vital Nutrients, Aunutra Industries Inc., Baseline of Health, Kamdhenu Laboratories, Globatic Herbs, Sandhu Products, and Natures Nectar LLC. Likewise, *F. roylei* is one of the most traded species around the globe, covering industrial demand worth US dollar 400 million in China (Luo et al. 2018). Though in India, it is sold locally at Rs. 10,000–15,000/Kg in bulb form (Table 8.1). While *S. rebaudiana* is an alternative to sucrose due to its low-calorie natural sweetening compound, SG, and widely used in various food preparations and formulations (Priya et al. 2011). In 2017, the global *stevia* market was USD 370 million and is expected to reach USD 640 million by 2023 (Anonymous 2017). *D. hatagirea* is a high value medicinal plant due to its underground tuber part containing dactylorhin. The market value of its dry tubers is in the range of Rs. 3000–3200/kg (Chaurasia et al. 2007) (Table 8.1).

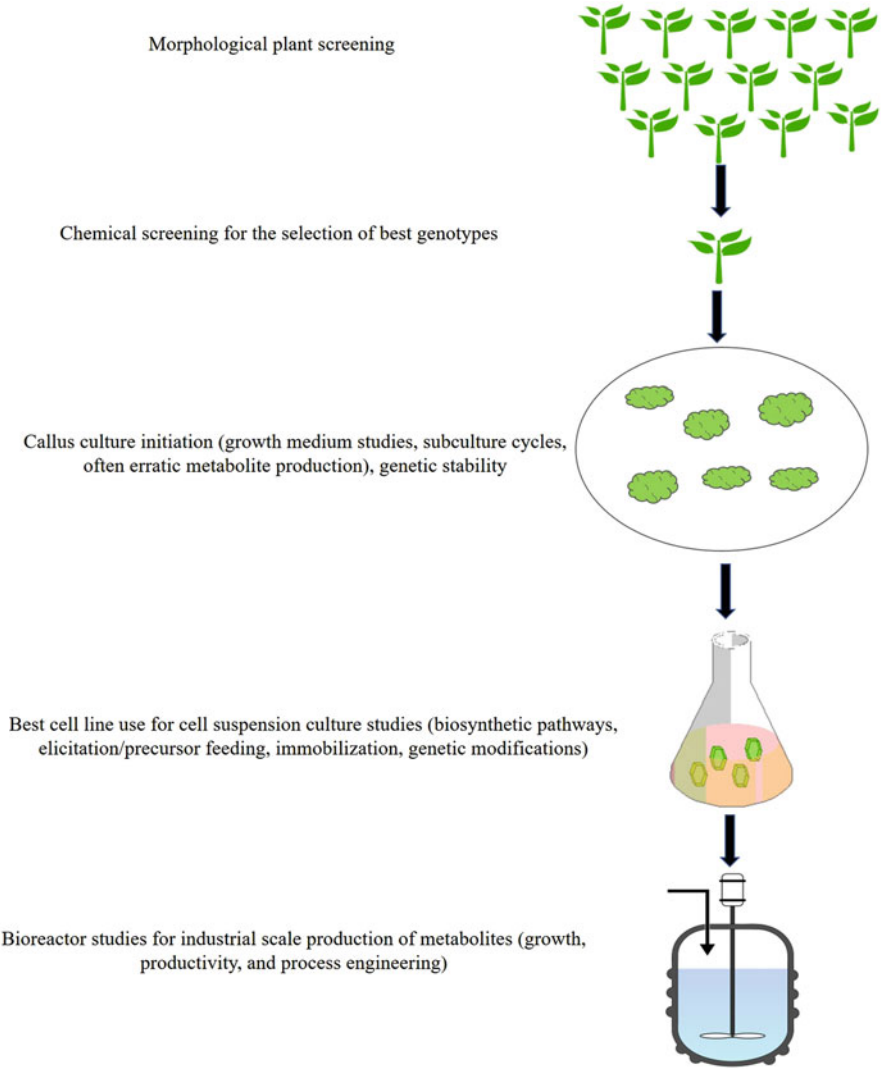

Fig. 8.1 Workflow depicting the stages for metabolite production

8.2.1 *Rhodiola imbricata* Edgew

Rhodiola imbricata Edgew is a perennial, herbaceous, medicinal herb belonging to Crassulaceae family. It inhabits places like stony cervices and wet places of high altitude region of Ladakh, India (Chaurasia et al. 2003). It is commonly referred as Himalayan rose root, Shrolo and Sanjeevani Buti (Bhardwaj et al. 2018a; Rattan et al. 2020). Since ages, the plant is being used due to its bioactives and well

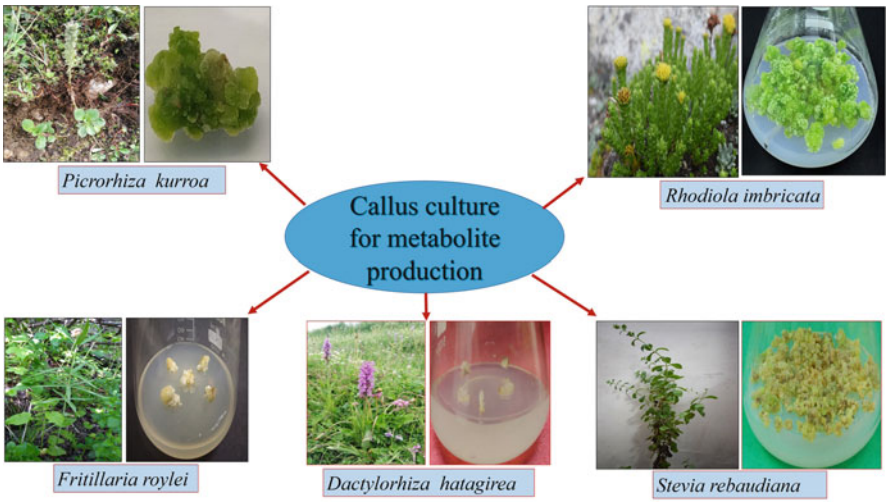

Fig. 8.2 Representation of callus culture. (**a**) For *Picrorhiza kurroa*: the image of callus adapted from Partap et al. (2020); (**b**) for *Rhodiola imbricata*: the image of callus adapted from Rattan et al. (2020); (**c**) for *Fritillaria roylei*: the plant image adapted from Kumar et al. (2021) and the image of callus adapted from Kumar et al. (2020)

Table 8.1 Market potential of commercially important plants

Plant species	Demand	Secondary metabolites	Uses
Rhodiola imbricata	20–30 tonnes raw material/year	Salidroside and rosavins	Nutraceutical, pharmaceutical, health care, aerospace, and sports sector
Picrorhiza kurroa	Global annual Demand 500 tonnes, and production 375 tonnes	Picrosides	Hepato-protective, anti-inflammatory, ant-cancerous, and anti-asthmatic
Fritillaria roylei	Rs. 10,000–15,000/ kg dry bulbs	Sipeimine and peimine	Lungs congestion, asthma, and stomach problems
Stevia rebaudiana	Market valued around USD 370–USD 640 million	Steviol glycosides	Antibacterial, antifungal and anticaries properties
Dactylorhiza hatagirea	Dry tubers Rs. 3000–3200/kg	Dactylorhin	Neurostimulant, astringent, antibacterial, aphrodisiac, immunomodulator and nutritional properties

recognized for its adaptogenic properties like freeze tolerance, anti-fatigue, anti-stress accompanied by other properties such as immunomodulation, radioprotection, anti-fatigue, antioxidant, anti-ageing, anti-cancerous, anti-depression, wound healing, high altitude sickness, headache, schizophrenia, sexual dysfunction and

also helps in radioprotection (Grech-Baran et al. 2015; Tayade et al. 2017; Bhardwaj et al. 2018b; Kapoor et al. 2018; Tao et al. 2018; Rattan et al. 2020). Medicinal properties of the plant are due to its bioactive components. The plant parts of *R. imbricata* is rich in rosavins, salidroside, tyrosol, vitamin, minerals, and dietary amino acids (Tayade et al. 2017; Bhardwaj et al. 2018a; Kapoor et al. 2018, 2019; Rattan et al. 2020). Salidroside and rosavins are used extensively in the nutraceutical, pharmaceutical, health care, aerospace, and sports sector (Tayade et al. 2017; Tao et al. 2018; Rattan et al. 2020).

Though in *Rhodiola*, callus culture technique is used previously for the production of metabolites, majorly salidroside and rosavins. Kapoor et al. (2018) described induction of callus from leaf explant as surface sterilized leaf was inoculated on MS medium, augmented with varying concentrations of NAA + BAP (0.1 mg/L-5 mg/L). As they found the highest callus induction frequency, i.e. 93% after 10 days on culture medium augmented with 5 mg/L NAA + 5 mg/L BAP. Furthermore, they studied callus induction, proliferation, and production of salidroside and tyrosol under continuous red, green, blue, and RGB LEDs. The production of salidroside was highest in blue light culture condition (2.82 mg/g DW) followed by white light culture condition (2.65 mg/g DW). Kapoor et al. (2019) used various concentrations of jasmonic acid to evaluate the salidroside production in cell suspension culture and content enhancement was observed at 100μM JA treatment, i.e. 5.25 mg/g DW.

However, recently, Rattan et al. (2020) studied callus induction from in vitro explants of *R. imbricata*. Efficient induction, proliferation, and compact to friable callus culture protocols were described using both leaf and root explant (Fig. 8.2). In this study, leaf explant showed maximum callus induction, i.e. 100% (14–16 days) and root explant, i.e. 87.50% (25–30 days) on MS media with TDZ and NAA and observed colour transition in prolonged callus cultures with respect to ageing process. Furthermore, phenylethanoids (salidroside and tyrosol) and phenylpropanoids (rosavin and rosarin) were quantified in leaf callus with the accumulation of salidroside content (3.59 mg/g DW). Further studies needed to be done in this important Himalayan medicinal plant for efficient production of valuable metabolites.

8.2.2 *Picrorhiza kurroa* Royle ex Benth

Picrorhiza kurroa is a significant medicinal herb growing in the north-western Himalayan region of India at an elevation of 3000–4800 m (Chettri et al. 2005). Various bioactive compounds such as glycosides, terpenoids, flavonoids, sterols, and phenolic compounds are accumulated in plant parts of *P. kurroa* (Debnath et al. 2020; Partap et al. 2020). Among these bioactive compounds, picrosides, i.e. P-I, P-II, P-III, and kutkoside are the most demanded and used for the preparation of herbal drugs. Major uses of this herb are hepato-protective, anti-inflammatory, ant-cancerous, and anti-asthmatic (Soni and Grover 2019). The demand for *P. kurroa* raw material encouraged overuse from natural habitats and resulted in their status as endangered (Debnath et al. 2020; Kumar et al. 2012). Therefore, more alternative strategies for the sustainable production of targeted metabolites are very

important. Though plant cell culture techniques provide an attractive and promising solution to reduce times and costs needed for safer production of desired metabolites (Partap et al. 2020). Previously, effect of quality of light, photoperiod, and temperature were studied and found as the major responsible factors for high-frequency callogenesis in *P. kurroa* (Partap et al. 2020; Gahlan et al. 2012; Kawasoo et al. 2010). Recently, Partap et al. (2020) and Chaudhary et al. (2019) studied the effect of thidiazuron (0.5 mg/L) + 0.3–0.5 mg/L (IBA) along with picloram (5 mg/L) and recorded callus induction, i.e. 97.78% (Fig. 8.2). Similarly, Sood and Chauhan (2009) reported 56.30% callus induction frequency on 2.0 mg/L 2,4-D + 0.5 mg/L IBA in *P. kurroa*. Moreover, Jan et al. (2010) and Parihar et al. (2018) also described that media with 0.25 mg/L (2, 4-D) + 0.25 mg/L (BAP) and 0.5 mg/L (BAP) + 0.75 mg/L (Kn) stimulated the overall induction rate. These variations in the *P. kurroa* callus response to different plant growth regulators could be due to different phytohormone and culture conditions. Nevertheless, Partap et al. (2020) reported the antioxidant activity (IC_{50}–40.88μg/mL), total phenol content (41.35μg/mg), and total flavonoid content (76.97μg/mg) in leaf derived callus of *P. kurroa*. However, Kant et al. (2013) and Bhandari et al. (2010) described the antioxidant activity in wild leaves and rhizome with IC_{50} value of 0.81–66.48μg/mL. Studies showed that picroside content in *P. kurroa* were ranged in 0.08–3.89% in P-I and 0.36–4.33% in PII (Kawasoo et al. 2010; Pandit et al. 2012; Partap et al. 2020). Similarly, Ganeshkumar et al. (2017), Rehman et al. (2014), and Partap et al. (2020) also reported picroside I, II and picroside III content in callus culture, which is comparable with wild grown *P. kurroa* plants. Biomass generated through callus culture was found very potent for production of secondary metabolite and it could be used for the large-scale industrial production of picrosides.

8.2.3 *Fritillaria roylei* Hook

Fritillaria roylei Hook. a bulbous, Himalayan perennial medicinal herb belongs to the family *Liliaceae* and is found at an altitude range of 3500–4600 m. The bulbous extract of this herb is an essential component of ayurvedic formulations such as Astavarga and Chyavanprash (Bisht et al. 2016). Its bulbs extract naturally contains steroidal alkaloids which have the potential to cure various diseases such as lung congestion, asthma, tuberculosis, and burns (Joshi et al. 2007; Kumar et al. 2020). The increasing demand for wild *Fritillaria* bulbs for herbal drug preparations facilitates the overexploitation of this species from natural habitats and therefore became critically endangered (Joshi et al. 2007; Chauhan et al. 2011). Due to its immense therapeutic value, conserving this species into its natural habitat through plant biotechnological procedure is at almost need (Joshi et al. 2007; Bisht et al. 2016). Nevertheless, plant cell culture especially callus culture is the most promising and sustainable method for targeted metabolite accumulation, scale-up production, and further selective enhancement (Kumar et al. 2020). For in vitro culture establishment, the first foremost and important requirement is the standardization and optimization of in vitro culture conditions, plant growth regulators, explant

sterilization, media forms, and explant type. Highly effective and consistent in vitro culture conditions in *F. roylei* have been optimized by Kumar et al. (2020). Contamination issues in culture media are very often during the callogenesis process particularly when underground plant parts (ex vitro/wild) are used as explants. The study reported by Kumar et al. (2020) highlighted the effective sterilization protocol with maximum survival, i.e. 79.67% in bulb scales using 0.1% mercuric chloride. This report was supported by previously reported literature, i.e. mercuric chloride at low concentrations is most effective disinfectant against bacterial and fungal contamination (Witomska and Lukaszewska 1997; Paek and Murthy 2002; Wang et al. 2016).

The high-frequency callus induction protocol was optimized using different concentrations and combinations of plant growth regulators, viz. TDZ, picloram, BAP, NAA, Dicamba, and 2, 4-D. Kumar et al. (2020) reported maximum callus induction (88.89%) on MS medium supplemented with TDZ—0.5 mg/L and picloram—2.0 mg/L after 5–6 weeks in dark condition at 25 ± 2 °C culture conditions (Fig. 8.2). This was the first report on callogenesis in *F. roylei* using bulb scale as explant (Kumar et al. 2020). However, prior information for alkaloid production in callus cultures and embryonic calli of various other *Fritillaria* species is well documented (Li et al. 2002; Wang et al. 2010; Hao et al. 1982; Wu and Tang 1992). In vitro callus culture has become a sustainable forum not only to accumulate and enhance the metabolite content but also for the regeneration process to save plants from critically endangered conditions and thereby preserve their natural environment (Espinosa-Leal et al. 2018). The optimized robust and effective in vitro callus culture system in bulbous *Fritillaria* species offers a sustainable way for the conservation, micro-propagation, and biologically active processing of steroid alkaloids for commercial usage in herbal formulations (Kumar et al. 2020).

8.2.4 *Stevia rebaudiana* Bertoni

Stevia rebaudiana Bertoni. is an economically important crop for food and pharmaceutical industries. The most abundant glycosides in *stevia* leaves are stevioside and several types of rebaudiosides (reb A–F) which are about 300 times sweeter than sucrose. These sweet compounds pass through the digestive process without chemical breakdown. Apart from its low calorific sweetening property, it helps in overcoming depression, cavities, hypertension, hyperglycaemia, obesity, sweet cravings, and urinary insufficiencies (Blinstrubiene et al. 2020). For rapid fulfilling the emerging demand of industry, plant cell culture is well exploited in case of *S. rebaudiana* (Fig. 8.2). Various biotechnological tools had been reported to utilize for generating quality biomass with enriched phenolics, flavonoids, antioxidant activity, and steviol glycoside content in *S. rebaudiana* cell cultures. As literature reviewed, *S. rebaudiana* leaves show very good response to a wide range of plant growth regulators in varying combinations, such as kinetin, 2,4-D, NAA, BAP. In recent reports, 1 mg/L NAA + 0.5 mg/L BAP (Golkar et al. 2019) and 2 mg/l 2,4-D

(Kumari et al. 2017) in combinations had been reported for compact green callus proliferation. Blinstrubiene et al. (2020) reported increased accumulation of steviol glycosides in NAA + proline treated callus cell lines. Although the cell line augmented with 2.0μM NAA + 5.0μM proline showed higher rebaudiosides accumulation as compared to stevioside. Similarly, addition of proline reduced the flavonoid content in callus cell lines, the phenolic content was significantly higher in callus cell lines augmented with 2.0μM NAA + 2.0μM proline. The antioxidant activity is reported higher in callus cells supplemented with 2.0μM NAA + 5.0μM proline. In a similar study, Golkar et al. (2019) studied the effect of salicylic acid and silver nanoparticle elicitation in callus cell lines of *S. rebaudiana*. They reported higher biomass in salicylic acid supplemented callus cell lines and observable biomass increment in silver nanoparticle supplemented callus cell lines. Although no significant effect of elicitation was observed in rebaudioside A content, AG nanoparticle elicitation resulted in increased stevioside accumulation on *stevia* callus. Javed et al. (2017) also reported increased antioxidant activity in ZnO and CuO nanoparticle treated callus cell lines of *S. rebaudiana*. Apart from chemical elicitors, effect of physical elicitors like various light spectra had also been studied on callus cultures of *S. rebaudiana* (Ahmad et al. 2016). The study resulted in enhanced antioxidant secondary metabolite production in coloured light. Polyethylene glycol (PEG) and proline proved to be effective in enhanced production of steviol glycosides (Gupta et al. 2015).

8.2.5 *Dactylorhiza hatagirea* (D. Don)

Orchidaceae is largest plant family that has a range of diverse bioactive secondary metabolites. *Dactylorhiza hatagirea* (D. Don) Soo is a valuable medicinal orchid endemic to North-Western Himalayan parts (3000–4500 m above sea level) and belongs to family Orchidaceae. Mostly, the tuber part of this plant is being used as it has neurostimulant, astringent, antibacterial, aphrodisiac, immunomodulator, and nutritional properties (Vij et al. 1992; Thakur and Dixit 2007). Distribution of *D. hatagirea* spreads to Afghanistan, Pakistan, India, China, and Bhutan. It inhabits in alpine meadows, humus rich soil amid grasses, nearby to snowy rivers with other herbs (Pant and Rinchen 2012). Medicinal properties of *D. hatagirea* are due to the presence of a glucoside, dactylorhin in the tuber. *D. hatagirea* is generally considered as high valued medicinal herb as the market value of its dry tubers is in the range of Rs. 3000–3200/kg. Hence, there is a need of sustainable strategy such as plant cell culture can play a vital role to fill the gap (Fig. 8.2). Extraction of dactylorhin using a solvent extraction method from dried rhizomes gave very low yield (Li et al. 2009). There are only one or two reports on the extraction of this pharmacologically very much relevant metabolite, dactylorhin from natural resources. Reported by Warghat et al. (2014) on *D. hatagirea* using seed as explants for plantlets regeneration and mass multiplication. Dactylorhins have been quantified and isolated in tuber parts of related genera of terrestrial orchids too (Morikawa et al. 2006; Sakuno et al. 2010). It was observed that content of

dactylorhin A, B, and E in in vitro cultures was less when compared to naturally grown parts. Further, as per the market demand of pharmaceutical industry, alternative approaches such as cell culture will be useful for the production of metabolites and reproductive parts such as flower as well as whole inflorescence could be used apart from underground parts like tubers/roots of *D. hatagirea*.

8.3 Conclusion and Future Prospective

Natural products are hard to synthesize. So, there is a need to fill this space with sustainable and cost-effective production of plant-based natural products. Hence, biotechnological production under in vitro system such as plant cell culture and suspension culture at shake flask and bioreactors level represents an effective means for the commercial scale mass production of bioactives. The ever-expanding knowledge about biosynthetic pathways of desirable products will lead to genetic interventions in plant cell culture to improve the total net yield. Although the full potential of plant cell culture is not yet exploited. Hence, there is a necessity to develop, restructure the plant cell culture with crucial augmentations for the year-round production of desired metabolites.

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Transgenic Implications for Biotic and Abiotic Stress Tolerance in Agricultural Crops

9

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Abstract

Plants encounter variable stresses in the environment which lead to huge crop losses worldwide. Environmental stresses that a plant can undergo are categorized into two categories as (a) biotic and (b) abiotic stress. Biotic stresses include attacks by different insects, nematodes, and microbial pathogens like fungi, bacteria, and viruses. While on the other hand, abiotic stresses include high salinity, heat, cold, drought, osmotic stress, and heavy metal. Plants are quite susceptible to both kinds of stressful situations and have adopted different mechanisms to encounter these situations. Plants sense these stresses and stimulated specific stress responses thereby activating different stress response signaling pathways and generating appropriate cellular responses helping in combating these stresses. This chapter gives an overview of the major stresses, plants encounter during growth and transgenic implications that have been made to modify these stress-tolerant properties to produce crops with improved crop yield and minimize crop losses.

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Keywords

Transgenics · Biotic · Abiotic · Stress · Environment · Crops

9.1 Introduction

The plant undergoes different kinds of stresses in nature that contribute to adverse growth and compromised plant productivity. Any kind of stress induces a series of adaptive responsive in plant-like alteration in gene expression and induction of defensive cell metabolism. Plant stress could be divided into two major categories termed as biotic (microbial pathogens, nematode, insects, and weed) and abiotic caused through various environmental cues (heat, cold). Both kinds of stressors contribute to both pre- and post-harvest losses. Furthermore, it is also noticeable that with increasing global warming and changing climate situations, agricultural crops significantly encounter an increased event incidence of both abiotic and biotic stresses compromising plant yield (Mahalingam 2015; Ramegowda and Senthil-Kumar 2015; Kumar and Srivastava 2016, 2020a, 2020b; Srivastava et al. 2016; Parmar et al. 2017; Gambhir et al. 2020; Kumar et al. 2018a, 2018b, 2018c). Both kinds of stresses concurrently cause more destruction when compared to either of the stress occurring solely. For instance, abiotic stress directly influences the occurrence, survival, and dissemination of the different pathogens, insects, and weed growth. Additionally, these stress situations also influence plant physiology and host-defense responses to promote plant–pest interactions. However, this effect is not always additive as the outcome could be influenced by the nature of the interaction between these stress factors (Pandey et al. 2015; Ramu et al. 2016).

Tackling any kind of stress is a bigger problem for gradually increasing world population, estimated to reach ten billion by 2050 (Bengtsson et al. 2006; Secretariat UNIS for DR 2015). All this needs a significant increase in crop production by minimizing crop losses in any kind of stressful situation. The improvement could either be achieved by a traditional breeding method like wide-cross hybridization, mutation breeding or by modern technology including transgenics. Despite several attempts, the conventional plant breeding methods have failed in combating this issue. Current studies propose that the tolerance to any kind of stress is multigenic and quantitative QTL (quantitative trait locus) in nature (Collins et al. 2008), which could be a possible reason for this failure. Modern transgenics serve as an alternative to tackle this issue as this includes the introduction of exogenous genes into the host or an alteration in the expression of a host's gene that can help in improving stress tolerance (Roy and Basu 2009). Besides, modern transgenics require comparably less time than conventional plant breeding methods with a benefit of transfer of only desired gene(s) to the host plant, it always has an upper hand over the conventional crop breeding methods (Yamaguchi and Blumwald 2005). Due to ease in the use of transgenics for crop improvement, the technology has been used extensively world-wide (Wani et al. 2016a). However, the employment of genetic engineering needs

the identification of the key genes underlying these processes of plant stress tolerance.

It is well known that any kind of stress induces a series of adaptive responses in the cells contributing to changes in the physiology and molecular makeup of the plants that could be dangerous to the cellular machinery. If we talk at the molecular level, any kind of stress leads to different molecular responses like the production of reactive oxygen species causing damages to cellular molecules like protein, nucleic acids, and lipids by the process of oxidation or peroxidation. In severe conditions, these stress situations may also lead to programmed cell death and thus cause the death of the entire plant (Sharma and Dubey 2007). Hence, exploring the underlying molecular mechanisms and key molecules contributing to stress signaling could be an alternative strategy for crop engineering for stress-tolerant properties, which could be done by application of the modern transgenic approaches like sequencing and functional genomics (Heidarvand and Amiri 2010). Usually, a cell signaling cascade is triggered upon any kind of stress upregulating the different transcription factors inducing various stress-responsive genes coding for proteins helping in stress-tolerant phenotypes. As stress-sensitive plants are compromised in synthesizing these proteins, they are susceptible to all these stressors impeding their overall growth. Several genes have been identified that code for stress-protective compounds and proteins in the different organisms (plants, animals, or microbes). The ideal genes for this kind of targeted insertion have been classified into three major categories; (a) associated with osmolyte synthesis like mannitol, glycine betaine, proline, and heat shock proteins, (b) associated with the uptake of ion and water, and (C) associated to transcriptional control and different signaling pathways. Progress in understanding the underlying mechanism of these stress responses has undergone major development in recent years (Jaspers and Kangasjärvi 2010). The transgenics have been successfully employed to produce plants with different attributes like an increase in grain yield, increase tolerance to high salt and drought stress in rice, barley, maize, etc. (Wani et al. 2016b). Following is a brief introduction of the different kinds of stressors contributing to significant crop losses and the genetic developments created to combat these losses associated with stressful situations. As losses due to both kinds of stressors cause severe crop losses worldwide, it is conceivable that the development of the plant with improved traits for abiotic and biotic stress requires identification and improvement of stress adaptive traits in these plants. This book chapter is a brief introduction about these stresses (biotic and abiotic) and the genetic improvement approaches that have been implicated for improving stress-responsive properties in plants.

9.2 Biotic Stresses

9.2.1 Insect Pest and Nematodes Resistance

The control of insect pests in commercially grown agricultural crops is predominantly based on the indiscriminate use of synthetic insecticides which is deleterious to human health and the environment. Transgenic crops have revolutionized modern agriculture and have become a major tool of integrated pest management leading to a reduction in insecticide use, protecting the environment and human health. Insect-resistant plants were also developed about two decades ago in other crops (Ansari et al. 2015). The search for novel useful genes encoding insecticidal proteins is still in progress and information deriving from the prolonged cultivation of commercialized GM products confirms the efficacy of this biotechnological tool. The new research area includes the use of novel transgenes and improved transformation protocols especially for the development of insect-resistant cultivars in economically grown agricultural crops.

The soil bacterium (*B. Thuringiensis, Bt*) produces a wide range of proteins (δ-endotoxins) that are included in crystals formed during sporulation and characterized by distinct insecticidal spectra (de Maagd et al. 2001; Sharma and Srivastava 2013). *Bt* spores contain high levels of δ-endotoxins harmful to specific insects of Lepidopteran, Dipteran, and Coleopteran orders which are the major pests of agricultural crops and perennial tree species. The *Bt* spores and the crystal (Cry) proteins are ingested by the insect and solubilized within the alkaline midgut. The protoxins are then activated by proteinases and finally, the active *Bt* toxin binds to specific molecular receptors causing the irreversible damage of the midgut epithelium by colloid osmotic lysis. *B. thuringiensis* has been used as a commercial insecticide for more than 50 years and to date, an extensive number of reports have demonstrated that *Bt* proteins have negligible potential adverse effects against humans, animals, and non-target invertebrates. More than 130 *Bt* genes encoding different δ-endotoxins have been isolated and, among this extremely large gene array, those coding for the *CryIA*(a) and *CryIA*(c) proteins have been used to develop transgenic crops resistant to Lepidoptera. Also, the *CryIIIA*(a) protein has been chosen by different research groups to specifically target Coleopteran pests. Insect resistance was firstly reported in tomato using *Bt.* gene in 1987. Cotton was the first commercially successful crop in which *cry* genes were incorporated to provide resistance against lepidopteron insect pest (Perlak et al. 1991). After the success of transgenic cotton, cry genes have been incorporated in many crops, viz., potato rice, canola, soybean, maize, chickpea, alfalfa, and tomato. Insect-resistant transgenic crops have the second largest area under cultivation which is 23.3 million hectares in 2017 (ISAAA 2017), 304 events have been approved in different crops worldwide for commercial cultivation. Out of these events, 208 events comprising various insect resistance (IR) genes in maize have been approved for cultivation. The commercialized crops having various IR genes are cotton (49 events), potato (30 events), soybean (6), rice (3), sugarcane (3), poplar (2), brinjal (1), and tomato (1).

Apart from *cry* genes, other insecticidal genes such as Proteinase inhibitors (PIs) are also used to develop insect-resistant GM crops. PIs are natural compounds abundantly found in seeds and storage organs of a wide range of plant species and contributing to the plant defense system against insect pests and pathogens (Schuler et al. 1998). Proteinase inhibitor families are specific for each of the four classes of proteolytic enzymes (cysteine, serine, aspartic, and metalloproteinases). Serpins and Cysteine are the most explored plant PIs against insect pests. Green and Ryan proposed this concept in a pioneer study reporting rapid accumulation of protease inhibitors in potato and tomato leaves attacked by Colorado potato beetles, both locally as well as systemically (Green and Ryan 1972). A few years later, a seminal study by Hilder et al. (1987) reported PI-expressing transgenic tobacco lines by utilizing the potentials of plant genetic transformation. With the advancement of gene transfer technology, this agronomically useful gene was introduced in rice cultivars that enhanced protection to stem borers (Xu et al. 1996) and wheat (Altpeter et al. 1999) to protect from leaf-feeding and storage pest. Since then, various research groups have reported numerous studies of enhanced herbivore protection in multiple plants involving bioassays, PI-expressing transgenic plants, and insect feeding assays. The inhibiting activity of PIs is due to the ability to form stable complexes with proteinases, blocking, altering, or preventing the access to the substrate-binding region of their catalytic site. To develop effective strategies for plant protection against insect pests based on PIs transgenesis, it is imperative to know the class of proteolytic enzymes present in the insect guts, which ultimately results in the extended developmental period, reduce fecundity and increase mortality due to amino acid deficiencies. Different proteinases predominate in different insects. Most of the Lepidopteran species have serine proteinases as the major digestive enzymes. Coleoptera has a wider range of dominant gut proteinases (Schuler et al. 1998). Broadway and Duffey (1986) suggested that the PI mediated inhibition of proteinases is responsible for hyperproduction of digestive enzymes, enhanced loss of essential amino acids, and finally inhibition of insect growth rates. However, some insect species seem to be able to modify dynamically the spectra of their digestive enzymes by the production of insensitive proteinases. Besides several reports of successful PIs-transformed plants enhanced protection to insect pests, this promising strategy of crop protection could not be successfully commercialized.

9.2.2 Nematodes Resistance

Plant-nematode parasitism is one of the most damaging uncontrollable biotic stresses on crops, and the cumulative effect on agriculture is severe. The majority of these losses are inflicted by relatively few species. The most damaging are root-knot nematodes (*Meloidogyne* species) and cyst nematodes (*Heterodera* and *Globodera* species), with root-knot nematodes the major contributors to yield losses (Koenning et al. 1999). Management of nematode parasitism is therefore imperative. Integrated use of chemicals, resistant varieties, and cultural and biological practices provide the most successful management strategy. These approaches are, however, becoming

increasingly unsatisfactory. Although conventional intensive farming methods rely largely on the use of nematicides, dependence on this approach must diminish as environmental and health concerns around these toxic chemicals increase. Crop rotation as a strategy to limit nematode infestation has limited utility against those species with cosmopolitan host ranges, such as *M. incognita*, which may potentially parasitize up to 3000 plant species (Abad et al. 2003). Resistance in plants is therefore an attractive approach for controlling nematode populations. This may be either naturally occurring or transferred to crop cultivars from wild relatives or breeding lines through conventional breeding methods or engineered through molecular techniques. Biotechnology offers several benefits for nematode control in integrated management strategies such as reducing risks to the environment and human health, accessibility for food producers in the developing world, and the possibility of achieving durable, broad-spectrum nematode resistance (Thomas et al. 2006).

Several nematode resistance (Nem-R) genes have been isolated from plants which confer resistance against sedentary endoparasites. The first nematode resistance gene to be cloned was Hs1pro-1 from sugar beet, which confers resistance against the sugar beet cyst nematode (Cai et al. 1997). The encoded protein does not have obvious similarities to known plant genes. However, other cloned Nem-R genes closely resemble known plant R-genes in their domain structure. Four of these genes, *Mi-1*, *Hero A*, *Gpa2*, and *Gro1–4*, all cloned from tomato or potato relatives, fall into the NBS-LRR class of R-genes. The tomato genes Mi-1 and Hero A confer broad-spectrum resistance against several root-knot nematode species (Milligan et al. 1998) and against several pathotypes of two potato cyst nematode species (Ernst et al. 2002), respectively. By contrast, the potato genes *Gpa2* and *Gro1–4* confer resistance to a narrow range of pathotypes of a single potato cyst nematode species. *Mi-1*, *Gpa2,* and *Hero A* are members of the NBS-LRR class of plant R-genes that does not contain an N-terminal toll-interleukin receptor-like (TIR) domain. The *Hero A* gene product is 32% identical to *Mi-1* and w22% identical to *Gpa2* at the amino acid level (Williamson and Kumar 2006).

9.2.3 Antifungal Resistance

Antifungal proteins like chitinase, glucanase, defensin, thaumatin-like proteins, osmotin-like protein, phytoalexins, RIPs, etc. are produced by different flowering plants (Vigers et al. 1991), fungi (Guo et al. 2008), invertebrates and vertebrates (Raj and Dentino 2002) to combat the fungal pathogens. Some of this plant protein comes under the pathogenesis-related protein group (PR). Genes of these antifungal proteins were transferred to different plants to increase fungal resistance against fungal pathogens. Below is a summarized different antifungal protein which was used to produce transgenic plant against fungi.

9.3 Chitinase and Glucanase

Most of the fungi contain chitin and glucan in their cell wall as major components. Chitinase and glucanases are hydrolytic enzymes that can degrade chitin and glucan, thus leads to the degradation of the cell wall of fungi. Chitinase comes under the glycosyl hydrolase family and it hydrolyzes glycosidic bond in chitin. On the basis of isoelectric pH, the sequence at N-terminal, enzyme localization, and signal peptides chitinase enzyme was found to belong to 18 and 19 families of glycosyl hydrolases. Classes III and V of chitinase come under glycosyl hydrolase 18 and I, II, and IV under family 19. These enzymes are PR-3 group members (PR-Pathogen-related proteins) are first described in the orchid bulb by Bernard as an antifungal factor (Sharma et al. 2011). These are probably the most frequently studied and described PR proteins. These hydrolytic enzymes can cause lysis of fungal hyphae, inhibition of fungal growth, and exhibit in vitro antifungal activity (Boller 1993; Neuhaus 1999). Chitinase was reported to be present in plants, animals, microbes, and human beings. But the most preferred one is microbial chitinase because they can be easily produced in bulk and also available is more. But the first group of PR families is plant chitinases which are abundantly present in the plant kingdom and found to be effective against many phytopathogenic fungi like Ascomycota and Basidiomycota phyla (Punja 2004). It was also reported that these hydrolytic enzymes can also be produced in plants in response to abiotic stress as well as growth conditions. The number of chitinase gene isolated from different sources has been studied, sequenced, cloned as well as transformed into plants to develop fungal resistance. It was found out that when glucanase and chitinase genes were expressed in transgenic plants, then it results in more resistance to fungal pathogens (Nishizawa et al. 1999). But the expression of these genes in transgenic plants at a low level is a key issue. Lee and Raikel (1995) reported that in rice and tobacco, the expression of chitinase genes increased the plant's resistance to phytopathogenic fungi. Jabeen et al. (2015) studied for the first time that transgenic tomato plants showed resistance to two major fungal pathogens, i.e. *Fusarium oxysporum* f. sp. lycopersici (Fol) causing fusarium wilt and *Alternaria solani* causing early blight (EB) when rice chitinase (*RCG3*) was expressed in tomato. *Agrobacterium*-mediated transformation of cotyledonary petioles with an endochitinase gene (*chit33*-cDNA) isolated from *Trichoderma atroviride* under CaMV35S constitutive promoter showed increased resistance against *Sclerotinia sclerotiorum* in canola (R line Hyola 308) (Solgi et al. 2015). *EuCHIT2*, a new chitinase gene that was isolated from *Eucommia ulmoides* Oliver was overexpressed in tobacco plants showed resistance to *Erysiphe cichoracearum* DC (Dong et al. 2017). Khan et al. (2017) also developed transgenic potatoes using the *Agrobacterium*-mediated method that overexpressed endochitinase gene and showed resistance against *Alternaria solani.* Novel chitinase gene *LOC_Os11g47510* from indica rice Tetep provides enhanced resistance against sheath blight pathogen *Rhizoctonia solani* in rice (Kamboj et al. 2017). Chitinase enzyme gained attention towards biocontrol of fungal pathogen, but glucanase enzymes are less studied as compared to chitinase. Only a few reports are available on glucanase gene transformation in plants, but some transgenic plants

overexpressing glucanase gene was successfully produced. Glucanase gene of tobacco was overexpressed in groundnut which showed tolerance to *Cercospora arachidicola* and *Aspergillus flavus* (Sundaresha et al. 2010). It was found out that transgenic groundnut was not only resistant to fungi but also produced less aflatoxin. In another report, grapevine, b-1,3-glucanase (*VvGHF17*) gene was overexpressed in *Arabidopsis* plants, showed resistance to *Colletotrichum higginsianum* and *Botrytis cinerea* (Fujimori et al. 2016). However, the synergistic action of these hydrolytic enzymes with each other as well as with other antifungal proteins has resulted in excellent in vitro and in vivo antifungal action (Melchers and Stuiver 2000). For example, when chitinase (*chi11*) and osmotin (*ap 24*) antifungal proteins encoding genes isolated from rice and tobacco, respectively, were expressed in tobacco, then this synergistic action can cause enhancement of sheath blight tolerance in transgenic rice (Sripriya et al. 2017).

9.4 Defensin

Defensin is a small antimicrobial cationic peptide that is present in various living organisms such as plants, microbes, and mammals. It contains about 45–54 amino acid residues which form a highly conserved structure scaffold with cysteine amino acid to forms αβ conformation. In plants, γ-thionin of wheat and barley was renamed as defensin based on structure and function similarity with insect defensin. The tertiary structure of plant defensin is formed of 3 antiparallel strands and 1 α- helix strand stabilized with disulfide bridges is highly conserved to form CSα/β (Cysteine stabilized α-helix β sheet motif) (Zhu et al. 2005). 3D Structures of different plant defensins are almost similar instead of low-level amino acid identity. Plant defensin is secreted in extracellular space in plant cells except for some floral defensin which is targeted to the vacuole. Plant defensin is naturally synthesized and present in every organ of plants. In plants defensing, major role is in the inhibition of phytopathogenic fungal growth at a very less concentration (Lay and Anderson 2005). Main mechanism of how defensin prevents fungal growth is not clear but it was found out that defensin bound to fungal cell membrane because of some electrostatic or hydrophobic interactions and at a very high concentration it causes membrane permeabilization which leads to the death of fungi (Sagaram et al. 2011; Thevissen et al. 2003; Valente et al. 2013; Hayes et al. 2013). The transgenic expression of defensins has enhanced plant resistance to phytopathogenic fungi. However, so far, there are no reports of enhanced resistance through the transgenic overexpression of defensin genes in those plants from which it was initially originated. Defensin gene isolated from plants was overexpressed in many plant species. For example, a defensin gene *Rs-AFP2* was isolated from *Raphanus sativus* and overexpressed in transgenic rice showed antifungal ability against *Rhizoctonia solani* and *Magnaporthe oryzae* (Jha and Chattoo 2010). This gene causes direct inhibition of theses pathogen (Lacerda et al. 2016). Spore germination and growth of obligate biotrophic fungi *Fusarium tucumaniae* and *Colletotrichum gossypii* var. cephalosporioides was inhibited in transgenic *Pichia pastoris expressing* rDrr230a

defensin protein gene. This gene also showed inhibition of the Asian soybean rust pathogen *Phakopsora pachyrhizi* and was used against cotton and soybean fungal diseases. A defensin gene J1–1 was overexpressed in transgenic pepper showed increased resistance against *Colletotrichum gloeosporioides* fungi which is the causal agent of fruit-specific anthracnose fungus (Seo et al. 2014). Transgenic poplar plant expressing a putative defensin gene showed enhanced resistance against *Septotis populiperda* (Wei et al. 2020).

9.5 Thaumatin-like Proteins

Thaumatin-like proteins are present in plants such as Kalemfe which is a tropical flowering plant. It is a very sweet tasting protein that is almost about 100,000 times sweeter than sucrose. These proteins also come under the PR-5 Proteins family. These are low molecular weight proteins of about 20–24 kDa with 200 residues and 16 conserved cysteine which are involved in 8 disulfide bond formation which gives stability to this protein (Fierens et al. 2009). TLPs are present in different kingdoms such as plants (angiosperms, gymnosperms), animals, and fungi also (Liu et al. 2010). These proteins also expressed in plants against biotic and abiotic stress (Muoki et al. 2012; Singh et al. 2013). TLPs are also found to be antifungal proteins when overexpressed in transgenic plants (Singh et al. 2013; Wang et al. 2011a, b; Liu et al. 2012; Mahdavi et al. 2012; Acharya et al. 2013). It's unclear how these thaumatin-like antifungal proteins interact with the fungal pathogen, and more research is needed. Thaumatin-like proteins possess the antifungal activity and overexpression of these proteins showed tolerance to fungal pathogens (Wang et al. 2011a, b; Liu et al. 2012; Mahdavi et al. 2012; Acharya et al. 2013; Singh et al. 2013). Thirty-three putative TLPs gene of grape was studied for grape disease resistance and it was found that overexpression of *TLP29 in Arabidopsis thaliana* causes powdery mildew resistance (Yan et al. 2017). ObTLP1 which is an ocimum thaumatin-like protein was found to be an antifungal protein and was reported to inhibit the growth of *Ceratonia sclerotiorum* and *Botrytis cinerea.* When this gene was overexpressed in transgenic Arabidopsis, then it led to resistance against these fungi and also against dehydration and salt stress; thus suggesting their role in abiotic stress also (Misra et al. 2016). *Agrobacterium*-mediated transformation of *Ostlp*, a thaumatin-like protein in cassava inhibits *Colletotrichum gloeosporioides* f. sp. *Manihotis* growth (Ojola et al. 2018).

9.6 Osmotin-like Proteins

Osmotin or osmotin like proteins is a multifunctional protein that comes under the PR-5 protein family because they are homologous to thaumatin. Osmotin structure shows three motifs with similar folding as thaumatin and other PR-5 proteins. It consists of three domains. Singh et al. (1987) characterized osmotin from salt adapted cultures tobacco (*Nicotiana tobaccum*) cells. Osmotin is a multifunctional

stress-responsive protein that enhances biotic and abiotic stress resistance in plants (Anu et al. 2015; Le et al. 2018; Su et al. 2017). Osmotin gene expression and protein formation is induced by any biotic stress such as fungal attack and its overexpression in transgenic plants leads to less disease symptoms (LaRosa et al. 1992; Liu et al. 1994; Zhu et al. 1996). Osmotin protein attacks specifically the plasma membrane of the pathogen which leads to signaling for cell death. Cell wall composition also determines osmotin toxicity because it governs osmotin protein access to the plasma membrane (Ibeas et al. 2000, 2001; Narasimhan et al. 2001, 2005). *Oryza sativa, Glycine max, Capsicum chinense, Vitis vinifera,* and *Sesamum indicum* are examples of plants in which osmotin gene was being transformed and showed increased resistance to fungal growth (Kim et al. 2004; Elvira et al. 2008; Weber et al. 2014; Katam et al. 2015; Chowdhury et al. 2017). *Ob*TLP1, which showed similarity to stress-responsive osmotin protein as well as to thaumatin-like protein, was isolated from *Nicotiana tabacum* and showed resistance to *Botrytis cinerea*, *Sclerotinia sclerotiorum,* and to salt stress and dehydration when expressed in *Arabidopsis* (Misra et al. 2016). Chowdhury et al. (2017) reported that *SindOLP* when overexpressed in sesame showed resistance against biotic as well as abiotic stresses. Transgenic lines of potato cultivar "Kufri Chipsona 1" were developed containing *OsmWS* osmotin gene isolated from *Withania somnifera*. These transgenic lines showed 22 fold expression of this gene within 3 days and inhibit *Alternaria solani* growth (Kaur et al. 2020).

9.7 Plant Ribosome-Inactivating Proteins

RIPs are RNA N-glycosidase which causes depurination of the highly conserved region, i.e. α-sarcin loop of 28s rRNA and thus inactivates ribosome by inhibiting the eF-Ia to bind with the ribosome. This inhibition leads to blockage of translation on the ribosome (de Virgilio et al. 2010). RIPs are widespread in nature and are distributed among different plant genera within different tissues. A number of RIPs are found to possess different antimicrobial activities in nature such as antifungal, antitumoural, antibacterial, and antiviral activities (Stirpe 2004; Puri et al. 2009; Bian et al. 2010). In agriculture, it is demonstrated in vitro and in transgenic plants that RIPs have been connected to defense by antifungal, antibacterial, antiviral, and insecticidal activities (Akkouh et al. 2015). For example, transgenic tobacco plant containing maize proRIP antifungal protein showed increased resistance against *R. solani* (Maddaloni et al. 1997). Yuan et al. (2002) reported that blast disease in transgenic rice was found to be inhibited by a type I RIP TCS (Yuan et al. 2002). Curcin-2 isolated from *Jatropha curcas* leaves was expressed in tobacco plants showed antifungal activity against R. *solani*, this protein was found to exhibit activity against different other stresses also (Huang et al. 2008). *Agrobacterium*-mediated transformation of the potato cultivar "Desirée" with Ribosome-Inactivating Protein (*rip30*) gene of barley produced a transgenic which showed enhanced resistance to *Rhizoctonia solani* in greenhouse condition (M'Hamdi et al. 2013). Plant RIPs also showed enhanced resistance when co-expressed with other

antifungal proteins, e.g. when rice basic chitinase (*RCH10*) and modified maize RIP (*MOD1*) were co-expressed in rice, it showed good resistance against *R. solani* (Kim et al. 2003). Transgenic plants of blackgram co-expressing chitinase gene from barley and RIP showed Corynespora leaf spot fungal growth inhibition (Chopra and Saini 2014). Transformation of RIP α-MMC gene into rice showed increased resistance to blast fungus (Qian et al. 2014). A transgenic potato lines expressing *PhRIP I* gene of *Phytolacca heterotepala* coding for a ribosome-inactivating protein was found to possess more resistance to *Botrytis cinerea* and *Rhizoctonia solani* fungal pathogens (Gonzales-Salazar et al. 2017).

9.8 Phytoalexin

Phytoalexin term was originally coined by Müller (1958) and they come under low molecular weight plant antibiotic group. These are naturally produced secondary metabolites that possess antimicrobial activity. These are produced naturally in plant cells as normal growth metabolites or can be induced in the presence of pathogen attack or other stress. They can inhibit bacteria, fungi, insects, nematodes, toxic against animals or plants itself. About 350 phytoalexins have been identified and characterized from 30 plant families, *Leguminosae* plant family produces maximum 130 phytoalexins. These phytoalexins are well diversified in the plant kingdom and are characterized among different classes of chemical compounds such as coumarins, diterpenes, flavonoids, alkaloids, phenolic compounds, luteolinidin, apigenidin, and apigeninidin. Pisatin was the first phytoalexin isolated and characterized from garden pea, *Pisum sativum* (Cruickshank and Perrin 1960). The molecules that signal plants to begin the process of phytoalexin synthesis are called elicitors. Elicitors of biotic origin may be involved in the interaction of plants and potential pathogens, whereas abiotic elicitors are not involved in normal host–pathogen interactions. Phytoalexins only showed resistance in a sufficient concentration which will be produced by one or more phytoalexins along with another component. Phytoalexins were biosynthesized by phenylpropanoid pathways mainly around resistant tissue and also in necrotic lesions. Also, the acetate-mevalonate and shikimate pathways are involved in flavonoid biosynthesis. These all pathways are interconnected and are involved in the synthesis of some important enzymes which play a crucial role in resistance such as chalcone isomerase (CHI), chalcone synthase (CHS), phenyl-alanine ammonia lyase (PAL) CoA ligase, and stilbene synthase. Overexpression of these potential enzymes shows resistance against different diseases. Stark-Lorenzen et al. (1997) expressed the stilbene synthase gene of grapevine in rice and found that disease resistance was increased. Resveratrol synthase and isoflavone methyltransferase gene was also expressed in alfalfa plants to increase disease resistance. Similarly, isoflavone reductase (*GmIFR*) isolated from soybean enhanced resistance against *Phytophthora sojae* in Soybean (Cheng et al. 2015).

9.9 Antibacterial Proteins

These are small-sized lytic peptides which are having amphipathic α-helical structure. These proteins produce pores in the bacterial cell membrane causing lysis of bacterial cells (Boman 1991). These antimicrobial proteins are produced by the different living organisms from bacteria to animals as defense proteins. Some of the antibacterial proteins which are transferred to plants to increase resistance against bacteria are summarized below;

9.9.1 Cecropins

These are positively charged antimicrobial peptides which are isolated from giant silk moth (*Hyalophora cecropia*) hemolymph. Cecropin term was given because of its source of isolation. They are proteinaceous in nature consisting of 31–39 amino acid residues and synthesized as lipid bodies in cells. Cecropin mainly lyses the cell membrane of bacteria, on interaction with bacterial membrane, it forms an α-helical structure interaction which causes ion channel formation. It also inhibits proline uptake and leads to leaky membranes. It acts as the main constituent of insects immune system of bacteria and at low concentration (0.1–5μM) inhibits many gram-positive bacteria as well as some gram-negative bacteria (Chen et al. 1997). Antibacterial activity of cecropins isolated from *Antheraea pernyi, Hyalophora cecropia*, and *Bombyx mori* has been demonstrated towards different genes (Jaynes et al. 1993; Sharma et al. 2000). *Agrobacterium*-mediated transformation of antimicrobial peptide cecropin P1 (cecP1) in rapeseed (*Brassica napus* L.) was done and it has shown that these transgenic plants showed resistance to the bacterial and fungal pathogens *Erwinia carotovora* and *Fusarium sporotrichioides* (Zakharchenkoa et al. 2020). Cecropin B isolated from Chinese tasar moth (*Antheraea pernyi*) has been expressed in transgenic citrus to eliminate the effect of Huanglongbing (HLB), associated with *Candidatus liberibacter asiaticus* bacteria (Zou et al. 2017).

9.9.2 Attacins

Attacins are also another type of antibacterial proteins that are much larger than cecropins, i.e. about 180–190 aminoacids. There are about six different types of attacins (A-F) that have been isolated from a moth, i.e. *H. cecropia*. These proteins, i.e. A-F attacins differ from each other because of the processing step during synthesis, protein from A-D constitute a basic group whereas E and F are acidic. Attacins are found to attack gram-negative bacteria but these proteins do not cause lysis but disrupt outer membrane structure. Attacin proteins are not broad spectrum like cecropins but it can inhibit the growth of some bacteria like *E. coli*, *Acinetobacter calcoaceticus*, and *Pseudomonas maltophilia* (Hultmark et al. 1983). Apples were transformed using cDNAs coding for attachin E which were coupled to plant promoters (Norelli et al. 1994). Transformed plants of a susceptible

apple rootstock N.26 possessed increased resistance to the fire blight pathogen *Erwinia amylovora* compared to the untransformed control, but were still more susceptible than the naturally resistant rootstock Liberty. Attacin expressed in transgenic potato enhanced its resistance to bacterial infection by *E. carotovora* subsp. *atroseptica* (Arce et al. 1999). Transgenic pear and apple expressing attacin genes have significantly enhanced resistance to *E. amylovora* in in vitro and growth chamber tests (Ko et al. 2000). Transgenic apple expressing attacin targeted to the intercellular space, where *E. amylovora* multiplies before infection, has significantly reduced fire blight, even in apple plants with low attacin production levels (Ko et al. 2000). Attacin A gene was transferred to citrus under the control of a phloem-specific promoter to control Huanglongbing disease (Tavano et al. 2019).

9.9.3 Lysozyme

This is a low molecular weight self-defense enzyme that was discovered in 1922 by Alexander Fleming. These enzymes come under antimicrobial proteins because they are hydrolytic in nature and attack the peptidoglycan layer of bacteria. It specifically cleaves between N-acetylmuramic acid and N-acetylglucosamine of cell wall peptidoglycan (Wohlkönig et al. 2010). The number of reports suggested that lysozyme can kill gram +ve and gram –ve bacteria. T4 lysozyme (T4L), human lysozyme, and Hen egg-white lysozyme (HEWL) are some of the classes of this antimicrobial protein gene, which have been cloned and transformed to different plants. Transgenic tobacco plants expressing these lysozyme genes were found to be more resistant against plant pathogenic bacteria (Trudel et al. 1995; Kato et al. 1998). *E. carotovora* causes soft rot disease in potato, resistance against this disease has been conferred in potato by expressing *T4L* gene from T4-bacteriophage (Düring et al. 1993). Fungal and bacterial growth was inhibited in transgenic tobacco expressing human lysozyme gene suggesting its potential use for controlling plant disease (Nakajima et al. 1997). A plant lysozyme was isolated from *Momordica charantia* L., which can be used further to increase bacterial resistance in plants. Resistance to these diseases could also be achieved by engineering potato with lysozyme gene (*chly*) from chicken (Serrano et al. 2000), and complete resistance was achieved by expression of the phage T4 lysozyme (Ahrenholtz et al. 2000).

9.10 Herbicide Resistance

Weeds are a major constraint to crop production because they compete for nutrients and other resources with the main crops, posing a serious threat to crops (Fartyal et al. 2018). Herbicide resistance is the most predominant trait that has been adopted for cultivating GM crops. In the early 1990s, GM crops resistant to broad-spectrum herbicides such as glyphosate and glufosinate have first been cultivated commercially. These GM crops are highly valued worldwide and have shown economic, social, and ecological benefits (Green and Castle 2010; Heap and Duke 2018). The

herbicide glyphosate is known to inhibit 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS), a key enzyme in the shikimate and phenylpropanoid pathway that is responsible for the biosynthesis of aromatic amino acids and several secondary metabolites. In 1996, the first glyphosate-tolerant soybean ("Roundup Ready") harboring *cp4epsps* gene was commercialized and many other commercialized crops harbor this gene. Glyphosate-resistant maize was introduced in the year 1998. Till now, maximum events, i.e. 210 have been approved for maize for herbicide resistance (ISAAA database 2020). Another important herbicide glufosinate also known as phosphinothricin inhibits glutamine synthetase (GS) enzyme that catalyzes the assimilation of ammonia with glutamate to form glutamine. The inhibition of glutamine synthetase leads to the assimilation of ammonia that inhibits photosystem I and II reactions and indirectly inhibits photosynthesis (Wang et al. 2018). Two glufosinate resistance genes *bar* and *pat*, were isolated from soil bacteria. Out of these two, pat (phosphinothricin N-acetyltransferase) gene was isolated from *Streptomyces viridochromeogenes* and *bar* gene from *S. hygroscopicus.* These genes encode resistance to phosphinothricin (PAT) and bialaphos (L-Alanyl-L-alanyl-phosphinothricin; Bar). Thus, researchers primarily focused on these two genes in basic research for the development of herbicide-resistant genetically engineered crops. In the past few years, the glyphosate and glufosinate resistant genes (*EPSPS* and *bar*) have been introduced into pepper, soybean, maize, millet, potato, and other crops (Zhao et al. 2020). Besides these two above-mentioned herbicides, transgenic crops specific to other herbicide groups such as 2,4-D (*aad*-1 and *aad*-12 genes), dicamba (*dmo* gene), isoxaflutole, mesotrione, oxynil, and sulfonylurea, have been commercialized recently. Currently, different multinational companies such as Monsanto, Dow, Bayer, Syngenta, and BASF are developing new herbicide-resistant traits in different crops. *Agrobacterium*-mediated gene transfer technology has been used to introduce the *EPSPS* and *bar* genes, which confers resistance to glyphosate, and glufosinate into castor (Zhao et al. 2020). Maize, soybean, cotton, and canola are among the most widely grown commercial crops that confer herbicide resistance (Brookes and Barfoot 2015).

9.11 Virus Resistance

Plant viruses have been a major threat to agricultural production and with the advent of transgenic technology, there has been a revolutionary enhancement in the production of GM crops resistant to various plant viruses through various strategies. Significant resistance to a variety of plant viral diseases has been accomplished by gene silencing techniques. Bonfim and co-workers in 2007 explored the concept of using an RNA interference construct to silence the sequence region of the *AC1* viral gene and generated highly resistant transgenic common bean plants (Bonfim et al. 2007). This method has also been adopted for the engineering of plum pox virus resistance (Scorza et al. 2013). Exceptional progress has been perceived for transgenic alfalfa, melon, potato, rice, tomato, and tobacco against a broad spectrum of plant viruses, including alfalfa mosaic virus, cucumber mosaic virus, potato virus X

(PVX), potato virus Y (PVY), and potato leaf roll virus (Parray et al. 2019). In recent years, non-coding RNAs (ncRNAs) have gained unprecedented attention for regulating cellular processes and engineering plant virus resistance (Taliansky et al. 2021).

9.12 Abiotic Stress Tolerance

Variable photoperiod, nutrient deprivation, starvation, drought, high salt conditions, temperature, and osmotic changes are some of the situations that contribute to abiotic stress (Hirayama & Shinozaki 2010; Zhu 2016). These are of critical importance as studies have estimated the loss caused to reach around 50% by 2050 by this kind of stress situation (Bengtsson et al. 2006; Ahuja et al. 2010; Thakur et al. 2010; Lobell and Gourdji 2012). Furthermore, it is also estimated that the frequency of drought, salinity, and heat will increase in the coming years (Easterling et al. 2000; Bernstein et al. 2008), standing as a challenge to agriculture production. Plant encounters various kinds of abiotic stresses that contribute to a significant crop loss worldwide. All the abiotic stresses are interconnected and include the following.

9.12.1 Salinity

High salt situations in the soil and drought are two main abiotic stresses affecting around 20% of the crop losses in irrigated fields (Qadir et al. 2014). The ions that contribute to high salinity situations are Na^+, Ca^{2+}, Mg^{2+}, K^+, Cl^-, $SO4^{2-}$, $HCO3^-$, $CO3^{2-}$, and NO3 (Flowers and Flowers 2005). The accumulation of either a single type of ions or more than one type of ion may result in a condition that is termed salination. High saline conditions can be caused due to various reasons like mineral weathering, precipitation followed by the movement of salt towards land surfaces are some primary causes of the salinity. High salt has a variable effect on crop plants, though the primary effect includes osmotic stress and toxicity. These primary effects may lead to another secondary effect on the cell-like compromised cell and membrane expansion thereby a compromised metabolism. It is quite difficult to assess which factor contributes to salinity tolerance in crops. It is conceivable that the proper correlation studies between a trait and salinity tolerance could help in easing out the direct relationship between the effects of any salt on crop productivity. In past, many studies have been proposed to characterize the response of any biological molecule, e.g. transcription factors and biomolecules (Negrão et al. 2017) towards any kind of salt stress, however, these studies are still very limited. We propose that engineering the biomolecules to produce stress-resistant crops could be a promising approach because they all play a significant role in the plant's adaptation to variable salt conditions. Some of the development in major food crops has been described in the coming sections.

9.12.2 Heat

Global warming has led to a significant increase in temperature. It has been estimated that average temperature rise by 3–4 °C has estimated to increase the crop losses to 15–35% in tropical regions like Africa and Asia and by 25–35% in the Middle East (Ortiz et al. 2008). Despite a significant increase in global food production, a food deficit still survives as global cereal production is declining because of increasing temperature (Fischer and Edmeades 2010). The most common effect of high temperature on any crop is an effect on their overall reproductive development due to pollen infertility (Zinn et al. 2010). Heat stress is a major abiotic stress that plant gets exposed in nature and affects every stage of plants life, however, the variability could be seen in the patter they affect the plant that differs from species to species (Sakata and Higashitani 2008). The noticeable effects of the increase of temperature on plants are leaf and stem scorching, abscission, and senescence, inhibition of root and shoot growth, finally causing the fruit damage and results in a decrease in plant productivity (Vollenweider and Günthardt-Goerg 2005). In certain cases, high temperature may also result in changes in plant architecture like elongated hypocotyls and petioles (Hua 2009). Like any other stress, in response to heat stress, plants undergo a series of cellular and metabolic responses that are necessary to survive in these high-temperature situations. This includes changes in a cellular organization like changes in cytoskeleton and membrane functions. These structural changes are also accompanied by production transcription faction producing biomolecules like heat shock proteins (HSPs) (Bray et al. 2000), and the production of biomolecules like phytohormones (abscisic acid; ABA) and antioxidants (Maestri et al. 2002).

9.12.3 Drought

Drought is a situation where the plant does not have a sufficient amount of water which is necessary for the optimum growth of the plant. Various reasons responsible for this kind of stress are low rainfall and compromised irrigation conditions. Drought has a variety of effects on plant growth. The first is a lack of germination and seedling establishment in various crops (Ashraf and Harris 2004; Kaya et al. 2006). It has been reported that in rice, drought during a vegetative state severely compromises overall plant growth (Manickavelu et al. 2006). Drought situations are often associated with compromised photosynthetic activity and finally wilting, thereby compromising plant yield which could be followed by plant death.

9.12.4 Cold

Sensitivity to low temperature is another important abiotic stress that is of critical importance contributing towards the significant yield loss in crops. Cold affects plant life in different aspects, however, an ability to tackle this kind of stress may lead to

cell death. Plant encounters temperature fluctuations in the natural environment and thus need a different mechanism to different responses to minimize cellular damages. Response to cold may involve an initiation of a signaling cascade to cause metabolic changes significantly adding to the increased tolerance to chilling temperatures (Chinnusamy et al. 2003). These changes are quite beneficial to plant because these changes ease a plant to cope up with cold stress. These changes/processes induce upon stress are collectively termed as "low temperature-induced signal transduction (LTST)." LTST leads to the upregulation of certain genes that produces specific proteins that help is an adaptation to freezing temperatures. From the above sections, it is clear that any kind of abiotic stress on plants induces a series of adaptive responses in the plant that are quite common in every kind of stress.

9.13 Genetic Engineering for Stress-Tolerant Properties

It is conceivable that the introduction of these alien/novel stress-responsive genes into plants to bring a stress-tolerant property is a promising approach to tackle these stress-sensitive phenotypes (Mittler 2002; Mittler and Blumwald 2010). Therefore, an extensive part of the current genetic research has been dedicated to producing stress-resistant plants by this kind of gene introduction. With the advancement of genetic engineering technology, the cloning and overexpression of stress-resistant genes has become an easy task. The conventional *Agrobacterium*-mediated gene introduction is the common method for gene introduction into the host plant. Besides, the *Agrobacterium*-mediated transformation other non-agrobacterium species that have been identified for genetic transformation in plants are *Rhizobium* sp. NGR234, *Sinorhizobium meliloti,* and *Mesorhizobium loti*. Herein, we describe some important genes/proteins that have been identified and modified in plants for their stress-responsive roles. Depending upon the type of response they belong to; these genes can be divided into two major groups; ones involved in cellular protection (osmoprotectants, membrane stabilization, detoxification), transcription factors, and signaling molecules (Vendruscolo et al. 2007).

9.14 Abscisic Acid (ABA) Response Genes

Abscisic acid is one of the most important plant hormones that serves variable functions in the plant. ABA is an important messenger that is involved in different adaptive responses like regulation of accumulation of osmolytes, LEA (Late Embryogenesis Abundant) protein synthesis, and antioxidant enzymes (Chaves et al. 2003; Verslues et al. 2006). ABA levels in plants increase in response to different stressors and result in stomatal closure to minimize the water loss occurring due to the process of transpiration from leaves. Different cellular responses are largely dependent on the ABA levels (Sreenivasulu et al. 2012), thus conceivable that engineering this trait for crop improvement could be a promising approach. *ERA1* is one such gene that has been identified in *Arabidopsis*, and ß-subunit of a

farnesyltransferase. It has been shown that plants that do not have this gene are shown to have increased drought tolerance. This has been also shown that downregulation of *ERA1* by expressing under a drought inducible promoter through antisense expression of *ERA1* in *Arabidopsis* and canola (Jalakas et al. 2017). Similarly, the mutants for ABA receptors like pyrabactin resistance 1-like 1 (*pyl1*), *pyl4*, and *pyl6* in rice have been shown to improve plant improvement for drought resistance (Miao et al. 2018). Following a similar strategy, a Canadian company is developing new transgenic plants under the name Yield Protection Technology™ and it has developed transgenic plants for maize, soybean, and cotton since 2011.

9.15 Gene Encoding Compatible Solute

Stress-induced biosynthesis and accumulation of various organic metabolites is the common and most effective defense mechanism plant display in response to any kind of stress. Osmoprotective adaptation to a stressful situation is a widespread response that is conserved in all kinds of living organisms (Saxena et al. 2013). These solutes also act as scavengers for free radicals and stabilize the plant proteins during stress (Nahar et al. 2016). These compounds have low molecular weight and do not inhibit normal cellular functions and are termed as compatible osmolytes. These chemicals are fundamental to all organisms from bacteria to plants and can be characterized into different categories depending on their biochemical nature (Khan et al. 2009; Jewell et al. 2010) (Table 9.1). The majority of these proteins are hydrophilic and uncharged in nature and function in a vast variety of functions including scavenging of the ROS (reactive oxygen species), as osmoprotectants, pH stabilizers, proteins, enzymes, and membrane. The first report of this type of gene to plant lies in the early 90s, where the introduction of these osmolytes has shown to confer the cold and salt resistance properties to the host plants. Glycine betaine, β-alanine, proline, and mannitol are some common compatible solutes that are conventionally used for metabolic engineering for stress-resistant properties. However, glycine betaine is regularly used for this purpose in different crops. Various transgenic plants that have been produced by this kind of gene introduction are listed in Table 9.2.

Table 9.1 Categories of plant protectants based upon their biochemical nature

S. no.	Categories	Sub-groups
1.	Amino acids	Proline, glutamate, glutamine, alanine
2.	Amino acid derivatives	Ectoine, hydroxyectoine
3.	Quaternary amines	Glycine betaine, polyamines, dimethyl sulfoniopropionate, DMSP
4.	Sugars	Trehalose, sucrose
5.	Polyols including sugar alcohols	Mannitol, sorbitol, galactinol

Table 9.2 Recent examples of the plant development through gene modification in agriculturally important crops

S. no.	Gene	Type of gene	Improved tolerance	References
Rice (*Oryza sativa*)				
1.	*Si*MYB56	R2R3-MYB transcription factor	Drought	(Xu et al. 2020)
2.	PheASR2	Transcription factor	Drought	(Wu et al. 2020, p 2)
3.	*Jc*MADS40	MADS-box family genes	Drought and salt	(Tang et al. 2020)
4.	*Os*ARD1	Acireductone dioxygenase (ARD) metal-binding protein family	Drought and salt	(Liang et al. 2019)
5.	*Os*ZFP350	Zinc finger protein	Heat, salt and drought	(Kang et al. 2019)
6.	*Os*MYB6	MYB family gene	Drought and salt	(Tang et al. 2019)
7.	*Os*JMJ703	Rice histone demethylase gene	Drought	(Song et al. 2018)
8.	*Os*CTZFP8	Zinc finger transcription factor	Cold	(Jin et al. 2018)
9.	*Zm*PIF3	Phytochrome-interacting factors (PIFs)	Drought	(Gao et al. 2018)
10.	PYL3	Pyrabactin resistance-like (PYL) gene family	Cold and drought	(Lenka et al. 2018)
11.	*Os*JAZ1	JAZ (JASMONATE ZIM-domain) proteins	Drought	(Fu et al. 2017)
12.	*Os*MAPK3	MAPK family	Cold	(Zhang et al. 2017)
13.	*Os*bZIP46	bZIP transcription factor	Drought and temperature stress	(Chang et al. 2017)
14.	*Os*LOL5	Zinc finger proteins (ZFPs)	Alkaline and salt	(Guan et al. 2016)
Wheat (*Triticum aestivum*)				
15.	*Ta*HsfA6f	Heat shock factors (Hsfs)	Salt	(Bi et al. 2020)
16.	*Ta*OAT	Ornithine amino transferase	Salt	(Anwar et al. 2020)
17.	*Ta*DREB3	DREB transcription factors	Heat, cold and salt	(Niu et al. 2020)
18.	*At*WRKY30	Transcription factor	Heat and drought	(El-Esawi et al. 2019)
19.	ERF1-V	AP2/ERF transcription factor	Salt and drought	(Xing et al. 2017)
Barley (*Hordeum vulgare)*				
20.	*Ta*HsfA6bT	Heat shock factor	Heat	(Poonia et al. 2020)

(continued)

Table 9.2 (continued)

S. no.	Gene	Type of gene	Improved tolerance	References
21.	*Hv*MYB1	Transcription factor	Drought	(Alexander et al. 2019)
22.	OSM	Osmotic response gene	Salt	(Viktorova et al. 2019)
23.	*Hv*SHN1	Ethylene responsive transcription factor	Salt and drought	(Djemal et al. 2018)
24.	*At*VHA-C	Vacuolar ATPase subunit C	Salt	(Adem et al. 2017)
25.	CPK2a	Calcium-dependent protein kinase	Drought	(Cieśla et al. 2016)

9.16 Free Radicle Scavengers

Exposure to any kind of stress situation finally leads to the production of reactive oxygen species, production of which negatively affects different processes like enzyme and biochemical activities and thus affects the biosynthesis of DNA, protein and carbohydrates, thus exceeding the oxidative stress in a cell. The exposure of plants to these environmental stresses leads to reactive oxygen species (ROS) (Verslues et al. 2006; Jewell et al. 2010). Production of the free radicle upon stress situation is another phenomenon of the stress response that is seen in plants upon waterlogging, drought, high salinity, and high temperatures. It is evident that ROS influences the expression of different genes that influence growth, cell cycle, response to pathogens, plant development, and even cell death (Gill and Tuteja 2010). Thus, modification and expression of the gene helping in scavenging these reactive oxygen species could be an alternative approach to produce stress-tolerant plants. Some of these genes that have already been shown to be successfully introduced in plants for imparting stress-tolerant properties are; ascorbate peroxidase, superoxide dismutase, and glutathione reductase.

9.17 Genetic Engineering of the LEA Proteins Coding Genes

The late embryogenesis abundant (LEA) protein is a stress-induced protein produced in vegetative tissues of the plants. Although the exact functions of these stress-induced proteins are not known, it has been known that these proteins are water-binding molecules that are dehydration and cold-responsive. These are the proteins that help in protecting the desiccation and protect the seed development during salt stress, dehydration, and cold. These proteins are encoded by different genes in different plant species, for instance, responsive to dehydration (RD), early responsive to dehydration (ERD), inducible to cold (KIN), regulated by cold (COR), and responsive to abscisic acid (RAB). Two members of this class of proteins are HVA1

(protein from barley) and LE25 (protein from tomato). The reports have shown that the introduction of the *LEA* gene from barley into rice imparts tolerance to salinity and water stress (Xu et al. 1996). A similar observation was made in yeast transformed with *LE24* from tomato (Imai et al. 1996). Similarly, expression of the *HVA* gene in wheat has also been reported to improve the quality of plants to grow in water-deficient situations with an ability to produce high biomass. A recent example of this kind of gene introduction for stress resistance was achieved by expressing Melon *Y3SK2*-Type *LEA* gene in tobacco. A recent study has shown that the introduction of this gene increases resistance to drought and salt (Aduse Poku et al. 2020).

9.18 Molecular Chaperones

Heat shock proteins (HSPs) are the to-date known molecular chaperons that help incorrect folding of the proteins. Besides these, conventions protein folding molecules other protein folding molecules that have been extensively studied are known as peptidyl-prolyl-isomerases. It has been reported that HSP is produced in response to rapid heat stress. Besides HSP's are also known to get produced under different stages of plant development like embryogenesis, seed germination, development of pollen, and fruit maturation (Prasinos et al. 2005). The modification of these molecules for developing stress-tolerant plants lies early in 1987, when the transgenic tobacco expressing HSP17 were produced, though no conclusion was made on the stress-tolerant property as a promoter was incompetent (Schöffl et al. 1987).

9.19 Proteins for Ion Homeostasis Across the Membrane

A high salt situation causes osmotic stress in the cell and leads to an increase in the high salt situations in the cytoplasm. To counter this, plant has to develop a mechanism where a plant can utilize these ions to minimize the adverse effects of these ions in the cell. This is regulated by genes that regulate the ion channels like Na^+/H^+ antiports and stress signaling through calcium- and calmodulin-dependent protein phosphatase calcineurin. The first successful example of this kind of gene introduction was achieved by overexpressing a single endogenous gene (*AtNHX1*) encoding a vacuolar Na^+/H^+ antiport protein in *Arabidopsis*. The transgenic Arabidopsis was found to be thriving well in the high salt situation, i.e. 200 mM sodium chloride, which correlated well with higher levels of the *AtNHX1* transcripts and protein and vacuolar Na^+/H^+ antiport activity. Similarly, expressing catalytic and regulatory subunit of yeast calcineurin in tobacco has also been shown to generate transgenic tobacco with salt-tolerant properties.

9.20 Transcription Factors

A number of genes that are activated upon abiotic stress are controlled by a complex network of transcription factors (Yamaguchi-Shinozaki and Shinozaki 2006). The stress-responsive genes can generally be classified into two groups; regulatory and functional (Shinozaki et al. 2003). Functional proteins include different enzymes, membrane proteins (water channel and transporters), heat shock proteins, all these proteins have a direct role in stress response. Regulatory proteins include different transcription factors, kinases, and phosphatases that regulate various stress responses. Furthermore, a response to any kind of stress on the plant is multigenic in nature, involving the role of two or more genes in a stress response mechanism. Thus, the introduction of only a single gene is not sufficient to induce a series of changes that may be necessary for a specific stress adaptation. Thus, engineering the different transcription factors involved in controlling specific traits together may be a promising alternative approach to achieve a specific stress-tolerant trait. Some successful initial examples of this kind of gene modification have been achieved by the overexpression of the HSPs have known to confer thermotolerance in *Arabidopsis* (Lee et al. 1995). Similarly, overexpression of the *CBF1* ("C-repeat binding factor"), a transcriptional activator has been shown to confer tolerance to freezing in *Arabidopsis* inducing the expression of four *COR* ("cold-regulated") genes (Jaglo-Ottosen 1998). Another transcription factor is the dehydration-responsive Element (*DREB1A*), this TF is also known to upregulate different stress-responsive genes. Both DRE and CBF factors are known as a cis-acting element that regulates the gene expression in response to variable dehydrating stressors (salt, cold, and drought) (van Rensburg and Krüger 1994). A member of the DRE family, *DREB1A*, is reported to impart drought resistance in *Arabidopsis thaliana* via inducing the expression of different stress-responsive genes (Pellegrineschi et al. 2003). Another family of a transcription factor that is involved in stress tolerance is the NAC gene family members. These transcription factors are known to get expressed in different stages of growth and response to the environment. SNAC1 is a member of this family, expression of which is known to improve drought resistance in rice (Hu et al. 2006). Other transcription factors like *bHLH*, *bZIP*, *NAC*, *AP2/ERF*, *MYB*, Zinc finger, *WRKY*, and kinases are associated with increase crop yield in rice (Dubouzet et al. 2003; Hu et al. 2006; Hossain et al. 2010).

9.21 Recent Advances in Plant Improvement for Abiotic Stress Tolerance

In the above section, we have successfully described the traditional transgenic methods to crop improvement. The current section focuses on the recent advancement that has been made in crop development programs to improve plant for the desired traits.

9.22 Development of Abiotic Stress-Tolerant Crops by miRNA

MicroRNA (miRNA) can be described as the single-stranded RNAs that are approximately 21–24 base pairs in length (Zhao et al. 2011). Studies have shown an involvement of these microRNAs in imparting stress resistance to variable stress on plants (Xia et al. 2012). These miRNAs inhibit the expression of target RNA by binding to the 3′ end of the RNA, thus inhibiting the translation (Meng et al. 2010; Li et al. 2011; Ding et al. 2011). These miRNAs are involved in different cellular processes like transcription, protein stability, and degradation (Shen et al. 2010; Ding et al. 2011). Several studies have shown that 11 miRNAs exert tissue-specific expression towards major abiotic stresses in *Arabidopsis thaliana*. According to the reports in *Arabidopsis thaliana*, miRNA-169 has been shown to contribute towards drought resistance. Similarly, other miRNA's like 159, 396, and 393 also showed to contribute to other abiotic stressors (salinity, cold, and heat) (Table 9.3).

Table 9.3 Recent examples of the plant development through miRNA technology

S. no.	Targeted miRNAs	Transgenic plant	Response	Reference
1.	miR398	Wheat	Cold tolerance	(Lu et al. 2020)
2.	MiR319	Rice	Salt stress tolerance	(Liu et al. 2019)
3.	miR393a	Creeping bentgrass	Salt, drought and heat tolerance	(Zhao et al. 2019)
4.	miR166	Rice	Drought tolerance in knocked-down mutants	(Zhang et al. 2018)
5.	miR5144	Rice	Salinity and mercury stress tolerance	(Xia et al. 2018)
6.	miR827	Maize	Drought tolerance	(Ferdous et al. 2017)
7.	miR159	Rice	Increased drought resistance	(Zhao et al. 2017)
8.	miR156	Rice	Reduced cold tolerance	(Cui et al. 2015)
9.	miR408	Chick pea	Enhanced drought tolerance	(Hajyzadeh et al. 2015)
10.	miR319	Bentgrass	Enhanced salt and drought tolerance	(Zhou et al. 2013)
11.	miR319	Rice	Enhanced tolerance to chilling stress	(Yang et al. 2013)
12.	miR395	Rapeseed	Enhanced tolerance to oxidative stress and heavy metal stress	(Zhang et al. 2013a, b)
13.	miR828	Sweet potato	Oxidative stress tolerance	(Lin et al. 2012)
14.	miR169	Tomato	Enhanced drought tolerance	(Zhang et al. 2011)

9.23 Development of Abiotic Stress-Tolerant Crops by CRISPR (Lustered Regularly Interspaced Short Palindromic Repeats)/Cas9

CRISPR-Cas9 is a recent development developed for crop improvement in recent years. This system depends on an RNA-DNA recognition system that employs a double-strand break in the host genome. The technology has an upper hand over the other crop improvement methods as this is a comparably fast, and efficient gene-editing method for crop improvement (Mao et al. 2013). Besides, it is preferred over the other gene-editing tools as this is simple in designing and efficiently introduces mutation with a targeted introduction at desired locations (Ma et al. 2015; Malzahn et al. 2017). The technique is preferred over other genetic improvement techniques as this is comparable to less tricky and avoids the tedious screening of the desired clone. Besides mutation, this technique can also be used to induce or repress the expression of the particular gene using modified CRISPR where an inactive form of Cas9 (dCas9) is fused with a transcriptional activator or a repressor (Bortesi and Fischer 2015). Due to its vast potential in genome improvement, it has the potential to replace other gene improvement methods. While the system is employed for the improvement of animal cells (Gilbert et al. 2013; La Russa and Qi 2015); the system has limited reports of successful use of this method in plant improvement (Piatek et al. 2015). Some recent examples of CRISPR-Cas mediated plant improvement are summarized in Table 9.4.

9.24 Conclusion

Overall, we have summarized the major biotic and abiotic stress mechanisms that can be engineered for imparting stress-resistant properties to different plants. Though, a huge effort has been already made in this area, there is a considerable challenge that remains to be addressed. As the plant undergoes multiple combinations of stress in field conditions, this area needs further addressing. We propose that the plant's response to multiple different stressors cannot be inferred from assessing the plant's response to individual stress. Thus, it is essential to test different improved varieties to multiple stressors in field conditions where all stressors occur at once. Another challenge to getting these improved varieties to farmers is their development expenses and the approvals these GM plants require for

Table 9.4 Some recent examples of crop improvement via employing CRISPR/Cas9 system

S. no.	TFs	Species	Response	Reference
1.	SST	Rice	Seedling salt-tolerant gene	(Lian et al. 2020)
2.	*Os*MYB30	Rice	Cold tolerance gene	(Zeng et al. 2020)
3.	*Os*GA20ox2	Rice	Lodging resistance	(Nawaz et al. 2020)
4.	ANAC069	Arabidopsis	Salt and osmotic sensitivity	(He et al. 2017)
5.	*Zm*WRKY17	Maize	Salt sensitivity	(Cai et al. 2017)

their field trials. As multiple precautions are already in the guideline to ensure the safety associated with the GM crops, precautions to ensure this safety should not become a barrier for future crop development programs.

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Production of Marker-Free Transgenic Plants

10

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Abstract

During the process of genetic transformation of crop plants with the desired transgene(s), certain selectable marker gene(s) are also employed for the selection of transgenic events. Normally, selective agents like antibiotics and/or herbicides and their corresponding resistant gene(s) are exploited for introducing agronomically important traits/genes into plants. Although these selection genes are imperative for efficient screening, they exhibit regulatory and public concerns. The marker genes present in commercial transgenic crops may be transmitted to pathogenic microbes in the gastrointestinal region/soil or weeds, making them tolerant to antibiotics or herbicides, respectively. Further, sexual breeding of transgenic plants evokes the issue of transgene expression since redundancy of transgenes in the DNA may induce homology-based gene silencing. Therefore, generation of a marker-free transgenic system has become a subject of paramount importance so as to maintain sustainability. Hitherto, various approaches for elimination of marker gene(s) from nuclear as well as chloroplast genomes (co-transformation-segregation, non-selected transformation, site-specific transformation, homologous recombination, transient co-integration) have evolved. In the present chapter, we describe the different marker excision strategies along with their merits and demerits. In addition, we discuss various developments made in marker-free technology and suggest possible directions for their safe and maximum usage in the coming future.

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Keywords

Biosafety · Marker gene elimination · Transgenic plants · Co-transformation · Site-specific recombination · Homologous recombination

10.1 Introduction

Genetic engineering of crop plants introduces a foreign gene(s) of interest that instills a desired trait in the plant machinery and a marker gene(s) that assists in the identification of the subsequent transformed cells. Selectable marker genes (SMGs) are dominant genes used to provide resistance to a particular antibiotic or herbicide that kills non-transgenic cells. The transformation efficiency for most crops is usually low; therefore, marker genes are used in almost all plant transformation procedures to differentiate among transformed and non-transformed cells. Table 10.1 showcases some of the selectable marker genes that are employed for plant transformation studies.

Once the transformed cells are selected, SMGs lose their utility. Further, the prolonged existence of marker genes in genetically modified (GM) crop plants may

Table 10.1 Marker genes used for transformation of plants

Marker gene	Gene product	Selective agent	References
Neo *nptII*	Neomycin phosphotransferase II	Kanamycin, neomycin, paromomycin, geneticin (G418), amikacin	Fraley et al. (1983)
aadA *nptIII*	Aminoglycoside-3-adenyltransferase Neomycin phosphotransferase III	Streptomycin, spectinomycin, neomycin, geneticin (G418), paromomycin, amikacin, kanamycin	Hare and Chua (2002)
hph (*aphIV*)	Hygromycin phosphotransferase	Hygromycin B	Waldron et al. (1985)
gox	Glyphosate oxidoreductase	Glyphosate	Barry et al. (1992)
cah	Cyanamide hydratase	Cyanamide	Weeks et al. (2000)
pat, *bar*	Phosphinothricin acetyl transferase	Phosphinothricin	De Block et al. (1989)
SPT	Streptomycin phosphotransferase	Streptomycin	Maliga et al. (1988)
AK *dhfr* *sul*	Aspartate kinase Dihydrofolate reductase Dihydropteroate synthase	High concentration threonine and lysine Methotrexate Sulfonamide	Yoder and Goldsbrough (1994)
EPSP synthase	5-Enolpyruvylshikimate-3-phosphate synthase	Glyphosate	Zhou et al. (1995)

evoke public and regulatory concerns and also have certain technological drawbacks like detection of pleiotropic effects and gene silencing (Chong-Pérez and Angenon 2013; Tuteja et al. 2012). Five major concerns related to genetically modified crops are (1) impact on natural ecosystems, (2) food safety, (3) gene transfer into non-target crops, (4) religious/moral issues, and (5) corporate control of food supply. Further, horizontal flow of antibiotic tolerance genes to human and animal gastrointestinal region and vertical flow of herbicide tolerance genes to wild/weedy relatives are considered as the main biosafety risks in GM crop plants (Dale et al. 2002).

Nonetheless, not all researchers are in favor of these theories. In fact, the lack of sufficient scientific proof regarding the safe nature of marker genes has hindered public acceptance of GM crops and their products to a considerable extent (https://www.isaaa.org/resources/publications/pocketk/36/default.asp). Thus, complete elimination of selectable marker genes is a better option so as to prevent any concerns related to GM crops. Besides, the elimination of marker genes in GM plants may also slash the costs incurred for generating transgenic plants and curtail the need for safety assessments that are time consuming, thereby hastening the commercialization of new transgenic plants.

10.2 Techniques to Develop Marker-Free Transgenic Plants

10.2.1 Excision of Selectable Marker Genes from the Nuclear Genome

10.2.1.1 Co-transformation

Since the advent of transformation technology, many approaches to get rid of selectable marker genes have been reported. One of the earliest and simplest elimination strategies is the co-transformation method. This technique is based on transformation of the gene of interest (GOI) and the selectable marker gene (SMG) using two independent plasmid vectors or binary vectors that deliver both the genes to unlinked genomic loci. Consequently, the SMG separates from the GOI by virtue of segregation and recombination that occur over sexual crossing thereby enabling selection of progenies possessing only the transgene of interest and not the marker. There are at least two key parameters, which must be deliberated to establish a functional co-transformation system: (1) sufficiently high co-transformation proficiency and (2) high frequency of non-linked integration of T-DNAs in the plant genome. Other factors such as the plant species, type of initial plant material, DNA construct, and stringent tissue culture protocol are equally imperative for designing an efficient co-transformation experiment. *Agrobacterium*- mediated system is potentially useful for this purpose since multiple T-DNAs can be readily integrated into plant cells either via a single-strain method (by utilizing a single *A. tumefaciens* strain) or mixture method (from a mixture of *A. tumefaciens* strains), and the likelihood of distinguishable integration events is fairly high as compared to direct gene transfer methods.

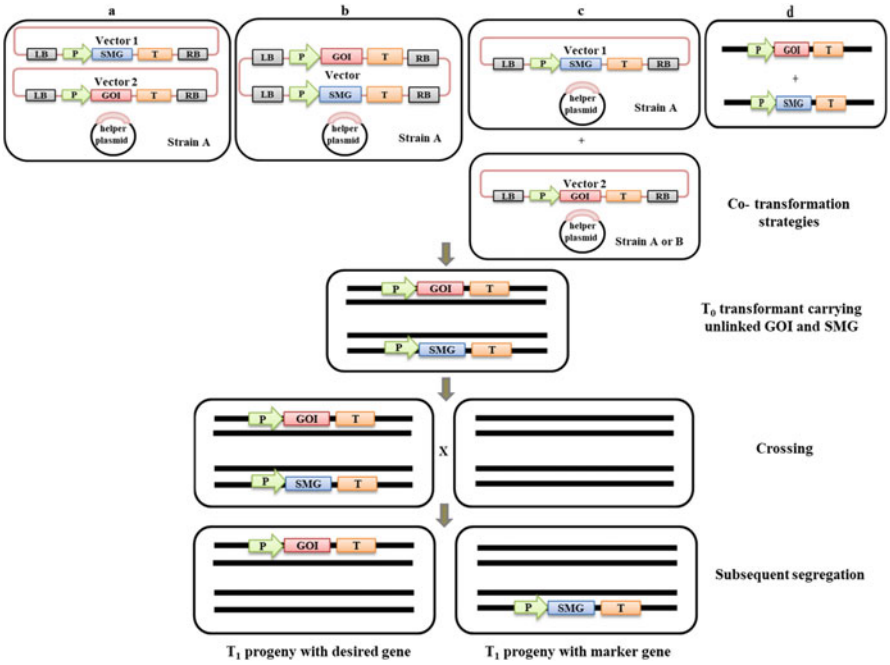

Fig. 10.1 Co-transformation approach to eliminate selectable marker genes. The SMG and GOI are introduced either on individual constructs (**a**) or on the same construct (**b**) in a single Agrobacterium strain, or on two separate plasmids present in two different *Agrobacterium strains* (**c**); the two genes can also be delivered directly through particle bombardment (**d**) (Chong-Pérez and Angenon 2013) *Note: LB* left border of T-DNA, *P* promoter, *SMG* selectable marker gene, *GOI* gene of interest, *T* terminator, *RB* right border of T-DNA

Different co-transformation strategies: The co-transformation system operates on the following strategies.

1. Use of a single *Agrobacterium* strain, either harboring two separate binary vectors, one containing the GOI and the other the marker gene (Fig. 10.1a) (de Framond et al. 1986; Daley et al. 1998) or carrying a single binary construct enclosing two different T-DNAs (Fig. 10.1b) (Depicker et al. 1985; McCormac et al. 2001).
2. Use of two separate *Agrobacterium* strains each with a binary construct bearing one T-DNA region (Fig. 10.1c) (McKnight et al. 1987; Poirier et al. 2000).
3. Sometimes direct delivery of the two genes via particle co-bombardment (Fig. 10.1d) (Shiva Prakash et al. 2009).

Even though outcomes of a few co-transformation experiments have demonstrated high efficiency of the single-strain method over mixture method (Depicker et al. 1985; Komari et al. 1996), the overall merits of these strategies remain elusive (Komari et al. 1996). Moreover, marker-free transgenic plants have

been developed by co-transforming plant DNA (P-DNA) carrying GOI and conventional T-DNA bearing SMG, thus manifesting that plant-derived P-DNA fragments can be employed in place of universally utilized *Agrobacterium* T-DNA in transformation practices (Rommens et al. 2004). Furthermore, Jeongmoo et al. (2004) improvised co-transformation strategy by including the negative selection step wherein positive (*GUS* gene) and negative (*codA* gene) SMGs placed in the same binary plasmid were inserted into *N. tabacum* cv. Samsun NN, followed by screening of transformants for presence of desired transgene and absence of negative marker gene (*codA*) by PCR analysis and GUS staining. The results depicted independent segregation of *codA* gene of the *GUS* gene. Therefore, co-transformation with negative SMGs was found to be a feasible approach to produce SMG-free transgenic plants. This modus allows experimenters to lessen their search for marker-free transgenic plants without having to perform extensive molecular analyses such as thousands of PCRs.

The system offers certain advantages: (a) high adaptability of standard *Agrobacterium*- mediated gene delivery methods, (b) promising transformation frequencies of 30–50% (McCormac et al. 2001) which even reached up to 85% in some cases (De Block and Debrouwer 1991), and (c) convenient handling of separate T-DNA constructs with regard to linked DNA fragments. However, it also has few disadvantages: (a) often both the T-DNAs exist as linked copies, thus making their segregation impossible, a phenomenon quite common while performing particle co-bombardment; (b) considering that the strategy centers around genetic segregation of genes, it cannot be employed in case of sterile and vegetatively-bred crops, and is infeasible in plants with long life span such as transgenic trees; (c) Also, co-transformation method calls for an abundant generation of transformants (to identify non-linked transgene loci), and further breeding procedures (to remove SMG) making it a cumbersome process.

10.2.1.2 Total Exemption of SMGs from Selection of Transgene Events

A direct strategy to generate marker-free transgenics is to circumvent selectable marker genes during plant transformation experiments. Since the transformants harbor T-DNA(s) with known sequence, identification of transgenic events among the non-transgenic ones using molecular techniques like polymerase chain reaction (PCR) is quite feasible. This approach was employed by Aziz and Machray (2003), wherein they identified tobacco transgenic plants via GUS assay and stated a collective transformation and expression rate of 15%. De Vetten et al. (2003) developed marker-free transgenic potato and cassava plants using *A. tumefaciens* strains. PCR analysis of the putative transgenic shoots revealed a transformation frequency of 1–5%. However, the authors also reported <2% chimeric transformants. Likewise, 2.2–2.8% tobacco plants were genetically modified using an effective binary construct under no selection pressure by Li et al. (2009). The work also demonstrated 28–56% chimerism in transgenic tobacco plants, which was mainly attributed to dependence of tobacco transformation system on shoot organogenesis process wherein a single shoot can arise from multiple cells and the leaf tissues obtained from such shoots comprise various layers. Of these layers, L2 is

responsible for generating male and female germ lines, and when L2 layer originates from non-transformed cells it normally produces chimeras. The method of non-selected transformation has been used in different plants such as citrus (Domínguez et al. 2002), barley (Holme et al. 2006), wheat (Doshi et al. 2007), *A. thaliana* (Kim and Veena 2007), white pine (Tang et al. 2007), etc. Although there are scientific proofs pertaining to attainment of transgenics via this approach, complete exemption of marker genes also has its downsides, viz. tedious transformation process due to presence of many non-transformants among the examined plants and applicability of the method in a limited number of crop species.

10.2.1.3 Excision of Marker Genes Via Site-Specific Recombination

Site-specific recombination is a biological phenomenon wherein DNA strand interchange takes place between segments having some degree of sequence homology (Bode et al. 2000). This repositioning of DNA segments is mediated by microbial site-specific recombinases, which cut DNA backbone at definite recognition sites, exchange the two target strands, and ligate the helices back together. The recombinase recognition (R) sites are usually 30–200 base pair long, and comprise two palindromes (to which the enzyme attaches) which flank 6–8 core nucleotides (where recombination occurs). Excision of these R sites takes place at the borders between the recombinase binding elements and the core crossover sequence. The crucial aspect of this mechanism is the point where DNA strands transpose since it determines the directivity of the recombination site. The eminently specific, swift, and proficient nature of this system has persuaded its widespread application in developing genetic engineering tools to manipulate complex eukaryotic DNA. Therefore, any undesirable transgenic material/selectable marker gene located within the directly repeated recognition sites can be efficiently removed from the plant genome using the site-specific recombination approach. In fact, the first documented evidence of marker gene excision in transgenic plants exploited the bacteriophage P1 Cre-*lox* recombination approach, involving Cre-mediated recombination within the *lox* sites (Dale and Ow 1991).

Site-Specific Recombination Systems

The site-specific recombination strategies have been established in plants with an aim to cut out selection markers and generate marker-free transgenics or to incorporate the gene of interest into a pre-determined genomic location to develop site-specific transgenic crops (Nanto and Ebinuma 2008). The three most well-described recombination technologies are Cre/*lox*, FLP/*FRT*, and R/*RS* systems. These systems belong to the tyrosine (Tyr) recombinase family and require no accessory proteins and/or accessory sites to function in plants.

(a) Cre/lox system: The most widely used technology is the bacteriophage P1 Cre/*lox* system (Hoess et al. 1982; Hoess and Abremski 1985), which comprises two main components: (i) the Cre (cyclic recombinase) gene encoding a 38 kDa Cre recombinase protein and (ii) two 34 bp *loxP* (locus of X-over P1) sites, consisting of two 13 bp inverted repeats, which flank a crossover sequence of 8 nucleotides.

The 34 bp wild-type *loxP* site is arranged in the order: 5'--ATAACTTCGTATAatgtatgcTATACGAAGTTAT-3′. The Cre recombinase specifically attaches to both the symmetric 13 bp 5'-ATAACTTCGTATA-3′ repeats that circumscribe an asymmetric 8 bp 5′-atgtatgc-3′ crossover region, and cleaves the target strand together with both the *loxP* sites. This system has been applied in various crops like *Arabidopsis* (Zuo et al. 2001), *Nicotiana* (Dale and Ow 1991; Gleave et al. 1999), *Zea mays* (Zhang et al. 2003), etc.

(b) FLP/FRT system: The FLP/*FRT* system has been derived from 2-μm plasmid of *Saccharomyces cerevisiae* (Senecoff et al. 1985). In this, FLP (flippase) enzyme which is a 48 kDa protein acts on its two directly oriented 34 bp *FRT* (flippase recognition target) sites to eliminate nucleotides between them; the minimal *FRT* site sequence is: 5'-GAAGTTCCTATTCtctagaaaGTATAGGAACTTC-3′. By virtue of controlled FLP expression and precise allocation of the *FRT* sites in transformation vectors, this technology can be used to remove marker genes from plants post-selection (Lyznik et al. 1996). Many plants such as tobacco (Woo et al. 2009), potato (Cuellar et al. 2006), maize (Li et al. 2010), etc., have been freed from marker genes using this approach.

(c) R/RS system: Another technology is the R/*RS* system acquired from pSR1 plasmid of *Zygosaccharomyces rouxii*, where 56 kDa R (recombinase) protein binds to a pair of 959 nucleotide long palindromic sequences, containing merely 58 bp sized *RS* (recombination site), in order to catalyze intra-molecular recombination (Araki et al. 1985). The 31 bp minimal *RS* site sequence is: 5'--TTGATGAAAGAAtacgttaTTCTTTCATCAA-3′. The very first report on the occurrence of large-scale chromosomal excision, inversion, and conversion exploited the pSR1 system of *Z. rouxii* in *Saccharomyces cerevisiae* (Matsuzaki et al. 1990). R/*RS* recombination tool has been applied in several crops including *Oryza sativa* (Nakagawa et al. 2001), potato (Kondrak et al. 2006) for elimination of SMGs.

Mechanism of Recombinase Enzymes

Cre, FLP, and R recombinases employ the type IB topoisomerase (also called eukaryotic topoisomerase; topo IB) like mechanism to carry out recombination of the target DNA molecules. The procedure comprises two steps: (a) formation of a Holliday junction intermediate (covalent 3′- phosphotyrosine intermediate), followed by (b) re-joining of the complementary sequences. The recombination reaction commences when a highly conserved tyrosine (Tyr) nucleophile chops the DNA helix and adheres to the 3′-phosphate present at the site of cleavage. The consequent 5'-OH end of the sliced DNA now behaves as a nucleophile and attaches to 3′-phosphate on the complementarily cleaved strand, thus resulting in a fruitful union of the cut sequences. Apart from the Tyr residue, a conserved catalytic pentad made up of one lysine (Lys β), two arginine (Arg I and Arg II), one histidine (His-II), and one histidine/tryptophan (His/Trp-III) residues is also present, which provides active sites for the recombinase enzymes; these additional residues ensure that the enzymes bind and position themselves in the correct orientation on the DNA strands.

Classification of Site-Specific Recombination Systems

The aforementioned systems can be parted into two classes with respect to location of the recombinase gene:

(a) Category I: Placement of recombinase gene and selection gene on separate vectors. The first stage of the experiment requires a plant that is already transformed with SMG cloned between the R sites (recognition sites). In the second stage, the recombinase gene construct is introduced into the transformed plant either via re-transformation (Odell et al. 1990; Lyznik et al. 1996) or sexual breeding (Kerbach et al. 2005) to produce primary transformants bearing the recombinase and SMG within two directly oriented recognition sites. On expression of the recombinase gene, the enzyme catalyzes recombination between the R sites, thus removing the intervening undesirable sequence (or marker gene) along with one of the recognition sites in the secondary transformants (Fig. 10.2). Alternatively, a transgenic plant of interest can be out-crossed with a plant that bears a recombinase gene, eventually leading to the segregation of the SMG in F_1 generation, followed by removal of the recombinase in the successive generation.

Even though the process has been applied to a couple of plants, it has two main shortcomings: (1) dependency on breeding practices which can be laborious as well as time consuming and (2) limited feasibility, i.e. it is useful only in case of plants that are amenable to re-transformation or plants that are sexually propagated.

(b) Category II: Transformation of recombinase gene and selectable marker gene on the same plasmid between the excision site boundaries. The experiment involves the use of inducible promoters that activate the recombinase gene upon exposure to intrinsic or extrinsic signals resulting in excision of the recombinase as well as the SMG present within the recombination sites (Fig. 10.3). This strategy is also known as "auto-excision" (Verweire et al. 2007) or "self-excision" (Moravčíková et al. 2008), and has been successfully demonstrated in plants via: (1) heat-shock mediated promoter-recombinase expression vectors (Hoff et al. 2001), (2) chemically-driven plant promoters like maize GST-II-27 (glutathione-S-transferase) promoter, which is triggered by the herbicide antidote "Safener" (Sugita et al. 2000),

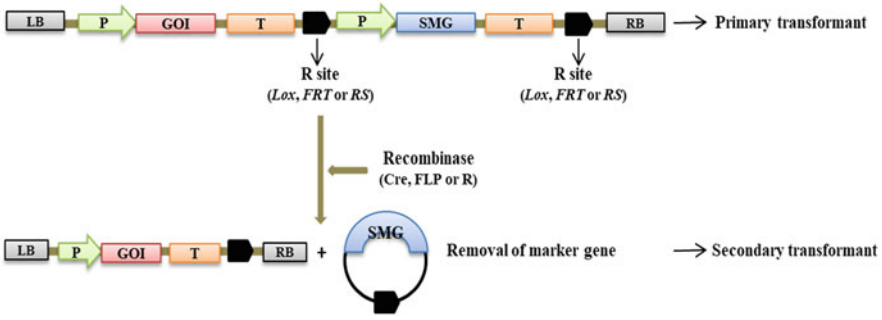

Fig. 10.2 General site-specific recombination strategy to remove marker genes from transgenic crops (Puchta 2003; Chong-Pérez and Angenon 2013) *Note*: *LB* left border of T-DNA, *P* promoter, *GOI* gene of interest, *SMG* selectable marker gene, *T* terminator, *RB* right border of T-DNA

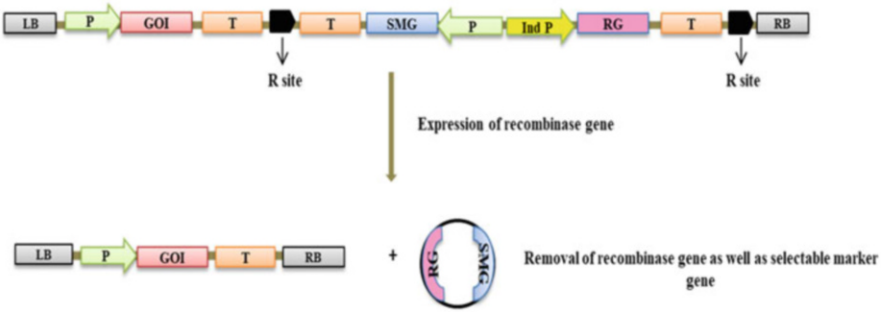

Fig. 10.3 Self-excision method for elimination of SMGs from transgenic crops (Chong-Pérez and Angenon 2013). The *SMG* along with a site-specific recombinase gene (*RG*) which is regulated by an inducible promoter (*Ind P*) is inserted within two directly repeated R sites. Expression of the activated recombinase gene results in the elimination of the marker gene and the recombinase gene from the plant genome. *Note*: *LB* left border of T-DNA, *P* promoter, *GOI* gene of interest, *SMG* selectable marker gene, *Ind P* inducible promoter, *RG* site-specific recombinase gene, *R* site recombinase recognition site, *T* terminator, *RB* right border of T-DNA

β-estradiol-regulated XVE hybrid transactivator to induce Cre/*lox* recombination (Zuo et al. 2001; Sreekala et al. 2005), etc. (3) plant endogenous signals (Verweire et al. 2007; Bai et al. 2008), (4) fusion of different target sites (Luo et al. 2007), and (5) tissue-specific promoters like *Arabidopsis* cruciferin C promoter (Moravčíková et al. 2008). Auto-excision is a more refined mechanism as it has following merits: (1) versatile nature that permits its use in a wide range of plant species, (2) capability to function at different levels of plant development (from early somatic embryo phase to late flowering/seedling phase), (3) applicability in vegetatively-bred as well as perennial plants, and (4) attainment of marker-free progenies in F_1 generation itself.

In general, site-specific recombination is a remarkably precise mechanism with recombination taking place only between specific sequences without causing any change or loss of DNA bases in the recombination site. Also, it is a simple procedure as it requires only a single recombinase for its completion. These systems tend to offer opportunities for resolving complex insertion patterns, such as integration of multiple copies of T-DNA at a single locus, to more simple and single copy DNA insertions at specific genomic locations (Srivastava et al. 1999). Moreover, post-recombination reaction, one of the recombinase recognition sites (*lox*, *FRT*, or *RS*), is retained within the plant DNA. This site can, therefore, act as a point for integrative recombination and help facilitate gene stacking in breeding programs (Ow 2007).

10.2.1.4 Homologous Recombination-Mediated Removal of SMG

Excision of SMGs after successful selection of transformed cells can be carried out using another promising approach called homologous recombination (HR). HR is the process where genetic information is exchanged among two highly similar or identical DNA sequences. This mechanism usually comes into play when the DNA

repair system of a plant attempts to fix any double-strand breaks (DSBs) in its genome.

Zubko et al. (2000) established a transformation system based on intra-chromosomal homologous recombination (ICR) to develop marker-free tobacco plants. The system constituted of two directly repeating recombination sites of bacteriophage λ, called as *attP* sites; *attP* sites contain high A + T content, which facilitates ICR. SMG excision was achieved by inserting the marker gene within the *attP* attachment sites. However, excision beyond *attP* sites was also observed in some marker-free tobacco plants. This was mainly attributed to the occurrence of non-homologous end joining post-recombination reaction. Whenever DSBs are induced at particular loci in DNA of a transgenic plant, a recombination hot spot is created which leads to an increase in the rate of HR as well as non-homologous end joining (NHEJ) [Puchta 2003]. These findings were utilized by Siebert and Puchta (2002) to remove SMG from the plant genome by HR as well NHEJ. For excision through HR, the marker gene was flanked by two restriction endonuclease sites (RE sites) and two homologous sequences. Upon expression of rare cutting I-SceI RE, homologous recombination occurred between the direct repeats leading to elimination of SMG as well RE sites from the plant DNA. Similarly, in case of marker gene elimination through NHEJ, SMG was placed within two I-SceI RE restriction sites. The action of I-SceI resulted in NHEJ among the break points and subsequently caused deletion of the marker gene (Fig. 10.4).

There are certain merits of this approach: (1) it exploits the plant's natural nuclear recombination machinery; (2) the frequency of homologous recombination might be enhanced by stimulating repair systems in plants; (3) efficacy of the mechanism corresponds to the size of homologous sequences; (4) heterologous recombinase expression and sexual out-crossing procedures are not essential; (5) it comprises a single selection step for transgenic calli, thereby saving time and lowering chances of somaclonal variations, and (6) absence of residual recognition sites in the plant DNA post-selection. Despite these, HR has some demerits too: (1) omission of non-target genes, (2) low efficiency, and (3) during selection many transformed events are likely to be lost since the process of recombination is beyond control. Moreover, the mechanism of HR is not fully understood consequently it cannot be applied to a wide spectrum of plant systems.

10.2.2 Excision of Selectable Marker Genes from the Plastid Genome

The plastid DNA, also referred to as ptDNA or plastome, represents an attractive target for genetic engineering since it offers various benefits: (1) significantly high degree of transgene expression due to presence of 10^4 copies of plastid genome per cell, (2) non-dissemination of transgene by pollination as chloroplast DNA is maternally inherited, and (3) no detected position effect, gene silencing, etc. The transplastomic plants are usually screened using SMGs like (a) *kan* or *nptII* or *aphA-6*, that confer resistance against antibiotic kanamycin, or (b) *aadA* that imparts tolerance to antibiotics streptomycin and spectinomycin. These marker genes are

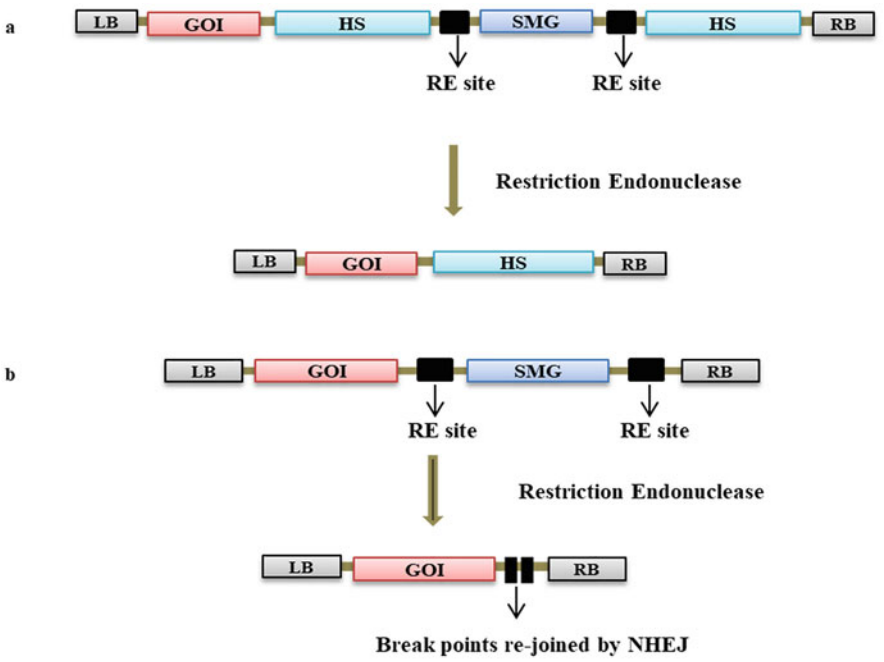

Fig. 10.4 (**a**) Elimination of selectable marker gene through homologous recombination post-induction of double-strand breaks in the plant genome with the help of a rare cutting restriction endonuclease (Puchta 2003), (**b**) Removal of selectable marker gene via non-homologous end joining after causing double-strand breaks in DNA using a rare cutter restriction endonuclease (modified from Puchta 2003) *Note: LB* left border of T-DNA, *GOI* gene of interest, *HS* homologous sequence, *SMG* selectable marker gene, *RE site* restriction endonuclease restriction site, *NHEJ* non-homologous end joining, *RB* right border of T-DNA

generally not needed once the desired transplastomic plant (plant in which genes are modified, inactivated, or new transgenes are incorporated into plastid genome) has been selected, as high expression of SMGs in the plastid genome may bear metabolic disadvantages (Chong-Pérez and Angenon 2013). Therefore, it is important to eliminate undesirable marker gene products from the ptDNA. Some of the techniques to excise transplastome marker genes are: (1) homologous recombination system, (2) site-specific recombinase-based elimination, (3) transient co-integration of SMG, and (4) co-transformation-segregation strategy.

10.2.2.1 Homologous Recombination System

In plastid transformation, gene of interest is integrated into the genome via homologous recombination (HR). Therefore, it is possible to eliminate SMG via HR within directly repeating gene promoters/terminators flanking the desired transgene and SMG. The foremost report demonstrating the occurrence of this phenomenon was on the unicellular alga *Chlamydomonas reinhardtii* wherein homology-directed SMG removal was observed under non-selective growth conditions (Fischer et al. 1996).

Iamtham and Day (2000) excised marker genes from tobacco chloroplasts by means of HR between identical direct repeats of chloroplast genome, after transformation with vector bearing three transgenes, viz. *uidA*, *aadA*, and *bar*. Once the antibiotic selection was removed, the elimination events accumulated significantly, rendering 25% of the transgenic lines homoplastic (containing only one type of recombinant ptDNA) and marker-free in the following generation. Further, Dufourmantel et al. (2007) also exploited this strategy to develop herbicide tolerant *aadA*-free soybean and tobacco plants. The experiment was conducted using a chloroplast transformation vector harboring *aadA* marker that interrupts the herbicide tolerance gene *HPPD* (4-hydroxyphenyl-pyruvate dioxygenase). Initial selection was performed using antibiotic spectinomycin, followed by elimination of *aadA* SMG via homologous recombination between 403 bp sized overlapping sequences of the 5′ and 3′ ends of the *HPPD* gene. The process yielded a transplastomic plant with complete herbicide tolerance and expression of *HPPD* gene. Moreover, Daniell et al. (2019) generated marker-free lettuce plants expressing pectinase enzymes (Pel B and Pel D) using homology-based SMG elimination scheme. The transformation construct used in the study comprised *aadA* marker gene that was placed within two 649 bp direct repeats of *atpB* (ATP synthase subunit beta). Post-selection with spectinomycin, the *aadA* gene cassette was excised by virtue of HR between the two *atpB* repeats.

10.2.2.2 Site-Specific Recombinase-Based Elimination

Removal of SMGs using site-specific recombinase enzymes is another promising technique to develop marker gene-free transplastomic plants. The system comprises two steps: (1) generation of transplastomic plants containing a marker gene placed within two directly oriented recombinase target sites followed by (2) nuclear transformation with a highly expressing plastid-directed recombinase gene that facilitates excision of the marker. The Cre gene can be introduced stably or transiently into transplastomic plants either via *Agrobacterium tumefaciens* or sexual crossing of transplastomic plants with Cre-expressing crop plants or transient Agroinfiltration (Fig. 10.5). The feasibility of this method was for the first time demonstrated in tobacco using the Cre/*lox* site-specific recombination system. In these studies, Cre recombinase was incorporated into tobacco genome through *Agrobacterium*-mediated transformation resulting in transplastomic plants free of SMGs. However, the so obtained events still had Cre and *npt II* genes within their DNA that needed to be removed through segregation in the seed progenies (Corneille et al. 2001; Hajdukiewicz et al. 2001). Further, Cre expression has also been introduced via pollen grains, in which Cre-induced non-specific rearrangements among the homologous plastome sequences were non-existent or took place at a significantly low rate as compared to the directly transformed tobacco plants. These findings demonstrated the dependence of this system on the method chosen for introduction of Cre gene into the nuclear DNA (Corneille et al. 2001). This is because when Cre was inserted via re-transformation, highly proficient HR events within identical gene sequences were observed, whereas Cre introduction through pollination/sexual crosses did not result in HR-mediated elimination events (Upadhyaya et al. 2010). Cre-mediated

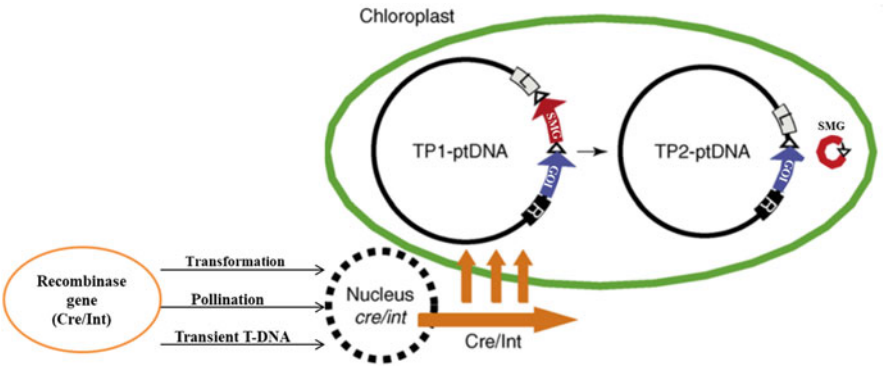

Fig. 10.5 SMG elimination from the chloroplast genome using Cre/Int site-specific recombinase genes (Lutz and Maliga 2007) *Note: L* left border of T-DNA, *SMG* selectable marker gene, *GOI* gene of interest, *ptDNA* plastid DNA, *R* right border of T-DNA

cleavage and joining of *loxP* sites induces unwanted recombination events that lead to deletions in the plastome (Corneille et al. 2001; Hajdukiewicz et al. 2001). Unlike native chloroplast enzymes, Cre/*loxP* recombination mechanism requires extra steps of first incorporating and then eliminating plastid-directed Cre gene from transplastomic plants. However, the timing of inserting recombinase gene into chloroplasts can be regulated and SMG removal is accelerated as soon as the recombinase reaches chloroplast.

Further, high expression of Cre gene under certain conditions might lead to crinkling of leaves and reduction in fertility of plants (Coppoolse et al. 2003). Alternatively, Kittiwongwattana et al. (2007) employed ΦC31 phage site-specific integrase (Int) for inserting the desired transgene into the tobacco plastome since Int mediates recombination within non-identical phage (*attP*) and bacterial (*attB*) attachment sites. The tobacco plastid in this case was engineered with a gene construct containing *aadA* marker surrounded by two genetically distinct recombination sites, viz. *attP* (215 bp) and *attB* (54 bp), that were detectable by the Int recombinase enzyme. The elimination of *aadA* gene was achieved with the help of a plastid-specific Int that was incorporated via *Agrobacterium*-mediated transformation. ΦC31/*attP*/*attB*-mediated plastid transformation is more advantageous than Cre/*loxP*-mediated transformation. This is because *attB* and *attP* are non-homologous sites, therefore ptDNA harboring *att*-flanked SMG is thought to be more stable as compared to plastome carrying *loxP*-flanked marker genes. Further, absence of pseudo-*att* sites in plastid genome lowers chance of undesirable crossover events facilitated by ΦC31 Int (Kittiwongwattana et al. 2007). Moreover, this strategy is unidirectional unlike Cre/*loxP* wherein Cre gene would be required for excision as well as re-incorporation of marker gene through *loxP* sites (Day and Goldschmidt-Clermont 2011).

10.2.2.3 Transient Co-integration of SMG

Stable integration of a SMG into ptDNA requires targeting sequences/arms to facilitate dual crossover/recombination events in the homologous sequences surrounding the SMG. Incorporation of SMG into the plastome occurs via one recombination event in either the left or right target sequences. This generates an unstable co-integrate structure due to presence of large direct repeats of both the arms. Later, homology crossover events result in either stable incorporation of both SMG and GOI or elimination of the integrated gene construct and generation of wild-type ptDNA (Fig. 10.6). This method was first employed by Klaus et al. (2004) where the SMG (*aphA-6*) was inserted external to the target region. This facilitated identification of co-integrate formed as a result of crossover by one of the target arms only. Further, when antibiotic selection is discontinued, the second crossover process can occur, and the SMG is excised (Fig. 10.7).

10.2.2.4 Co-transformation-Segregation Strategy

Co-transformation includes engineering with two constructs that direct target insertions into two different sites in the same plastome. One vector bears the SMG and the other one GOI/non-selected gene and these are introduced into the plastid via biolistic transformation. This step yields heteroplastomic ptDNA population carrying either or both of the genes. Antibiotic selection for the SMG produces transplastomic events harboring the non-selected gene as well. Finally, post-segregation marker-free transplastomic plants can be obtained (Fig. 10.8). The feasibility of co-transformation-segregation method was observed for the first time in *C. reinhardtii*, a unicellular alga having single plastid (Kindle et al. 1991; Newman et al. 1991). The approach depends on the heteroplastomic events produced by co-transformation of two separately targeted genes. This strategy was developed with an aim to obtain marker-free plants that are devoid of the antibiotic tolerance gene and are resistant against herbicides like glyphosate or phosphinothricin. However, transplastomic plants cannot be straightaway selected via glyphosate or phosphinothricin resistance post-transformation with the tolerance genes, viz. *CP4* or *bar*, since cells carrying only a few transgene copies die. Nevertheless, co-transformation using a vector carrying *CP4/bar* gene along with the spectinomycin tolerance gene *aadA*, and successful insertion of these genes in majority of the plastome copies render cells/tissues and regenerated plants resistant to great amount of herbicides.

10.3 Status of Marker-Free Transgenic Plants

The development of marker-free transgenic plants has undergone considerable advancement over the years. A wide range of approaches have been employed to generate SMG-free plants with agronomically valuable gene(s) in order to achieve sustainable crop development. Jia et al. (2007) for the first time reported the use of Agroinfiltration strategy for direct production of selectable marker-free *Nicotiana tabacum* plants. In this, leaf discs of tobacco were infiltrated with *A. tumefaciens*

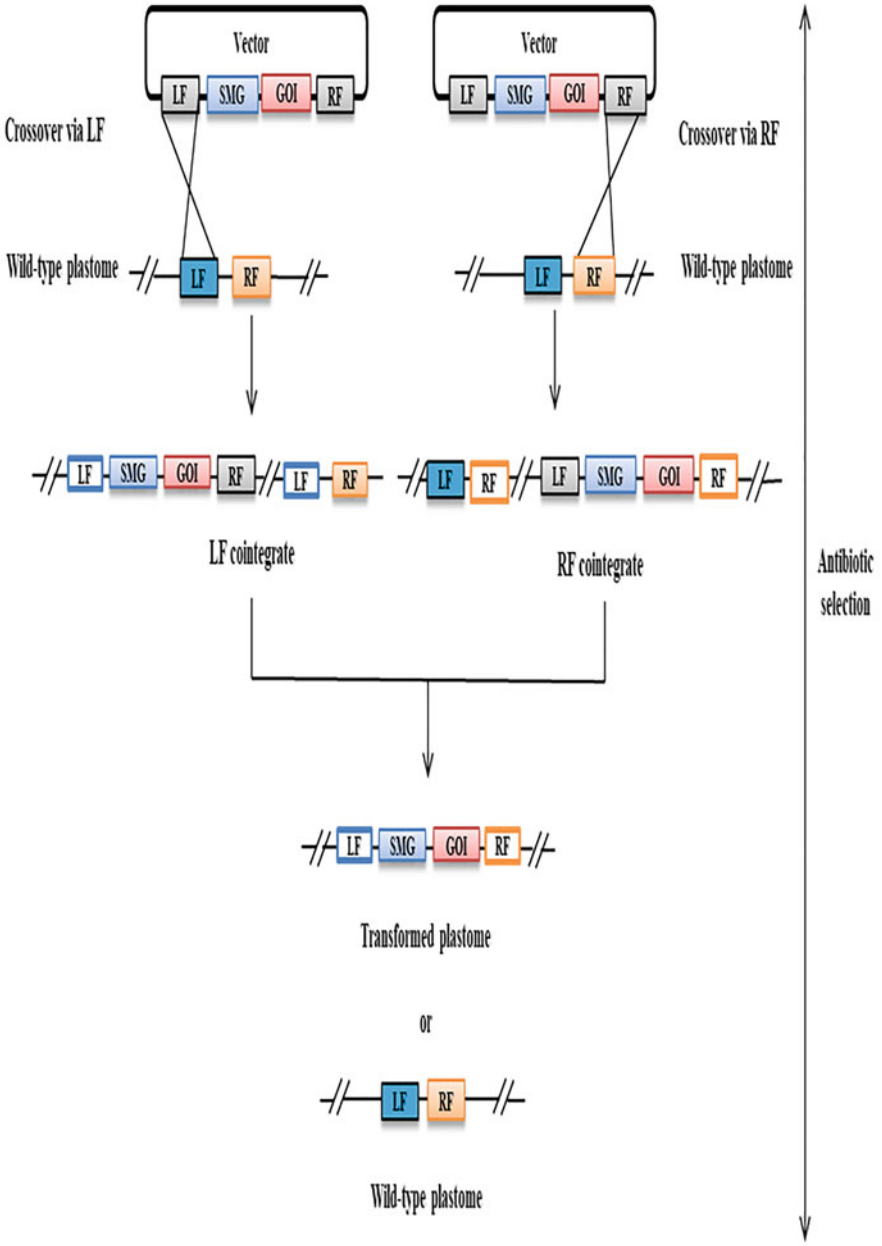

Fig. 10.6 Standard chloroplast transformation process using a gene construct with SMG placed between the homologous flanks (Chong-Pérez and Angenon 2013) *Note: LF* left flank, *SMG* selection marker gene, *GOI* gene of interest, *RF* right flank

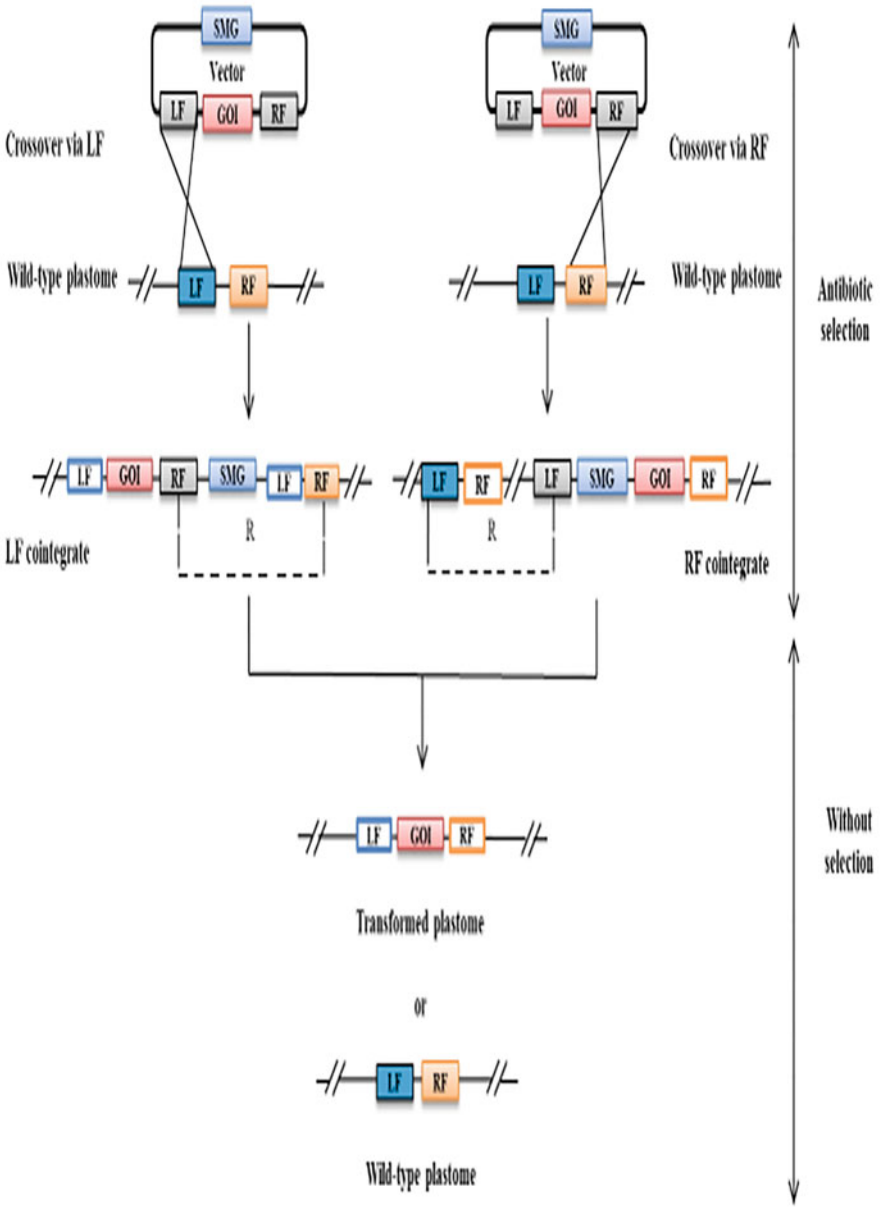

Fig. 10.7 Chloroplast transformation process using a gene construct with SMG placed outside of the homologous flanks (Chong-Pérez and Angenon 2013) *Note: LF* left flank, *SMG* selection marker gene, *GOI* gene of interest, *R* recombination, *RF* right flank

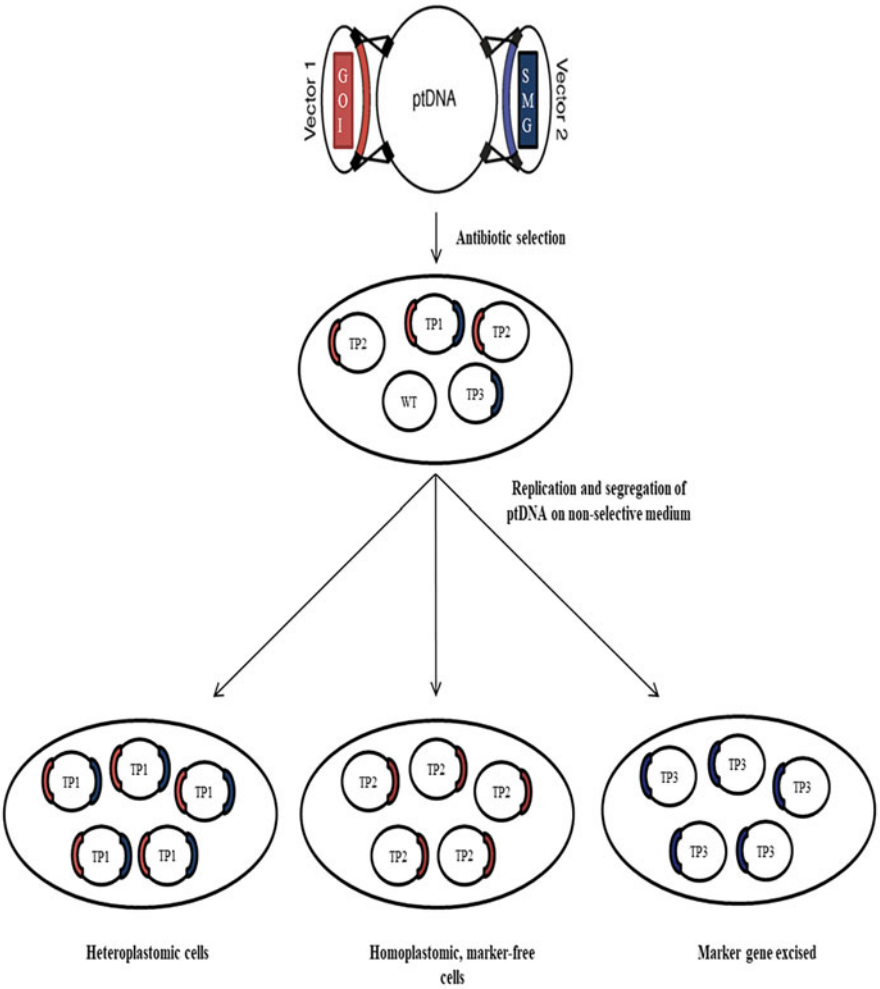

Fig. 10.8 Co-transformation-segregation approach to produce SMG-free transplastomic plants (Lutz and Maliga 2007; Chong-Pérez and Angenon 2013) *Note: GOI* gene of interest, *SMG* selection marker gene

cells carrying marker-free plasmid coding for *β-glucuronidase* (GUS) and a transformation frequency of 15% was recorded. Moreover, Li et al. (2007) developed a self-activating system for elimination of *hygromycin phosphotransferase* (*hpt*) gene using Cre/*loxP* site-specific technique in transgenic soybean plants. The scheme involved an embryo-specific promoter, *app1*, which facilitated the removal of marker gene from over 30% of the stable transgene events. This strategy, therefore, demonstrated the potential of developmentally-regulated gene promoters in mediating SMG excision. In potato, *nptII* marker was also removed using transient

Cre/*loxP* system wherein PVX virus vector was used to introduce Cre protein which upon expression rendered 20–27% potato plants free of *nptII* gene (Kopertekh et al. 2012). Marker-free potato plants have also been produced using heat-inducible Cre/*loxP* excision method which successfully demonstrated complete SMG removal in 71% of the treated explants (Orbegozo et al. 2016). Likewise, *Mirafiori lettuce big-vein virus* (MLBVV)-tolerant SMG-free transgenic lettuce was produced using a binary plasmid with one T-DNA carrying *nptII* marker gene and the other carrying lettuce ubiquitin promoter and terminator and IR (inverted repeats) of coat protein of *Mirafiori lettuce big-vein virus* (Kawazu et al. 2016).

Qi et al. (2013) produced *hpt* marker-free transgenic rice demonstrating high tolerance towards stem borers and leaffolder by virtue of *cry1Ab* gene which was stably introduced into the crop genome via mixtures of *A. tumefaciens* strains. Further, transgenic rice was also freed of *hpt* marker gene using twin T-DNA approach where twin T-DNA plasmids were employed to individually target 400 base pairs of rice stripe virus (RSV) coat protein (CP) and special-disease protein (SP) genes (Jiang et al. 2013). Srivastava et al. (2017) developed transgenic rice containing *β-glucuronidase* (GUS) gene which was transformed with a vector expressing Cas9 and two guide RNAs to target both the ends of GUS gene. Furthermore, Toda et al. (2019) delivered Cas9–gRNA ribonucleoproteins (RNPs) into rice zygotes, followed by culturing without any antibiotic selection, leading to regeneration of rice plants having targeted mutations, which accounted for over 14–64% plants. Dong et al. (2020) reported a standardized CRISPR/Cas9-based approach for targeted insertion of a carotenoid biosynthetic construct at the genomic sites of interest. The study yielded marker-free rice plants possessing high carotenoid content and no negative impact on yield or morphology. Some more studies on advances in production of marker-free transgenic plants have been summarized in Table 10.2.

10.4 Conclusions and Future Perspectives

With the rise of various biotechnological approaches, genetically engineered (GE) crop plants are being produced that are tolerant to different stress conditions, like soil erosion, drought, salinity, pollution, cold, varying climate, etc. However, biosafety regulations on these GM crops are stringent which hinder their global production. For instance, big markets such as the European Union have enforced strict guidelines on the release and import of GM food products. Therefore, development of methods for generation of "clean" SMG-less GM crop plants is the need of the hour. In this direction, plant biotechnology can play a key role in both improving crops as well as reducing future risk hazards. Moreover, value-added attributes have been incorporated into many essential crops which will enhance quality production. Still the occurrence of SMGs in transgenic plants is a major concern and cannot be neglected. Different techniques to remove selectable markers have come up over the years and further optimization is in motion. Recently, scientists have defined methodologies for elimination of leftover recognition

Table 10.2 Marker-free transgenic plants

Strategy	Crop/ genotype	Remarks	References
Co-transformation	Tobacco	Super-binary plasmids harboring two different T-DNAs developed for co-transformation	Komari et al. (1996)
	Tobacco	Feasibility of single-strain method of co-transformation demonstrated	Daley et al. (1998)
	Tobacco	Co-transformation using single *Agro* strain containing two T-DNAs of cointegrate and binary constructs, respectively	Jacob and Veluthambi (2002)
	Tobacco	Negative selection marker used for obtaining SMG-free plants	Park et al. (2004)
	Tobacco	Marker gene removal in T_0 generation using a non-conditional negative SMG, MYMV *TrAP*	Ramana Rao and Veluthambi (2010)
	Tobacco	Development of an improved double T-DNA binary plasmid system	Leng et al. (2020)
	Rice	Super-binary plasmids harboring two different T-DNAs employed for co-transformation	Komari et al. (1996)
	Rice	First report on production of SMG-free cereal crop	Tu et al. (2003)
	Rice	Sheath blight tolerant, marker-free plants; single *Agrobacterium* strain carrying two T-DNAs of cointegrate and binary plasmids, respectively	Sripriya et al. (2008)
	Rice	Sequential co-transformation with same marker gene via *Agrobacterium* to develop SMG-free, transgene-stacked plants	Ramana Rao et al. (2011)
	Rice	*AmA1* gene transferred in to rice via *Agrobact.* harboring a twin T-DNA binary construct to improve nutritional content	Xu et al. (2017)
	Rapeseed	Feasibility of single-strain method of co-transformation demonstrated	Daley et al. (1998)
	Rapeseed	Marker-free plants expressing and secreting *phytase* gene produced	Xu et al. (2019)
	Rapeseed	Development of marker-free *B. napus* plants with three different co-transformation systems	Liu et al. (2020a)
	Barley	*Agrobacterium*-mediated co-transformation with construct carrying adjacently placed T-DNAs	Matthews et al. (2001)
	Wheat	Marker-free wheat plants developed for the first time with construct bearing two separate T-DNAs	Wang et al. (2017)
	Wheat	FISH technique used for identification of marker-free wheat lines	Liu et al. (2020b)

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Table 10.2 (continued)

Strategy	Crop/ genotype	Remarks	References
	Broccoli	Marker-free plants with enhanced shelf-life produced via *A. rhizogenes*-mediated co-transformation	Higgins et al. (2006)
	Grapevine	Positive and negative SMGs used for producing marker-free grapevine	Dutt et al. (2008)
	Cucumber	Co-transformation of *gfp* and *nptII* via *A. tumefaciens* followed by their subsequent segregation	Khidr and Nasr (2018)
	White clover	Co-transformation of three transgenes using a single T-DNA	Narancio et al. (2020)
Site-specific recombination Cre/*lox* system	Tobacco	TMV-based construct used for driving Cre expression	Jia et al. (2006)
	Tobacco	First report on marker removal from transgenic plants expressing insecticidal protein	Chakraborti et al. (2008)
	Tobacco	Excision of transgene via developmentally controlled *Cre* recombinase	Kopertekh et al. (2010)
	Tobacco	ß-estradiol-inducible Cre and a conditional SMG used for marker removal	García-Almodóvar et al. (2014)
	Tobacco	Delivery of Cre gene using potato virus X expression vector	Kopertekh and Schiemann (2017)
	Tobacco	Generation of recombinase-based founder line for gene targeting	Aslam et al. (2019)
	Rice	Chemically-inducible Cre recombinase eliminates SMG	Sreekala et al. (2005)
	Rice	Cre/lox self-excision method for obtaining marker-free events	Bai et al. (2008)
	Rice	Cre/*lox* recombination employed for elimination of *hpt* marker gene placed between two *lox* sites	Sengupta et al. (2010)
	Rice	Cre/*loxP* regulated hpt-ΔEn removal and waxy reactivation	Terada et al. (2010)
	Rice	Marker-free events produced by chemically controlled Cre/*lox* system	Qiu et al. (2010)
	Rice	Heat shock-regulated Cre facilitated conditional elimination of SMG	Khattri et al. (2011)
	Potato	*nptII* gene removed using heat-inducible Cre/*lox* system	Cuellar et al. (2006)
	Potato	CPMV-flanked Cre gene used for excising *nptII* SMG	Kopertekh et al. (2018)
	Tomato	β-Estradiol-mediated Cre recombinase to eliminate *npt* gene	Zhang et al. (2006)

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Table 10.2 (continued)

Strategy	Crop/ genotype	Remarks	References
	Tomato	Salicylic acid-mediated expression of Cre facilitated removal of *npt* gene	Ma et al. (2009)
	Tomato	Estradiol-induced Cre/lox mechanism deployed for marker removal	Zhang et al. (2009)
	Indian mustard	Cre/*loxP* system used for excision of *gfp* or *nptII* genes	Arumugam et al. (2007)
	Oilseed rape	Auto-excision Cre gene cassette developed for removal of *bar* gene	Kopertekh et al. (2009)
	Apricot	ß-estradiol inducer used for Cre/*lox* recombination to produce marker-free plants	Petri et al. (2012)
	Orange	Cre/*loxP* system employed for removal of *ipt* gene	Zou et al. (2013)
	Tobacco	cre^{INT}, a cre gene variant, developed for generation of marker-free transgenic plants	Mlynarova and Nap (2003)
	Tobacco	Heat shock-inducible Cre system to produce marker-free transgenic tobacco	Wang et al. (2005)
	Arabidopsis thaliana	Heat-inducible Cre used for removal of *nptII* marker gene	Deb Roy et al. (2008)
	Maize	Heat-inducible Cre employed to eliminate *egfp* visual selection marker	Du et al. (2019)
	Maize	Inducible Cre method determined for removing marker and morphogenic genes	Wang et al. (2020)
	Wheat	Simultaneous use of cold-inducible Cre/*lox* system and minimal gene construct	Mészáros et al. (2015)
	Barley	Cold-inducible Cre/*lox* system utilized for SMG elimination	Éva et al. (2018)
FLP/*FRT* system	Tobacco	GM-gene-deletor system (heat shock-regulated FLP with two *loxP*/*FRT* sites) developed to remove *ipt* gene	Luo et al. (2008)
	Tobacco	Oxidative stress-inducible FLP used for removal of *hpt* marker	Woo et al. (2009)
	Maize	SMG-free maize plants with enhanced salt tolerance produced	Li et al. (2010)
	Rice	FLPe used for SMG removal from a transgenic locus generated via Cre/*lox* regulated gene integration	Akbudak and Srivastava (2011)
	Rice	Oxidative stress-inducible FLP for removal of *hpt* gene	Woo et al. (2015)
	Apple	CRISPR/Cas9-FLP/*FRT*-mediated genome editing technology for marker gene elimination	Pompili et al. (2019)
	Maize	*Agrobacterium*-mediated SMG excision from elite inbred lines	Anand et al. (2019)
	Sugarcane	Removal of nptII gene using FLPe/*FRT* system	Zhao et al. (2019)

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Table 10.2 (continued)

Strategy	Crop/ genotype	Remarks	References
R/*RS* system	Potato	PROGMO binary vector developed for production of backbone- and SMG-free plants	Kondrak et al. (2006)
	Apple	Marker removal system optimized using dexamethasone-induced recombinase	Righetti et al. (2014)
	Apple	Supersweet protein-expressing marker-free plants developed	Timerbaev et al. (2019a, b)
	Pear	Dexamethasone-induced recombinase for excision of codA-nptII protein-encoding SMG	Righetti et al. (2014)
	Tomato	pMF vector system used for developing marker-free plants carrying supersweet thaumatin II protein	Timerbaev et al. (2019a, b)

sequences at the recombination sites. This may encourage the use of site-specific recombination as the method of choice to excise undesirable genomic regions. Further, researchers are looking for ways to pace up the selection process of marker-less progeny plants post-co-transformation. Moreover, new SMG removal techniques based on homologous recombination and gene targeting have been demonstrated. With these advancements, the concern pertaining to uncontrollable transmission of antibiotic and herbicide tolerance genes in the environment will most likely have no relevance in the coming years. Development of marker-free GM plants would significantly support crop improvement programs with far-reaching applications in the field of fundamental research and biotechnology. All in all, genetically modified crops are likely to add up globally to food security.

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Conflict of Interest The authors declare no conflict of interest.

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Recent Progress in Cereals Biofortification to Alleviate Malnutrition in India: An Overview

11

Pankaj Kumar, Arun Kumar, Karuna Dhiman, and Dinesh Kumar Srivastava

Abstract

As the world's population increases, food insecurity and malnutrition due to essential micronutrient(s) deficiency are emerging as the two foremost challenges, and need urgent attention. Micronutrient deficiency among women, children, and adolescents is a big challenge in developing countries like India. People in such countries suffer not only from hunger but more from hidden hunger due to a lack of essential vitamins and minerals (micronutrients). Malnutrition is a major food-related primary health problem worldwide, including India, where the main staple food crops are cereals. Cereals contribute a significant part

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to human nutrition and are a vital source of energy for human diets. Supplements, a balanced diet, fortifications, and biofortification are strategies to alleviate micronutrient malnutrition. Biofortification is a new nutritional revolution to deliver nutrient-rich food to every individual. Cereal crop biofortification is a promising way to serve a larger section of our society, including rural and poor populations. Cereal biofortification can provide a comparatively cost-effective, sustainable, and long-term means of delivering sufficient micronutrients to rural communities in developing nations. Conventional breeding, application of genomic tools, agronomical and transgenic approaches are some of the common strategies for crop biofortification. Therefore, this chapter provides insights from the cited literature on recent progress in cereals biofortification to alleviate malnutrition in India using different crop breeding and transgenic approaches.

Keywords

Biofortification · Genetic engineering · Cereals · Malnutrition · Micronutrients

11.1 Introduction

In developing countries like India, people are largely dependent on staple food crops such as rice, wheat, maize, millets, and sorghum. Today, our nation has attained self-sufficiency in food supply due to the green revolution that significantly increased the food grain production from 50.82 million tonnes in 1950–1951 to 284.83 million tonnes in the year 2017–2018 (Table 11.1). However, more than two billion people, especially women and pre-school age children, are micronutrient malnourished caused by a deficiency of micronutrients in the diet. More than half of the human population of developing countries of Asia and Africa are deficient in micronutrients such as Fe and Zn (White and Broadley 2009; Gomez-Galera et al. 2010). According to National Family Health Survey (NFHS), Ministry of Health and Family Welfare (MOHFW), Government of India, International Food Policy Research Institute

Table 11.1 Indian Statistical report on total cereals and total food grain production. (In Million Tonnes)

Year	2015–2016	2016–2017	2017–2018	2018–2019	2019–2020[a]
Rice	104.41	109.70	112.76	116.48	118.43
Wheat	92.29	98.51	99.87	103.60	107.59
Maize	22.57	25.90	28.75	27.72	28.64
Total cereals production	235.22	251.98	259.60	263.14	273.50
Total food grain production	251.57	275.11	285.01	285.21	296.65

Sources: Agricultural Statistics Division, Directorate of Economics and Statistics, Ministry of Agriculture and Farmers Welfare

[a]Fourth advance estimates of production of food grains for 2020–2021

Table 11.2 Essential nutrients for well-being of human life[a]. (modified from Bouis and Welch 2010; Garg et al. 2018)

Air, water, and energy	Amino acids/ proteins	Fats/ lipids	Essential macro elements	Essential trace elements	Vitamins
Oxygen	Histidine	Linoleic acid	Na	Fe	A (retinol)
Water	Isoleucine	Linolenic acid	K	Zn	D (calciferol)
Carbohydrates	Leucine		Ca	Cu	E (α-tocopherol)
	Lysine		Mg	Mn	K (phylloquinone)
	Methionine		S	I	C (ascorbic acid)
	Phenylalanine		P	F	B1 (thiamin)
	Threonine		Cl	Se	B2 (riboflavin)
	Tryptophan			Mo	B3 (niacin)
	Valine			Co (in B12)	B5 (pantothenic acid)
				B	B6 (pyroxidine)
					B7 (biotin)
					B9 (folic acid, folacin)
					B12 (cobalamin)

[a]Various additional valuable substances in foods are also known to contribute to better health

(IFPRI), and World Health Organization (WHO)/World Bank Group-Joint Child Malnutrition Estimates-2017, two billion people are malnourished, and 795 million are undernourished worldwide. Also, around 155 million children (<5 years) are stunted (low height-for-age), 52 million wasted (low weight-for-height), and 17 million severely wasted. Malnutrition contributes to a loss of 11% GDP in Asia and Africa. Indian scenario of malnutrition showed that 195 million people (15.2%) of the population is undernourished. Human beings require around forty known nutrients in their diet to live healthy and productive lives (Table 11.2). But unfortunately, major staple cereals contain insufficient amounts of essential nutrients such as vitamin A, iron (Fe), zinc (Zn), calcium (Ca), manganese (Mn), copper (Cu), iodine (I), or selenium (Se) to meet daily requirements (Neeraja et al. 2017). Supplementation, dietary diversification, fortification, and biofortification adapted to conditions in different countries, and regions are the comprehensive strategies to alleviate micronutrient malnutrition (Zimmerman and Hurrel 2007; Stein 2010). Key food crops with enhanced nutrients can be obtained by biofortification, which involves the genetic enhancement of micronutrients (Bouis et al. 2013). Unlike fortification (addition of exogenous nutrients as in iodized salt), biofortification methods increase the nutrients of crops at source through agricultural interventions,

viz. agronomy, breeding, and biotechnology. Also, growth and production in soils with depleted or unavailable minerals can be improved using these biofortified crops (Cakmak 2008; Borg et al. 2009). Staple food crops can be biofortified to enhance micronutrient concentrations in edible parts to address hidden hunger, with the potential to reach the neediest of the population (Haug et al. 2007; Bouis and Welch 2010; Lyons and Cakmak 2012). Thus biofortification, which links nutritious agricultural products with human health, can be more effective and sustainable than the other approaches used to combat mineral malnutrition (Lyons 2014). Along with national and international biofortification programs, the Indian Council of Agricultural Research, Government of India, has taken leads for biofortification of cereal crops by targeting the enhancement of nutrients in staple food crops.

11.2 Biofortification Approaches

Biofortification approaches focus on enhancing the nutritional contents of the crop through agricultural interventions, viz. agronomy, breeding, and biotechnology (Fig. 11.1). These approaches are discussed as follows:

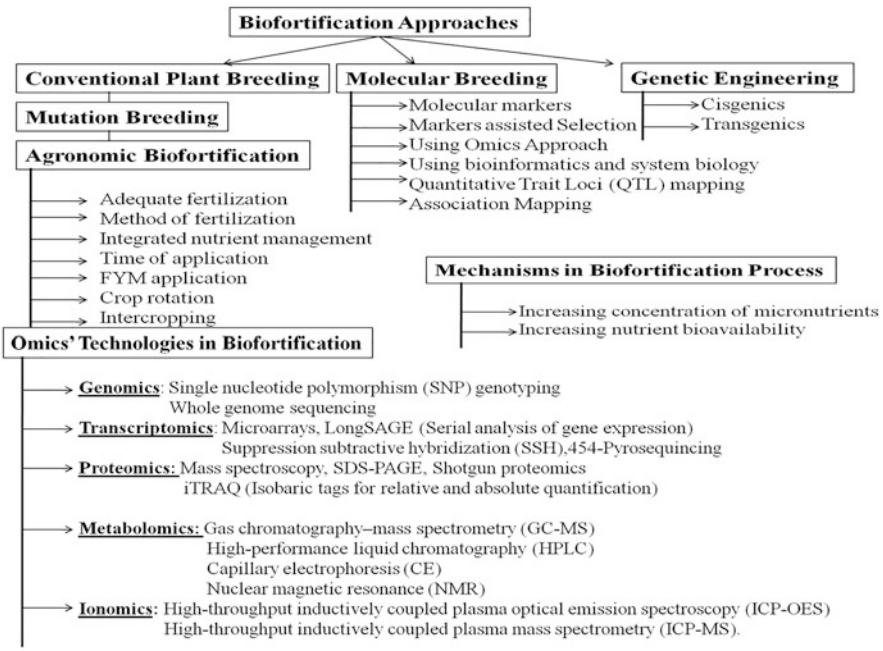

Fig. 11.1 Overview of biofortification approaches, mechanism, and omics technology as biofortification tools in cereals biofortification (modified from Carvalho and Vasconcelos 2013)

11.2.1 Biofortification Through Agronomic Approaches

The utmost demand for nutritional security of the developing world can be met by enhancing the dietary values of important staple food crops for certain essential micronutrients using agronomic biofortification. The physical application of nutrients to temporarily improve the nutritional and health status of crops through agronomical biofortification can improve human health by the consumption of such crops (Cakmak and Kutman 2017). Poor rural masses will never have money to buy mineral supplements. Even they cannot afford to improve the components of their diet by incorporating animal products; thus, agronomic biofortification will be a boon to such people. The agronomic biofortification approach generally relies on the application of mineral fertilizers, and either solubilization/or mobilization of nutrients from the soil to the edible parts of plants. Microbial cultures of plant growth-promoting soil microorganisms can also be used to enhance the nutrient mobility from soil to edible parts of plants and improve their nutritional status, in addition to fertilizer. Phyto-availability of these mineral elements can also be increased by using soil microorganisms like different species of *Bacillus*, *Pseudomonas*, *Rhizobium*, *Azotobacter* (Smith and Read 2007). Crop productivity of crops grown in nitrogen-limited conditions can be increased using N_2-fixing bacteria (Hardarson and Broughton 2004). Organic acids, siderophores, and enzymes capable of degrading organic compounds and increasing mineral concentrations in edible produce are released by mycorrhizal fungi associated with many crops (Rengel et al. 1999; Cavagnaro 2008). In developing countries of Asia and Africa, cereals are the staple food crops, so agronomic biofortification is the easiest and fastest way for grain's micronutrient (Fe and Zn) enhancement. Agronomic addition of the appropriate nutrient as an inorganic compound to the fertilizer increases the mineral content of the plant as demonstrated successfully in crops like rice, wheat, and maize (Bouis and Welch 2010).

11.2.2 Biofortification Through Conventional Breeding

The most accepted method of biofortification is conventional breeding. As compared to transgenic and agronomic-based strategies, this approach is sustainable and cost-effective. India is one of the mega centers of agro-biodiversity, but little effort has been made to evaluate the promising germplasm for enhanced nutrients in crops. With some identified donors for high nutrients, varieties are being developed through conventional breeding by crossing with popular varieties. Conventional breeding can be feasible if there is sufficient genotypic variation for the trait of interest. Levels of minerals and vitamins in crops can be improved by utilizing these variations in breeding programs. In conventional plant breeding, plants with desired nutrient and agronomic traits can be produced by crossing parent lines (with high nutrients) with the recipient lines with desirable agronomic traits over several generations. In case of limited genetic variation present in the gene pool crossing to distant relatives and moving the trait slowly into the commercial cultivars can be

done. Alternatively, mutagenesis of commercial varieties can be done to introduce new variations. HarvestPlus is the program that was launched by the Consultative Group on International Agricultural Research (CGIAR) along with the International Center for Tropical Agriculture (CIAT) and the International Food Policy Research Institute (IFPRI) to breed biofortified staple food crops. This program is investing heavily to boost three key nutrients—vitamin A, Fe, and Zn and is targeting the staple crops, wheat, rice, maize, cassava, pearl millet, beans, and sweet potato in Asia and Africa (Bouis and Welch 2010). It is directed to produce staple food crops with enhanced bioavailable essential minerals and vitamins that will have a measurable impact on improving the micronutrient status of target populations, primarily resource-poor people in the developing world. The Biocassava Plus program had been initiated to improve the nutrition status of the cassava crop. The breeding lines with adequate amounts of nutrients and promising yield thus developed are evaluated under the Indian Council of Agricultural Research (ICAR)-All India Coordinated Research Projects (AICRP) for varietal release. Recent approaches for biofortification include identification of genomic regions/candidate genes for high nutrients through tagging/identification of major genes or mapping of quantitative trait loci (QTL) followed by their introgression into popular varieties. Being a genetic solution, growing biofortified crops does not require any additional expenditure for farmers as this approach uses the intrinsic properties of crops. Since biofortified crops are developed through conventional breeding, regulatory constraints are not applicable for their release (Bouis and Welch 2010).

11.3 Transgenic Approach for Biofortification

Genetic engineering offers an alternative for increasing the concentration and bioavailability of micronutrients in the edible crop tissues when there is not sufficient variation among the genotypes for the desired character/trait within the species, or when the crop itself is not suitable for conventional plant breeding (due to incompatibility barrier) (Prasad et al. 2015). Thus, the transgenic approach can be an appropriate alternative in case of limited or no genetic variation in nutrient content among plant varieties. Plant transformation may be faster than conventional breeding to achieve the nutritional target, and this can be a valid alternative, where breeding approaches are not successful (Brinch Pedersen et al. 2006; Zhu et al. 2007). Genes from novel sources for desirable target traits can be introduced using this approach with unlimited access to the genes of interest, targeted expression in tissues of interest, rapid and direct application by introduction into popular varieties, and stacking of different genes. In the development of transgenic crops, the ability to identify and characterize gene function and then utilize these genes to engineer plant metabolism is essential (Christou and Twyman 2004).

Furthermore, metabolic engineering can be exploited to transplant alternative pathways using pathways from bacteria and other organisms (Newell-McGloughlin 2008). Genetic modifications can also be used to reduce the concentration of antinutrients which limit the bioavailability of nutrients in plants for redistribution of

micronutrients between tissues, enhancing the micronutrient concentration in the edible portions of commercial crops, increasing the efficiency of biochemical pathways in edible tissues, and/or even the reconstruction of selected pathways (Shewmaker et al. 1999; Agrawal et al. 2005; Yang et al. 2002). Unlike nutrition-based organizational and agronomic biofortification programs, the transgenic approach is an alternative and sustainable (White and Broadley 2005; Hefferon 2016). Transgenic lines were developed for β-carotene, high zinc, high protein, high iron, low phytate, and folic acid. Successful examples of transgenic products are high lysine maize, high unsaturated fatty acid soybean, high provitamin A and iron-rich cassava, and high provitamin A Golden rice (Garg et al. 2018).

11.3.1 Cereals Biofortification to Alleviate Malnutrition in India

The principal cereal food grain cultivated in India is rice, followed by wheat, maize, sorghum, and pearl millet. To date, a lot of research work which has been carried out in cereals crops with proof of concept using various biofortification approaches through national and international biofortification collaboration programs are discussed as follows:

11.3.1.1 Rice

Staple food crops such as rice (*Oryza sativa*) play a pivotal role in the Indian economy. Rice consumption is ~220 g per day, and thus, rice is a significant calorie supplement for two-thirds of the Indian population. Polished rice is a poor source of micronutrients (Eric and Eddie 2012). Various rice-growing countries, including India, have a primary consideration of emphasizing increasing the nutritional quality of rice. Biological and genetic enrichment of food products with vital nutrients, vitamins, and proteins aimed at rice biofortification program. Production of nutrient-packed rice grains in a sustainable way so that the product reaches the malnourished population in rural India can be possible with rice biofortification with vital nutrients so that the farmer can grow the variety indefinitely without any additional input. Conventional breeding can be used to enhance Zn and protein contents in polished rice, whereas transgenic technology appeared to be the only viable solution for increasing β-carotene and Fe.

Fe and Zn

Iron deficiency is one of the most prevalent micronutrient deficiencies affecting more than two billion people worldwide (World Health Organization 2016). Low dietary intake of Zn puts one-third of the world population at risk, including two billion people in Asia and four hundred million in Saharan Africa (Hotz and Brown 2004; Myers et al. 2015). Iron and zinc concentrations in brown rice are in the range of 6.3–24.4μg/g and 13.5–28.4μg/g, respectively. Therefore, there was an approximately twofold difference in iron and zinc concentrations, suggesting a vast genetic potential to increase the concentration of these micronutrients in rice grains (Gregorio et al. 1999). Many promising donors were identified by screening

thousands of rice germplasm lines for Fe and Zn contents in brown and polished grain across the world. However, approximate loss of micronutrients Fe and Zn during the polishing is about 90% and 40%, respectively (Babu 2013; Pinson et al. 2015). A total of 126 accessions, including cultivated *indica* and *japonica* rice cultivars, germplasm accessions, and wild rice genotypes of brown rice, were analyzed for Fe and Zn concentration in brown rice by Anuradha et al. (2012) in which Fe concentration ranged from 6.2 ppm to 71.6 ppm, and Zn concentration ranged from 26.2 ppm to 67.3 ppm.

Similarly, Roy and Sharma (2014) analyzed rice landraces (84 cultivars), which were collected from various agro-ecological regions of West Bengal and adjoining areas for Fe and Zn. The concentration of Fe and Zn varied from 0.25μg/g to 34.8μg/g and 0.85μg/g to 195.3μg/g, respectively. Identification of these micronutrient-rich genotypes opens up the possibilities for the linkage mapping of genomic regions or QTLs responsible for mineral uptake and translocation, which can be subsequently used as a donor for developing nutrient-enriched varieties. Genes associated with Zn metabolism and QTL for grain Zn concentration have been reported in rice (Swamy et al. 2016). ICAR-Indian Institute of Rice Research (IIRR), Hyderabad has released "DRR Dhan 45" using a donor from the HarvestPlus program. Chhattisgarh zinc rice-1, one of the high nutrient content varieties in polished rice, was released for the state of Chhattisgarh by Indira Gandhi Krishi Viswavidyalaya (IGKV), and "Mukul" (CR Dhan 311) was released for the state of Odisha by ICAR-National Rice Research Institute (NRRI). Expression of ferritin genes, nicotianamine synthase genes (NAS), or ferritin in conjunction with NAS genes to increase the Fe concentration of rice endosperm using transgenic technology could lead to a twofold and sixfold increase via single-gene and multi-gene approaches, respectively. Expression of Fe storage protein ferritin under the control of endosperm-specific promoters can increase the Fe storage capacity of rice grains. This approach can increase the concentration of Fe in the seeds of transformants by approximately twofold in polished seeds. Iron stored in ferritin is an important source for humans to avoid iron deficiency. Therefore, transgenic rice with 3–4 times as much Fe than wild-type rice was developed using different sources of the ferritin gene (Masuda et al. 2012; Paul et al. 2014).

Protein

Essential amino acids are crucial for normal growth and metabolism and cannot be synthesized de novo by humans, especially lysine (Lys) and methionine (Met) (Lee et al. 2003; Ufaz and Galili 2008; Cohen et al. 2014). Milled rice grains are a poor source of essential amino acids. Therefore, one of the main goals of breeders is to increase the lysine content in cereal grains, especially rice, to enhance the nutritional value of grains and prevent nutrient deficiency diseases such as kwashiorkor (Toride 2004). Lysine content has been enhanced in some cereals by a combination of conventional breeding and mutagenesis approaches. Moreover, it is challenging to improve this trait in most grains due to the limited availability of lysine-rich germplasm resources, particularly in the case of rice (Sun and Liu 2004). Lysine levels in crops can be increased with the development of molecular biological

techniques, and three strategies have been developed (Birla et al. 2015). The direct approach is over-expressing lysine-rich proteins in grains of rice (Wong et al. 2015), maize (Yu et al. 2004), and sorghum (*Sorghum bicolor* L.) (Zhao et al. 2003). Another approach is to modify seed storage proteins, e.g. silencing of 13 KDa prolamin encoding genes increased total lysine content by 56% and thus altered nutritional quality in rice (Kawakatsu et al. 2010). The last approach is to use metabolic engineering to regulate the key genes involved in lysine metabolism to increase lysine content in plants (Zhu and Galili 2003; Long et al. 2013). Several landraces and released varieties have been characterized for their protein and amino acid profiles in rice, and "Heera," an old variety of rice, was found to have >10% protein. The mean crude protein content of the varieties, as estimated using the Kjeldahl method, was in the range of 6–8% (Juliano 1993). CR Dhan 310 with >10% protein in polished rice developed by NRRI has also been nationally released. Genomic regions and genes associated with protein in rice have been deciphered (Rawat et al. 2013; Bao 2014; Garg et al. 2018).

Provitamin A

Vitamin A is a fat-soluble vitamin playing an essential role in vision, bone growth, reproduction, and in the maintenance of healthy skin, hair, and mucous membranes (FAO/WHO 2002). Among 118 countries primarily in Africa and South-East Asia, Vitamin A deficiency (VAD) is a global public health problem (Rostami et al. 2007). The vitamin A status of the poor can be addressed by an emerging strategy of biofortification of staple crops with provitamin A, and carotenoids (Tanumihardjo 2008; Tanumihardjo et al. 2008). For example, a bioengineered provitamin A enriched rice in India, Philippine, and Brazil is Golden Rice. In Asian countries, up to 73% of energy intake can be from rice. So vitamin A intake of vulnerable groups in developing countries can be increased by the enrichment of rice with vitamin A. Three genes for biosynthesis of β-carotene in grain were used to create golden rice, and the latest version is GR2R with >20 ppm of total carotenoids (Rawat et al. 2013). IARI, IIRR, and Tamil Nadu Agricultural University (TNAU) in India were the three research groups which were involved in the development of Indian versions of golden rice from the original prototype in collaboration with the International Rice Research Institute (IRRI) supported by the Department of Biotechnology (DBT), India.

11.3.1.2 Wheat

Wheat is the second important staple cereal after rice in India and the foremost target crop under the biofortification program and has significantly contributed in reducing hunger and malnutrition. Low genetic variation in cultivated wheat has been observed for Zn and Fe. However, wild relatives (*Triticum spelta*, *Aegilops tauschii*), emmer wheat, and different landraces are known to have wide variation for grain micronutrient (Zn and Fe) concentrations up to 190 ppm, and have been exploited for improvement of modern elite cultivars using biofortification approaches (Monasterio and Graham 2000; Cakmak et al. 2004; Ortiz-Monasterio et al. 2007; Garg et al. 2018).

Fe and Zn

Huge genetic variability for grain yield and micronutrient (Zn and Fe) has been observed in wheat germplasm. Wheat germplasm screening studies in hexaploid wheat genotypes revealed twofold variation, i.e. 25–55 ppm for micronutrient (Zn and Fe) contents, whereas in diploid wheat genotypes, it was fourfold (up to 100 ppm) (Chhuneja et al. 2006; Ortiz-Monasterio et al. 2007). The wheat varieties developed before the green revolution showed higher Zn and Fe contents than that of varieties developed after the green revolution, as reported by ICAR-Indian Institute of Wheat and Barley Research (IIWBR). The possible reason could be the selection for high-yielding varieties rather than the varieties with high nutritional quality (Neeraja et al. 2017). But because of today's need for nutritional security, Department of Biotechnology, Government of India, has started a wheat biofortification program for enhanced micronutrients using conventional and molecular breeding, and different biofortified varieties have been released in India. Indian Council of Agricultural Research, in collaboration with HarvestPlus wheat Biofortification program, has released different biofortified wheat varieties with 4–10 ppm higher Zn content. Biofortified wheat (high Zn content) varieties such as "BHU 1, BHU 3, BHU 5, BHU 6, BHU 7, and BHU 18" were released in India in 2014.

Along with high zinc content, BHU 1 and BHU 6 also had shown higher yield and disease resistance. Punjab Agricultural University, Ludhiana, India has also recently released high Zn wheat variety "PBW1Zn." Indian Institute of Wheat and Barley Research, Karnal, India, has also developed and released high Zn and Fe content wheat variety "WB2" (Rawat et al. 2013; Garg et al. 2018). Using the transgenic breeding biofortification approach, the Fe content in wheat genotypes has been enhanced by the expression of the ferritin gene (*TaFer1-A*) from wheat (Borg et al. 2012) and soybean (Xiaoyan et al. 2012).

Low Phytate

To decrease the phytate or phytic acid content in wheat, sizeable genetic variability in synthetic wheat hexaploid genotypes up to sixfold for the phytase level has been reported. Therefore, the Indian Institute of Wheat and Barley Research (IIWBR) developed a microlevel test to transfer the high phytate level traits into the high-yielding backgrounds (Ram et al. 2010). Expression of the phytochrome gene (*phyA*) resulted in increased phytase activity, leading to enhanced iron bioavailability (Brinch-Pederson et al. 2000). Bhati et al. (2016) reported a decrease in phytic acid by silencing of wheat *ABCC13* transporter.

Protein

Wild tetraploid emmer wheat (*Triticum turgidum* ssp. *dicoccoides*) has been identified with high grain protein content and micronutrient using molecular breeding approaches. Distelfeld et al. (2007) identified quantitative trait loci (QTL) (*Gpc-B1*) in *dicoccoides* wheat for high grain protein content and transferred into cultivated bread and durum wheat. In India, under the AICRP-Wheat biofortification program, Punjab Agricultural University (PAU), IIWBR and IARI transferred the *Gpc-B1* QTL into different high-yielding wheat accessions and developed genotypes

are being tested. Using transgenic breeding approaches of biofortification, transfer of the *Amaranthus* albumin gene (*Ama1* gene) into elite wheat cultivars resulted in increased wheat grain protein content, particularly essential amino acid content (lysine, methionine, cysteine, and tyrosine) (Tamas et al. 2009).

Provitamin A

Provitamin A is also an important targeted nutrient under wheat biofortification through breeding approaches. Indian Agricultural Research Institute (IARI), New Delhi has commercially released the high provitamin A durum wheat variety "HI 8627." By expressing bacterial *psy* gene and carotene desaturase genes (c*rtB, crtI*) using transgenic technology, wheat provitamin content was enhanced (Cong et al. 2009; Garg et al. 2018).

11.3.1.3 Maize

Maize is utilized as a human food and livestock feed, and thus it assumes worldwide significance. India is the second-most important maize growing country in Asia and is the world's sixth-largest producer and the fifth largest consumer of maize (Prasanna 2014). Around 73% of farmland dedicated to maize production worldwide is located in the developing world. In India, 10% of the total production of maize is used for human food, while 60% is utilized for poultry- and animal-feed (Yadav et al. 2015). Important breakthroughs in maize biofortification are because of the vital role of maize in global diets and the rich genetic diversity of the crop. Thus biofortification of maize, including enhancement of protein quality coupled to the enrichment of micronutrients like provitamin A, Fe, and Zn in grain assumes great significance.

Fe and Zn

Fe and Zn contents are low in maize, a staple crop of Southern and Eastern Africa (CIMMYT 2000). Banziger and Long (2000) reported genetic variability for Fe and Zn in white grained tropical maize germplasm ranging from 16.4–22.9μg/g and 14.7–24.0μg/g, respectively. Accessions (1814) were also evaluated in 13 trials over 6 years, and a range in the grain of 9.6–63.2 mg-Fe/kg and 12.9–57.6 mg-Zn/kg was reported (Neeraja et al. 2017). Fe content was 15–159 ppm in mid-altitude and 14–134 ppm for low land inbred lines, and Zn content was 12–96 ppm for mid-altitude and 24–96 ppm for lowland inbred lines in maize germplasm, showing that sufficient genetic variation is available in maize germplasm (Pixley et al. 2011). In India, wide genetic variation for kernel Fe and Zn in a diverse set of normal and quality protein maize (QPM)-inbreds was reported by Chakraborti et al. (2009), Chakraborti et al. (2011), Prasanna et al. (2011), Agrawal et al. (2012), Guleria et al. (2013), Goswami et al. (2014), Mallikarjuna et al. (2014), and Pandey et al. (2015). The presence of ample variability for kernel Fe and Zn indicates the possibility of genetic enhancement of these micronutrients in maize. Accumulation of Fe and Zn in the maize kernel is governed by polygenes (Gorsline et al. 1964; Arnold and Bauman 1976). QTLs governing the accumulation of these micronutrients in

maize have also been reported (Lungaho et al. 2011; Simic et al. 2011; Qin et al. 2012; Baxter et al. 2013).

Low phytate

Phytic acid/phytate, which is an anti-nutritional component, plays a major role in reducing the bioavailability of Fe and Zn; thus, it is an important target for biofortification in maize. Phytate in the seed has a primary function to store phosphorus as an energy source and antioxidants essentially required for the germinating seeds, and 80% of the total phosphorus in the maize grain is present as phytic acid (Raboy et al. 2000). But the positively charged minerals like Fe and Zn get chelated by the negative charge of the phytic acid and make them unavailable in the animal gut (Raboy 2001). Undigested phytic acid, when released into the environment, causes environmental pollution because phytic acid in grains reduces the availability of phosphorus to poultry since monogastric animals cannot digest it (Cromwell and Coffey 1991). Low phytic acid mutants can be produced by the mutations in genes encoding myo-inositol-3-Pi synthase (*MIPS*) and inositol polyphosphate kinases. Myo-inositol-3-Pi synthase (*MIPS*) followed by inositol phosphate kinases convert glucose 6-phosphate to inositol-3-phosphate for phytic acid synthesis in plants. Chemical or radiation-induced mutagenesis is being used to develop low phytic acid (*lpa*) mutants. These lpa mutants include *lpa1* mutant of maize (Raboy et al. 2000), barley (Larson et al. 1998; Rasmussen and Hatzack 1998), and rice (Larson et al. 2000). In maize, *lpa1*, *lpa2,* and *lpa3* are three low phytic acid (*lpa*) mutants with 66%, 50%, and 50% reduction in phytic acid content. The seeds of lpa mutants have been found to be viable and normal. In India, scientists at Tamil Nadu Agricultural University (TNAU), Coimbatore, successfully introgressed the *lpa2–2* allele into elite inbreds and marker-assisted introgression of *lpa1* and *lpa2* mutants in early maturing inbreds, viz. "CM145" and "V334," has been carried out at Vivekananda Parvatiya Krishi Anusandhan Sansthan (VPKAS) (Kumar et al. 2014; Gupta et al. 2015). Breeding for low phytate maize also offers several advantages both as food and feed.

Protein

In many developing countries of Latin America, Africa, and Asia, maize is the major staple food and often the only source of protein. Early work on maize biofortification was mainly focused on improving protein quality (Vasal 2000; Krivanek et al. 2007). There is a low level of essential amino acids such as lysine and tryptophan in traditional maize and thus possess poor endosperm protein, which is essential to humans and monogastric animals (FAO 1999). *Opaque* 2 mutant maize with high lysine and tryptophan content was discovered in Connecticut, USA, while screening for maize lines with better amino acid (Mertz et al. 1964). Several modern maize varieties collectively referred to as quality protein maize (QPM), which have improved protein quality, and agronomic characteristics were produced by conventional breeding approach and are currently being actively disseminated, particularly in Sub-Saharan Africa (Krivanek et al. 2007). With the discovery of maize mutant *opaque2* having enhanced nutritional quality, the number of open-pollinated

varieties and hybrids in quality protein maize (QPM) genetic backgrounds have been released worldwide, and to develop locally adapted QPM germplasm, marker-assisted selection has been applied (Danson et al. 2006). In India, in comparison to more than a hundred non-QPM/normal maize hybrids, only a dozen QPM hybrids have been released (Yadav et al. 2015). In 1970, India released its first soft endosperm-based nutritious maize composites, then in 1997, first hard-endosperm QPM composite was released, and the first QPM hybrid was released in 2001. Since then, various ICAR institutes and SAUs in India are engaged in the development of the QPM version of elite commercial hybrids for different agro-ecologies of the country (Gupta et al. 2009). The first report of marker-aided selection (MAS) of *opaque2* was the commercial release of "Vivek QPM." QPM provides a valuable model for the development, evaluation, targeting, and dissemination of biofortified crops, as most biofortification efforts are still in the early stages of research and development.

11.3.1.4 Pearl Millet

Pearl millet is one of the most important staple food crops grown in the arid and semi-arid tropical regions of Africa and Asia, and can be used to achieve food and nutritional security. The advantage of growing pearl millet is that it can tolerate high temperature, adaptation to soil salinity, and drought that increase the significance of this crop in varied adverse climatic conditions. Pearl millet also has inherent high nutritional values (dietary carbohydrates, energy, protein, and minerals (calcium, Fe, and Zn) and climate-resilient nature (drought and heat). To a great extent, pearl millet research progress is ongoing to assess the magnitude of genetic variability, optimizing efficient germplasm screening procedures, development and adoption with the objective of improvement in the breeding efficiency for pearl millet grain iron (Fe) and zinc (Zn) contents. Pearl millet is one of the key crops under the HarvestPlus biofortification challenge program, and most of the research work for its nutrition enhancement (high in Zn and Fe) is ongoing at ICRISAT in collaboration with AICRP at ICAR institutes/universities in India. The Indian government has also added pearl millet to the cereals of the public distribution system under the National Food Security Act/Mission (Govindaraj et al. 2018).

Pearl millet germplasm has been screened and explored for genetic diversification for high Fe and Zn contents at national and international biofortification programs. Large genetic variability (30–140 mg/kg Fe and 20–90 mg/kg Zn) for genetic improvement of grain Fe with Zn micronutrients content was reported in pearl millet populations, and parental lines were effectively utilized to develop high-yielding cultivars with high Fe and Zn contents in advanced breeding lines and hybrids (Rai et al. 2012). "Dhanashakti" is the first biofortified pearl millet cultivar rich in Fe that has been released in India and included in the Nutri-Farm Pilot Project of the Government of India for addressing Fe deficiency in India (Rai et al. 2014). Dhanshakti and Chakti (high Fe and Zn contents) are the open-pollinated varieties of pearl millet with hybrids (ICMH 1202, ICMH 1203, and ICMH 1301) which have shown high grain yield (>3.5 tons/ha) and high levels of Fe (70–75 mg/kg) and Zn (35–40 mg/kg) contents. Presently, India is growing more than 70,000 ha of

biofortified pearl millet hybrids/varieties. This increased adoption is due to significantly higher yields with enhanced nutrients, resistance to downy mildew disease, and tolerance to drought. Clinical research on biofortified "Dhanshakti" pearl millet cultivar has shown that 200 g of its daily consumption meets 100% of the recommended daily allowance (RDA) of Fe in adult men and children and 60% of the RDA in non-pregnant and nonlactating women in India (Neeraja et al. 2017). In India, the ICAR-AICRP-Pearl millet biofortification program has led to the development of various biofortified breeding lines with enhanced micronutrient (>80 ppm Fe and >50 ppm Zn) content, and currently being evaluated for their consistent performance. High-Fe (62–65 ppm) pearl millet cultivars "ICTP 8203" has been commercially grown in the Maharashtra state of India on more than 200,000 hectares. Biofortified pearl millet hybrid "MH 1928" with high Fe (>61 ppm) along with higher grain yield has been released at the national level, and various other hybrids with high Fe and Zn (>70 ppm) contents are in the pipeline and at testing stage (Govindaraj et al. 2018).

11.3.1.5 Sorghum

Sorghum is one of the top ten crops that feed this world and an important cereal crop in hot and dry agro-ecologies. After pearl millet, sorghum is the second cheapest source of energy and micronutrients and fifth most important cereal crop globally, and in the semi-arid tropics (Parthasarathy et al. 2006; Reddy and Reddy 2018). Sorghum has high photosynthetic efficiency as it is a C_4 species, and also it has inherent high biomass yield potential. Under the climate change scenario, sorghum has proved to be more relevant for food security because of the high levels of tolerance to drought and high temperature and adaptation to soils. Therefore, the biofortification of sorghum by increasing protein and mineral micronutrients (especially Fe and Zn) is of high priority as sorghum is among the cheapest sources of micronutrients. Therefore, to tackle India's double burden of malnutrition, a biofortified variety of unpopular staple food has attracted the attention of scientists. In India, sorghum contributes around 50% of the total cereal intake (75 kg grain per head per year), especially by rural consumers in the major production regions. Public bred cultivars, and parental lines showed wide variability for grain Fe (12–68 ppm) and Zn (11–44 ppm) in the studies at ICAR-Indian Institute of Millets Research (IIMR) (Hariprasanna et al. 2014). The Indian national program on sorghum with comprehensive testing in co-ordinated trials has released over 31 hybrids and 25 varieties for commercial cultivation (Reddy and Reddy 2018).

Increasing mineral micronutrients (especially Fe and Zn) in the grain is of widespread interest, which can be achieved by biofortification of sorghum (Pfeiffer and McClafferty 2007; Zhao 2008; Kumar et al. 2009). Kumar et al. (2013) observed that rainy season-specific sorghum commercial hybrids possess better Fe and Zn contents than post-rainy sorghums. Agronomic attempts by external application of Fe and Zn fertilizers were also made for biofortification, but there was no significant increase in grain Fe or Zn (Mishra et al. 2015). Bioavailability of grain Fe and Zn was affected by high variability for anti-nutritional factors like polyphenols, phytate, and fibers (Hariprasanna et al. 2015). It was reported that the bioaccessibility of Fe

and Zn from sorghum was very low at 4.13% and 5.51%, respectively (Hemalatha et al. 2007). A total of 2267 core germplasm accessions were screened at ICRISAT, and promising donors were identified with Fe ranging from 20–70 ppm and Zn from 13–47 ppm under the HarvestPlus program. They developed improved breeding lines with high yield and high grain Fe and Zn by exploiting the large variability in core collections. In the Maharashtra state of India, ICRISAT-bred biofortified sorghum line ICSR 14001 with 50% higher Fe and Zn than base-level out-yielded all other entries in the state multi-location trials. All India Coordinated Sorghum Improvement Project (AICSIP) proved ICSR 14001 superior under on-farm testing, and it is under testing towards its commercialization. Parbhani Shakti is being touted as India's first biofortified variety of sorghum, a plant from which grain and other crops are grown. Sorghum hybrids with high Fe and Zn are being developed at national and international programs. Recently in Nigeria, a sorghum variety "12KNICSV-188" with three times higher Fe (129 ppm) content and high yield was released.

11.3.2 The Recent Breakthroughs in Cereal Biofortification in India

For achieving nutritional security, biofortification, along with dietary supplementation and diversification, is a sustainable approach. India has executed an "enormous scale-up" of two national projects, i.e. Integrated Child Development Services and National Health Mission, to address nutrition. Still, these do not seem to accomplish sufficient inclusion (Menon et al. 2017). In India, National Nutrition Strategy (NNS), which envisages a Kuposhan Mukt Bharat, was launched on 5th September 2017, by National Institution for Transforming India (NITI Aayog). The main strategies of this program are to provide nutritious food, income and livelihood, health service, and drinking water and sanitation, which will possibly contribute to national food nutrition security. HarvestPlus is the Consultative Group on International Agricultural Research (CGIAR) Biofortification Challenge program. The main objective of this program is to tackle the global hidden hunger/malnutrition issues by a collaborative approach, including different national and international organizations/institutions/agencies (Bouis and Saltzman 2017). This biofortification program worked as a collaborative effort that involved multi-CGIAR teams including various plant scientists, plant geneticists/breeders, nutritionists, food scientists, economists, and social specialists. The goal of this group was to address micronutrient malnutrition by producing staple food crops with improved levels of bioavailable essential nutrients (minerals and vitamins) to enhance the nutrient status of target populations, primarily resource-poor people in the developing countries, such as India. Three essential issues were recognized to make biofortification fruitful: (I) development of biofortified varieties/hybrids must be having high yields and that are advantageous to the agriculturist/farmers, (II) the developed varieties should have a bioavailability of nutrients and effective in reducing hidden hunger problems due to micronutrient deficiencies, and (III) the biofortified crop should be as per farmer's and consumer's

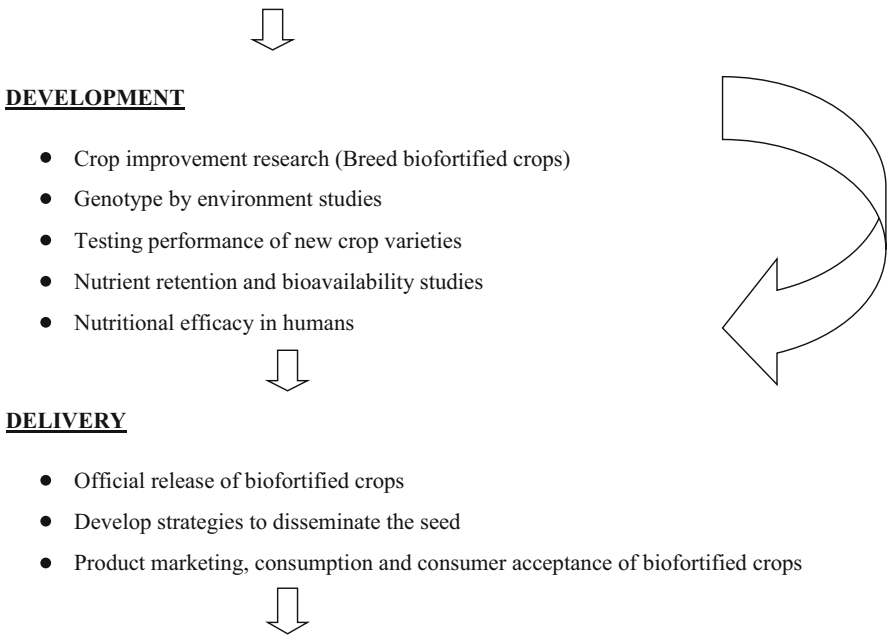

Fig. 11.2 HarvestPlus pathway for biofortification (modified from Bouis et al. 2011)

acceptance in target regions where people are severely suffered from micronutrient malnutrition {(Fig. 11.2) (Hotz and McClafferty 2007; Bouis and Saltzman 2017)}.

In India, initially, most of the scientific research efforts were made in agriculture to achieve food grain' self-sufficiency. But today, to resolve the major issues of the country's nutritional security, staple food crop biofortification to overcome the hidden hunger problem has become of utmost importance (Neeraja et al. 2017). By understanding the importance of crop biofortification, the Indian Council of Agriculture Research (ICAR), Govt. of India has endorsed the Consortia Research Platform (CRP) to enhance the nutritional status of the country's major staple food crops such as rice, wheat, maize, sorghum, pearl millet, and small millet by collaboration among different ICAR institutes, state agricultural universities (SAU), traditional universities, and Indian Council of Medical Research (ICMR). Consortia Research Platform (CRP) has focused on developing cereals biofortified with enhanced β-carotene, quality protein, Fe, and Zn using different biofortification approaches (Neeraja et al. 2017; Yadava et al. 2017). All human beings on this

earth have a right to nutritious food, so biofortification is a cost-effective and sustainable approach to address the hidden hunger/malnutrition problem. So far, the Indian Council of Agricultural Research (ICAR) has significantly improved the nutritional quality in high-yielding varieties of the country's staple food crops and released a large number of biofortified crop varieties as a sustainable way to alleviate malnutrition which could integrate into the food chain and ensure nutritional security (Table 11.3). For the rice biofortification program, ICAR consortia research platform, HarvestPlus, Department of Biotechnology (DBT), Department of Science and Technology (DST), and other supporting agencies, along with the Indian Institute of Rice Research (IIRR), Hyderabad, National Rice Research Institute (NRRI), Cuttack, Odisha, Indian Agricultural Research Institute (IARI), New Delhi, Indira Gandhi Krishi Vishwavidyalaya (IGKV), Raipur, University of Agricultural Sciences (UAS), Bengaluru, and Tamil Nadu Agricultural University (TNAU), Coimbatore have screened and identified rice germplasm with high Zn, Fe, and protein contents. Different rice breeding lines with improved nutritional content are being evaluated under the All India Coordinated Research Project (AICRP) on Rice.

Similarly, for wheat biofortification programs, the Indian Institute of Wheat and Barley Research (IIWBR), IARI, Punjab agricultural university (PAU), Chaudhary Charan Singh Haryana Agricultural University (CCSHAU), UAS, Dharwad have screened and identified wheat germplasm with higher Fe and Zn contents and are being tested at multi-locations. Different biofortified wheat lines high in Zn content developed through the HarvestPlus biofortification program and ICAR are under field trials in India and Pakistan. Globally, particularly in India, various biofortified QPM hybrids with improved lysine and tryptophan contents have been released and commercially cultivated by farmers. Various experimental QPM hybrids are currently under different stages of the All India Coordinated Research Project (AICRP) on Maize. Recently, the Indian Agricultural Research Institute (IARI), New Delhi has developed a provitamin A-rich maize hybrid by introgression of *crtRB1* into parental lines of elite hybrids under AICRP-Maize. By combined introgression of *opaque2*, *crtRB1,* and *lcyE* genes, IARI led to the development of multi-nutrient maize (Neeraja et al. 2017). Biofortified pearl millet variety "Dhanashakti" rich in iron (71 ppm) largely adopted by farmers in India has been released by the All India Coordinated Research Project (AICRP) on Pearl millet along with the private seed sector.

However, the critical concern for the developed biofortified food crops is the bioavailability of enhanced nutrients like Fe and Zn in human beings which are primarily affected by anti-nutritional factors like phytate/phytic acid or phytin (Raboy 2001). Bioavailability means a measurable amount of the nutrient quantity ingested that experiences intestinal absorption and utilization. Therefore, the bioavailability of nutrients from biofortified food crops is often the key to success (Neeraja et al. 2017). Recently, biofortified rice pure line varieties, i.e. CR Dhan 310 (protein-rich variety) and DRR Dhan 45 (Zn rich variety); biofortified wheat pure line varieties, i.e., WB 02 (Zn and Fe rich variety), and HPBW 01 (Fe and Zn rich variety); biofortified maize hybrid, i.e., Pusa Vivek QPM9 Improved (provitamin A, lysine and tryptophan-rich hybrid) and Improved Pusa (HM4,

Table 11.3 Cereals biofortified varieties developed by the Indian Council of Agriculture Research (ICAR), India as a sustainable way to alleviate malnutrition

Biofortified varieties/ hybrid	Improved nutrient content	Grain yield	Maturity	Adaptation	Developed by
Rice					
CR Dhan 310 (protein-rich pure line variety)	10.3% protein in polished grain as compared to 7.0–8.0% in popular varieties	45.0 q/ha	125 days	Odisha, Madhya Pradesh, and Uttar Pradesh	ICAR-National Rice Research Institute, Cuttack, Odisha
DRR Dhan 45 (Zn rich pure line variety)	High in Zn content (22.6 ppm) in polished grains in comparison to 12.0–16.0 ppm in popular varieties	50.0 q/ha	125–130 days	Karnataka, Tamil Nadu, Andhra Pradesh, and Telangana	ICAR-Indian Institute of Rice Research, Hyderabad
Wheat					
Wheat WB 02 (zinc and Fe rich pure line variety)	Rich in Zn (42.0 ppm) and Fe (40.0 ppm) in comparison to 32.0 ppm Zn and 28.0–32.0 ppm Fe in popular varieties	51.6 q/ha	142 days	Punjab, Haryana, Delhi, Rajasthan (excluding Kota and Udaipur division), Western UP (except Jhansi division), Jammu and Kathua district of J & K, Paonta Valley and Una district of HP and Tarai region of Uttarakhand	ICAR-Indian Institute of Wheat and Barley Research, Karnal, Haryana
HPBW 01 (Fe and Zn rich pure line variety)	Contains high Fe (40.0 ppm) and Zn (40.6 ppm) in comparison to 28.0–32.0 ppm Fe and 32.0 ppm Zn in popular varieties	51.7 q/ha	141 days	Punjab, Haryana, Delhi, Rajasthan (excluding Kota and Udaipur division), Western UP (except Jhansi division), Jammu and Kathua district of J & K, Paonta	Punjab Agricultural University, Ludhiana under ICAR-All India Coordinated Research Project on Wheat and Barley

				Valley and Una district of HP and Tarai region of Uttarakhand	
Maize					
Maize Pusa Vivek QPM9 Improved (provitamin A, lysine and tryptophan-rich hybrid)	Country's first provitamin A rich maize • High provitamin A (8.15 ppm), lysine (2.67%) and tryptophan (0.74%) as compared to 1.0–2.0 ppm provitamin A, 1.5–2.0% lysine and 0.3–0.4% tryptophan content in popular hybrids	55.9 q/ha [Northern Hills Zone (NHZ)] and 59.2 q/ha [Peninsular Zone (PZ)]	93 days (NHZ) and 83 days (PZ)	Kharif season in J&K, Himachal Pradesh, Uttarakhand (Hill region), North Eastern states, Maharashtra, Karnataka, AP, Telangana, and Tamil Nadu	ICAR-Indian Agricultural Research Institute, New Delhi
Pusa HM4 Improved (lysine and tryptophan-rich hybrid)	Contains 0.91% tryptophan and 3.62% lysine which is significantly higher than popular hybrids (0.3–0.4% tryptophan and 1.5–2.0% lysine)	64.2 q/ha	87 days	Kharif season in Punjab, Haryana, Delhi, Uttarakhand (Plain), Uttar Pradesh (Western region)]	ICAR-Indian Agricultural Research Institute, New Delhi
Pusa HM8 Improved (lysine and tryptophan-rich hybrid)	Rich in tryptophan (1.06%) and lysine (4.18%) as compared to 0.3–0.4% tryptophan and 1.5–2.0% lysine in popular hybrids	62.6 q/ha	95 days	Kharif season in Maharashtra, Karnataka, Andhra Pradesh, Telangana, Tamil Nadu	ICAR-Indian Agricultural Research Institute, New Delhi
Pusa HM9 Improved (lysine and	Contains 0.68% tryptophan and 2.97% lysine compared to	52.0 q/ha	89 days	Kharif season in Bihar, Jharkhand, Odisha, Uttar Pradesh (Eastern	ICAR-Indian Agricultural Research Institute, New Delhi

(continued)

Table 11.3 (continued)

Biofortified varieties/ hybrid	Improved nutrient content	Grain yield	Maturity	Adaptation	Developed by
tryptophan-rich hybrid)	0.3–0.4% tryptophan and 1.5–2.0% lysine in popular hybrids			region), and West Bengal	
Pearl Millet					
HHB 299 (Fe and Zn-rich hybrid)	High Fe (73.0 ppm) and zinc (41.0 ppm) as compared to 45.0–50.0 ppm Fe and 30.0–35.0 ppm Zn in popular varieties/ hybrids	32.7 q/ha	81 days	Kharif season in Haryana, Rajasthan, Gujarat, Punjab, Delhi, Maharashtra, and Tamil Nadu	CCS-Haryana Agricultural University, Hisar in collaboration with ICRISAT, Patancheru under ICAR-All India Coordinated Research Project on Pearl millet
AHB 1200 (Fe rich hybrid)	Rich in Fe (73.0 ppm) in comparison to 45.0–50.0 ppm in popular varieties/ hybrids	32.0 q/ha	78 days	Kharif season in Haryana, Rajasthan, Gujarat, Punjab, Delhi, Maharashtra, and Tamil Nadu	Vasantrao Naik Marathwada Krishi Vidyapeeth, Parbhani in collaboration with ICRISAT, Patancheru under ICAR-All India Coordinated Research Project on Pearl millet
Sorghum					
Improved variety (higher Fe and Zn) ICSR 14001, released as "Parbhani Shakti"	Fe concentration of 45 ppm and Zn 32 ppm compared to varieties that are currently being cultivated in India (Approx 30 ppm Fe and 20 ppm Zn). Higher	Yield levels are higher (>5.0 tons/ha) in post-rainy and summer seasons with irrigation	Tolerate higher temperatures (41 °C) at flowering and seed setting, but the flowering may be delayed (80 days)	Maharashtra and different sorghum growing regions of India It was released as a rainy season variety (*Kharif*), but it can be	ICRISAT under the HarvestPlus sorghum biofortification project under All India Coordinated Sorghum Improvement Project and released for

	protein (11.9%) and low phytate content (4.14 mg/100 g) compared to 10% protein and 7.0 mg/ 100 g phytate content in most sorghum cultivars			grown in post-rainy (*Rabi*) and summer seasons	cultivation by Vasantrao Naik Marathwada Krishi Vidyapeeth (VNMKV), Maharashtra

HM8, HM9) (lysine and tryptophan-rich hybrid); biofortified Pearl millet hybrid, i.e., HHB 299 (Fe and Zn rich hybrid) and AHB 1200 (Fe rich hybrid) were released for commercial cultivation (Yadava et al. 2017; Neeraja et al. 2017).

In July 2018, India's first biofortified sorghum (jowar) variety ICSR 14001 was released as "Parbhani Shakti," which is rich in Fe and Zn than traditionally grown sorghum varieties. It was developed under HarvestPlus biofortification programs by International Crops Research Institute for the Semi-Arid Tropics (ICRIAT), Patancheru, Hyderabad, and All India Coordinated Sorghum Improvement Project and released for large-scale seed production, dissemination, and commercial cultivation by Vasantrao Naik Marathwada Krishi Vidyapeeth (VNMKV), Maharashtra. Biofortified sorghum "Parbhani Shakti" having Fe concentration 45 ppm and Zn 32 ppm as compared to other varieties that are at present cultivated in India (Approx 30 ppm Fe and 20 ppm Zn). Improved biofortified varieties have also shown higher protein (11.9%) and low phytate content (4.14 mg/100 g) compared to 10% protein and 7.0 mg/100 g phytate content in most sorghum cultivars. It also tolerates higher temperatures (41 °C) at flowering and seed set, but the flowering may be delayed (80 days). Yields are higher (>5.0 tons/ha) in post-rainy and summer seasons with irrigation. It was released as a rainy season variety (Kharif), but it can also be grown in Rabi and summer seasons in different sorghum growing regions. Recently, in 2020, different nutritionally enriched cereals biofortified varieties were released, viz. rice (CR Dhan 315, high zinc); wheat (HI 1633, rich in protein, iron and zinc; HD 3298, protein and iron-rich; DBW 303, protein-rich and DDW 48, protein-rich); maize (Ladhowal Quality Protein Maize Hybrid 1, 2, and 3, lysine and tryptophan-rich); finger millet (CFMV1 and 2, rich in calcium, iron, and zinc) and little millet (CLMV1, iron-zinc rich). ICAR has introduced the Nutrition-Sensitive Agricultural Resources and Innovations (NARI) program to promote family farming that linked agriculture to nutrition, nutrition-smart villages to boost nutritional stability, and KVKs are designing and promoting unique nutrition garden models to ensure access to locally accessible, balanced, and diversified diets with adequate macro and micronutrients. To alleviate malnutrition and make India Kuposhan Mukta by naturally enriched food ingredients, the production of biofortified crop varieties will be upgraded and connected to government programs of mid-day meal, Anganwadi, etc. This would also contribute to increased farmers' incomes and open up new means of developing entrepreneurship.

11.4 Conclusion

India is one of the richest agro-biodiversity countries, and scientific research is continuously evaluating the promising germplasm for enhanced nutrients in staple food crops to alleviate malnutrition. Biofortification is a promising, cost-effective, and sustainable agricultural strategy for improving the nutritional status of malnourished populations. With proper planning, collaboration, execution, and implementation by different organizations (National and Internationals) like ICAR-AICRP, DBT, ICMR, and HarvestPlus, biofortification of cereal crops for product

development, testing, and validation will help in achieving the nation's nutritional security. It will have a significant impact on the lives and health of a large number of poor individuals/malnourished populations of the country.

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Potential and Perspective of Plant Proteinase Inhibitor Genes in Genetic Improvement of Economically Important Crops

12

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Abstract

The importance of protease inhibitors has been realized as one of the important family of genes with inhibitory activity against devastating insect pests. These are small defense proteins reported to occur in storage organs of plant (up to 10% of protein). The size of plant protease inhibitor proteins varies from 4 kDa to 45 kDa which include kazal (4 kDa), BBPI (8 kDa), Kunitz (20–24 kDa), and Serpin (40–45 kDa). Protease inhibitrors are capable to regulate endogenous functions like regulation of endogenous proteases, mobilization of storage proteins, cell metabolism and physiology, modulation of apoptosis and programmed cell death, stabilization of defense proteins, targeting the digestive proteases of insects, pathogen, and microorganism. Increasing environmental concerns compelled the scientific community to look for non-hazardous, effective, affordable, and eco-friendly solution to control insect pest damage to agronomically important crop plants. Use of protein molecules as natural entomotoxic metabolites has gained momentum as pragmatic approach for insect pest management of crops along with better quality of food.

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Keywords

Plant protease inhibitor · Kazal · BBPI · Kunitz protease inhibitor · Serpins · Storage proteins · Phytophagous insects · Entomotoxic metabolites

12.1 Introduction

The development of economically important plants with improved quality traits such as pest, disease, and drought resistance and prolonged shelf-life by conventional breeding is extremely time consuming (Delaney 2015). Due to an ever-expanding global population and changes in eating habits, the demand for more nutritionally balanced food and feed crops has increased immensely. In fact, the provision of sufficient food to feed approximately 9.7 billion people by 2050 (FAO 2017) and 11.0 billion by 2100 (James 2015) is one of the major challenges of this century. One possible way of relieving food scarcity problems and increasing food security is to develop improved plant varieties rapidly. Genetically modified (GM) crops are going to provide right set of opportunity to increase food and feed production efficiently by generating plants with higher yields, better climatic adaptions, and higher nutritional benefits (The Guardian 2016; Ahmar et al. 2020). GM crops have revolutionized agricultural commodities by allowing breeders to introduce specific genes from a wide variety of sources to produce more useful and productive crops (Tarafdar et al. 2014). The rapid acquisition of GM crops within the agricultural sector led to increased agricultural productivity that contributed to economic growth (James 2015). Crop protection plays a vital and integral role in modern-day agricultural production to minimize the loss of agricultural crop produce. The worldwide loss of crop yields by insects is estimated to be 15% of the total crop production (Maxmen 2013). The extensive use of pesticides and insecticides has increased the cost of pest control and also resulted in hazards to organisms, pollution of the environment, loss of effectiveness, and public concern over pesticide residues in foodstuffs (Christou et al. 2006; Oerke 2006). Further, the parallel evolution of insect populations resistant to chemical insecticides is also a major concern. Efforts have been made to reduce the broad-spectrum of toxicant release in the environment to keep the equilibrium for the sake of health issues. Therefore, the use of eco-friendly, sustainable, and effective protein molecules as natural entomotoxic metabolites has gained importance to obtain a better quality of food and protection of the environment and would be considered as a pragmatic approach for insect pest management of crops (Jaber et al. 2010; Jamal et al. 2013). The natural defense mechanism enables plants to protect themselves against pests by synthesizing specific macromolecules such as protease inhibitors (PIs), α-amylase inhibitors, lectins, and phenolic compounds.

Plant protease inhibitors (PPIs) are generally small defense proteins and have been reported to occur in storage organs (up to 10% of protein) (Tamhane et al. 2009). The size of PPIs ranges from 4 kDa to 45 kDa for various molecules such as kazal (4 kDa), BBPI (8 kDa), Kunitz (20–24 kDa), and Serpin (40–45 kDa). They

are enriched in cysteine residues that significantly contribute to the formation of disulfide bridges thereby conferring stability to heat, pH, and proteolysis (Chye et al. 2006). Protease inhibitors have a role in regulating endogenous proteases and as defensive genes in response to wounding, pathogenic stress, and insect pest. Methyl jasmonate (MeJA) is one of the key regulators of plants' defensive response to insect herbivores and up-regulation in its concentration in response to herbivore attack leads to induction of protease inhibitors synthesis (Singh et al. 2016).

Protease inhibitors are one of the important families of genes with highly proven inhibitory activity against insect pests and known to improve the nutritional quality of food (Bhattacharjee et al. 2012). The PPIs have a role in exogenous as well as endogenous functions like regulation of endogenous proteases, mobilization of storage proteins, cell metabolism and physiology, modulation of apoptosis and programmed cell death, stabilization of defense proteins, targeting the digestive proteases of insects, pathogen, and microorganism (Hörger and van der Hoorn 2013). PPIs have been found to target almost all classes of digestive proteases in insects. They have been classified on the basis of sequence homology, assignment of the inhibitory site(s), reactive sites, and interaction with the protease(s) according to a standard mechanism (Laskowski Jr 1986).

In phytophagous insects, PIs interact with midguts proteases of insect larvae, resulting in the formation of a stable protease–inhibitor complex that is incapable of enzymatic activity. Following inhibition of enzymes, the target proteases can no longer cleave peptide bonds causing a detrimental disruption of dietary protein assimilation in herbivorous pests. The reduction in the availability of amino acids that are considered necessary for insect growth and development ensures developmental delay, mortality, and reduced fecundity (Chougule and Bonning 2012; Rakashanda et al. 2012; Zhu and Zeng 2015). Currently, the substantial emphasis of studies on PPIs is to identify potential inhibitors of digestive proteinases of the target insects along with understanding the dynamic nature of insect midgut proteinases at the molecular level (Lopes et al. 2004). Lepidopteron insects have serine proteinases as a major component of their digestive complement and among them, trypsin-and/or chymotrypsin-like are the most commonly found proteinases (Srinivasan et al. 2006). To date, there is no evidence that protease inhibitors have toxic or deleterious effects on mammals (Sharma 2015). Protease inhibitor genes are gaining popularity in becoming an ideal choice for developing transgenic crop plants resistant to insect pests. The protease inhibitors act specifically against proteases of a particular group of insects, therefore, identification of the right candidate gene is very important before the transformation of crop plant with protease inhibitor gene. Major midgut proteases of the Lepidoptera and Diptera insect orders are serine protease types (Srinivasan et al. 2006); while Homoptera and Coleoptera insect orders contain cysteine protease types (Schluter et al. 2010) as reported by Sharma and Suresh (2015) using *In-Silico* genome-wide identification and structure-function studies of proteases as well as PIs genes from chickpea genome. Mainkar et al. (2020; data not published) identified 35 members of protease inhibitor from pigeon pea genome database and further nine genes were characterized and validated based on the artificial diet insect feeding bioassay. Five PI genes were observed with 72%

of mortality against Helicoverpa and aphids. These findings will help in selecting candidate genes from chickpea and pigeon pea and further characterize their function by manipulating them for the enhanced stress tolerance capacity of these economically important legume crops. Commercially available insect-resistant transgenic plants use only *Bt* toxin genes, however, the development of transgenic plants expressing PIs has emerged as an additional strategy for pest control. The transgenic plants having PI genes may, therefore, be a useful adjunct to the use of *Bt* as a biopesticide. Combinations of *Bt*- and PI- plants could be used to manage resistance development, either as seed mixes in adjacent plots or through "pyramiding," whereby the two genes are engineered into a single plant (Christeller et al. 2005). Given their wide versatility and broad-spectrum biotechnological applications, many plant PIs have been characterized (Volpicella et al. 2011). This chapter emphasizes the potential and perspectives of plant PIs in the genetic improvement of economically important crops and their applications in the agriculture field.

12.2 Distribution and Localization of PIs

Protease inhibitors have been found extensively distributed throughout the plant kingdom. Most of the plant's PIs that have been well characterized are from the *Leguminosae*, *Gramineae*, and *Solanaceae* families (Brzin and Kidric 1995). Protease inhibitors are generally found in storage tissues, such as seeds (Valdés-Rodríguez et al. 1993), endosperm (Cordero et al. 1994), tubers (Melville and Ryan 1972), and non-storage tissue as a consequence of several stimuli such as flower, leaves, and roots (De Leo et al. 2002; Sin and Chye 2004; Jamal et al. 2013. Their cellular localization is in the protein bodies of cotyledon, cell walls, intercellular spaces, and cytoplasm. Differences in localization of PI have been found according to legume type, i.e. mung bean trypsin inhibitors are localized in the cytoplasm of cotyledonary cells, while soybean trypsin inhibitors (BBI and KTI) found in the cotyledons and nucleus (Chrispeels and Baumgartner 1978; Hernández-Nistal et al. 2009). Protease inhibitor II of *Solanum americanum* was found to be expressed in phloem of leaves, stems, and roots suggesting a novel endogenous role of PIN2 in phloem sap (Xu et al. 2001).

12.3 Classification of Plant Protease Inhibitor (PPIs)

The PPIs have been classified based on the presence of amino acids in their reactive sites and interaction with the protease(s) (Table 12.1). The protease inhibitors were grouped into four crucial families, in view of the specific reactive site present in the PIs sequences (Laskowski Jr. and Kato 1989). These families are: Serine PIs, Cysteine PIs (Cystatins/Multicystatin), Aspartic PIs, and Metallocarboxypeptidase inhibitors. Based on structural and biochemical properties, PIs were classified as Serpin PIs, Bowman–Birk inhibitors (BBIs), Kunitz PIs, Squash PIs, potato protease

Table 12.1 Classification of protease inhibitors based on target proteases

Family		Mol. wt (kDa)	MEROPS Family/ Subfamily	Target Protease	References
Serine	Kunitz	18–24	13A, 13B	Trypsin, chymotrypsin, subtilisin, α-amylase, α-chymotrypsin, aspartyl protease	Botelho-Junior et al. (2014); Smith et al. (2016); Arnaiz et al. (2018)
	Kazal	8	I1	Trypsin and elastase	Pariani et al. (2016)
	Serpin	39–43	I4	Trypsin, chymotrypsin and cathepsin G	Huntington et al. (2000); Gettins (2007); Barrett et al. (2012); Heit et al. (2013); Rawlings et al. (2018); Mainkar et al. (2020)
	Pin I	8	I13	Trypsin, chymotrypsin, subtilisin	Cai et al. (1995); Turrà et al. (2020)
	Pin II	20–21	I20	Trypsin, chymotrypsin	Beekwilder et al. (2000); Dunse et al. (2010)
	Squash	3.0–3.5	I7	Trypsin	Heitz et al. (2001)
	Bowman-Birk	8-dicot and 16/8-monocot	I12	Trypsin, cathepsin G, elastase, chymotrypsin	Bowman (1946); Bowman (1946); Qi et al. (2005)
Cereal	Trypsin/ α-amylase	13	I6	Serine/α-amylase protease	Odani et al. (1983); Mahoney et al. (1984); Gourinath et al. (2000); Christeller and Laing (2005)
Cysteine protease inhibitor	Cystatin, multicystatin	11–80	I25B	Cysteine proteinase, cathepsin L, cathepsin B	Martinez and Diaz (2008); Zhang et al. (2008); Green (2013); Je et al. (2014); Martel et al. (2015)
Mustard (synapsis) TI	Trypsin inhibitor	7	I18	Serine protease	Ceciliani et al. (1994); De Leo et al. (2001)
Aspartyl and Metallocarboxypeptidase inhibitors	Aspartyl PI and metalloprotease inhibitor	4.2		Trypsin, chymotrypsin, elastase, cathepsin D, carboxypeptidase A and carboxypeptidase B	

inhibitors I (PPi-I), potato protease inhibitors II (PPi-II), Cereal trypsin/amylase inhibitors, and Mustard (Sinapis) trypsin inhibitor (MSI) (Rustagi et al. 2018).

12.3.1 Serine PIs

This is the most abundant and widely studied family of PIs that antagonize the activity of serine protease (Santamaria et al. 2014; Rawlings et al. 2018). They were further classified into different types based on the amino acid sequence present at their reactive site and functional domains, their function of structural and biochemical properties such as, Kunitz PI, Bowman–Birk inhibitors (BBIs), Serpins, potato protease inhibitors (PPi I), potato protease inhibitors II (PPi II), Squash inhibitors, cereal trypsin/amylase inhibitors, Sinapis trypsin inhibitor (Bateman and James 2011; Rustagi et al. 2018).

12.3.1.1 Kunitz PI

The Kunitz trypsin inhibitor was the first inhibitor protein characterized and isolated from soybean by Kunitz in 1945. The Kunitz protease inhibitors contain ~200 amino acid residues with molecular weight ~ 20 kDa with two disulfide bonds and one reactive site in their structure. These proteins classically defined as serine PIs and inhibit serine proteases such as trypsin, chymotrypsin, and subtilisin (Smith et al. 2016). They have potential role in defense as their expression is up-regulated in response to wounding by herbivores, methyl jasmonates and in response to pathogen (Botelho-Junior et al. 2014).

12.3.1.2 Bowman–Birk Inhibitors (BBIs)

Bowman–Birk PI family genes were the first to be identified and characterized from soybean (*Glycine max*) (Bowman 1946). The BBIs from dicotyledonous species (legumes) consist of a single polypeptide chain with molecular mass of 8 kDa with two domains bearing a separate reactive site to inhibit independently but simultaneously the trypsin and chymotrypsin proteases (Qi et al. 2005). In cereals (monocots), two kinds of BBIs, one consisting of a single polypeptide chain with molecular mass of 8 kDa with a single reactive site for trypsin/chymotrypsin and another has molecular mass of 16 kDa with two reactive sites for trypsin and chymotrypsin. The reactive site pattern of BBIs is stabilized by seven disulfide bonds in the dicots. Four cysteine residues were found lost in monocot BBIs corresponding to C3–C13 and C10–C11 of dicot BBIs. Only five disulfide bonds are found in monocots BBIs (Prakash et al. 1996).

Key Point
BBIs from the dicots are double-headed inhibitors, have molecular weight of 8 kDa with two reactive sites while monocot double-headed inhibitors have molecular mass of 16 kDa with two reactive sites and monocots, single headed BBIs have molecular mass of 8kda with one reactive sites.

12.3.1.3 Serpins (SERine Protease Inhibitors)

Serpins are the sizeable and widely distributed in nature, super family of PIs (Rawlings et al. 2018). Plant Serpins have a molecular mass of 39–43 kDa. Serpin inhibitors irreversibly inhibit their target serine protease (trypsin, chymotrypsin) by a large conformational change to disrupt the active site of the enzyme (Gettins 2007). In contrast to common competitive mechanism of PI, Serpin PI is involved in the cleavage of an appropriate peptide bond in the reactive center loop of the inhibitor that triggers a conformational change so that catalysis of enzyme does not happen beyond the formation of an acyl–enzyme complex. Hence, these inhibitors are also called as a suicide inhibitor (Huntington et al. 2000). Serpins represent a superfamily of PIs and derive their nomenclature from **SER**ine **P**rotease **IN**hibitor (Heit et al. 2013). Serine PIs are further classified into different divergent groups based on the substrate specificities, such as trypsin-like, chymotrypsin-like, elastase-like, and pronase-like (Barrett et al. 2012). Recently, a total of seven Serpin genes were identified from the available six legume genomes database and phylogenetically divided them into two major clades (clade-I and clade-II) based on the conserved reactive center P2 -P1' and exon-intron junctions in gene structure (Mainkar et al. 2020).

Key Point
Almost all the PPIs are reversible inhibitors except Serpins from the **Ser**ine **PI**s family which are irreversible inhibitors, also called Suicidal Inhibitors.

12.3.1.4 Kazal PI

Kazal protease inhibitors are double-headed inhibitors which inhibit trypsin and chymotrypsin serine proteases simultaneously and were first isolated as a trypsin inhibitor from pancreas by Kazal et al. (1948). Two putative Kazal serine PIs were characterized from *Arabidopsis thaliana* and reported their transient induced expression in response to leaf infection by *Botrytis cinerea* (Pariani et al. 2016).

12.3.1.5 Potato Protease Inhibitor I (PPI 1)

The inhibitors of this family are extensive in plants and have been reported in many species such as potato, chickpea, pigeon pea, squash, and tomato. These inhibitors have molecular mass of 8 kDa and are generally monomeric. Potato and cucurbit protease inhibitor proteins contain a single disulfide bond, while in general the PPI 1 lacks disulfide bonds (Cai et al. 1995). Potato type I (Pin1) serine PIs associated with plant storage organs and have known insecticidal and nematicidal activities. Overexpression of the PPI2C4 and PPI3A2 potato type I inhibitor genes led to a considerable reduction in the propagation and symptom development produced by *Pseudomonas syringae,* bacterial pathogen (Turrà et al. 2020).

12.3.1.6 Potato Protease Inhibitor II

It is one of the most studied wound-inducible PI at gene, protein and functional level. Pin II is a heat stable dimeric protein with a molecular weight of ~21 kDa containing two reactive site domains for inhibition of trypsin and chymotrypsin (Tamhane et al. 2009). An effective insect resistance gene, potato protease inhibitor II (PINII-2x) from a diploid potato, *Solanum phurejia* L has an important application in crop breeding for insect resistance (Bu et al. 2006). Heterologous expression of Pin II genes (*CanPI-7*) with 1–4 IRDs from *Capsicum annuum* had strong inhibitory results against *Helicoverpa armigera* growth and development (Tamhane et al. 2009).

12.3.1.7 Squash Inhibitors

The potent canonical serine protease inhibitors isolated from the gourd family were reported with the typical knottin fold cross linked with three disulfide bridges (Chiche et al. 2004). Squash inhibitors are found to be highly stable and rigid proteins. They inhibit serine proteases such as trypsin, chymotrypsin, Cathepsin G. They are the smallest proteins with 30 amino acid residues of single peptide chain with molecular mass of 3–3.5 kDa (Heitz et al. 2001).

12.3.1.8 Cereal Trypsin/α-Amylase Inhibitors

The cereal trypsin inhibitors encompass a single polypeptide chain having five disulfide bonds with a molecular mass of 13 kDa (Christeller and Laing 2005). These inhibitors inhibit the activity of serine and α-amylase protease (Gourinath et al. 2000). A large number of inhibitors have only α-amylase inhibitory activity; however, barley and rye PIs showed inhibitory activity against trypsin (Odani et al. 1983). In maize and ragi PIs, dual inhibitory activities were noticed against the serine and α-amylase proteases (Mahoney et al. 1984).

12.3.2 Mustard Trypsin Inhibitors (MTIs)

These are small single polypeptide chain serine PIs with molecular weight of 7 kDa, mainly found in the *Brassicaceae* family. The inhibitors form a compressed binding complex with trypsin and follow the standard mechanism of inhibition (Ceciliani et al. 1994).

12.3.3 Plant Cysteine PIs

The plant cysteine PIs have notable characteristics, which allow them to be classified in a special class called Phytocystatin. PhyCys are present in almost all plants and have also been observed in the *Chlorophyceae* algae. The unique inhibitory properties of PhyCys are a consequence of a tight and reversible interaction with their target enzymes. It involves a conserved tripartite wedge formed by the partially flexible N-terminus alpha helix containing one or two highly conserved glycine, G58

residues, and two hairpin loops consisting QVVAG motif, tryptophan residue, and PW motif. These inhibitor proteins are involved in the regulation of several processes of defense against pests and pathogens endogenously. The plant cysteine PIs are known to respond to abiotic stresses. Phytocystatin genes are reported to get expressed in *Arabidopsis thaliana* plants when exposed to high salt, drought, and heat stress (Zhang et al. 2008; Je et al. 2014).

12.3.3.1 Cystatins

The cystatins are reversible and tight-binding inhibitors of cysteine proteases such as Cathepsin C, papain, and legumain. Most of the PhyCys are small proteins with molecular weight in the range of 12–16 kDa, lack disulfide bonds, and show significant sequence identity among them and with cystatins of animal origin (Barrett 1987). Some members have a molecular mass of 23 kDa with a carboxy-terminal extension involved in the inhibition of cysteine protease of legumain type and are of group two phytocystatins, found in Taro and sesame (Martinez and Diaz 2008).

12.3.3.2 Multicystatins

The plant cystatins have molecular mass in the range of 85–87 kDa and belong to multicystatins type. These comprise eight cystatin repeating units each one capable of inhibiting cysteine protease and are mostly found in potato and tomato (Green 2013). Microarray analysis of tomato responses to the spider mite (*Tetranychus urticae*) feeding revealed the up-regulation of a multicystatin as a defense protein (Martel et al. 2015).

Key Point
Based on the studies on cystatins of animals, cystatin superfamily includes three classes of inhibitors, namely stefins, cystatins, and kininogens. However, plant cystatins are more similar to the type II cystatins of animals and they are allocated as an independent family.

12.3.4 Aspartate PI

This PI has a molecular mass of 27 kDa and helps to inhibit the aspartyl protease cathepsin D and serine proteases trypsin and chymotrypsin; however, it does not inhibit cathepsin E, rennin, and pepsin, despite all being aspartyl proteases. It helps to protect the plants by inhibiting the proteases of insects and pathogens.

12.3.5 Metalloprotease Inhibitor

These are small peptide inhibitors of approximately 39 amino acid chain with a molecular weight of 4.2 kDa. These inhibitors competitively inhibit carboxypeptidases only from animals and microorganisms, and not from the yeast and plants.

12.4 Regulation of plant PIs

PPIs perform two main functions. Firstly, they regulate endogenous proteases to control protein turnover and avoid an indiscriminate degradation (Volpicella et al. 2011; Martínez et al. 2012). Secondly, they regulate the activity of exogenous proteases to protect the plants against insects and pathogens (Santamaria et al. 2012; Hörger and van der Hoorn 2013). Production of these inhibitors is highly regulated by a signal transduction pathway that is initiated by predation and transduced as a wound response (Fig. 12.1). The production of PIs occurs via the octadecanoid pathway (OD), which catalyzes the breakdown of linolenic acid and the formation of jasmonic acid to induce PI gene expression (Koiwa et al. 1997). There are four systemic signals responsible for the translocation of the wound response, viz. systemin, abscisic acid (ABA), hydraulic signals, and electric signals (Malone and Alarcon 1995). These signals are translocated from wound site to all over plant through xylem/phloem as a consequence of hydraulic dispersal. The systemin, an 18 amino acid long peptide first isolated from wounded tomato leaves, is capable of systemic movement within the plant. Systemin initiates a cascade that triggers the release of linoleate from membrane lipids for synthesis of jasmonic acid to activate the expression of PI genes (Ryan 2000).

12.5 Mechanisms of Inhibition of Protease Inhibitors

PPIs can interact with target proteases in two different manners as narrated below (Bateman and James 2011; Joshi et al. 2013).

12.5.1 Standard Mechanism of Inhibition

12.5.1.1 Reversible Inhibition

The PPIs of serine family, viz. the Kazal, Kunitz, PPi-I and PPi-II, Bowman–Birk protease inhibitors were observed to exhibit the standard (canonical/competitive) mechanism of inhibition where an inhibitor tends to compete with substrate binding through direct competition or deformation of active site of an enzyme and prevents the substrate from binding (Fig. 12.2). The reversible inhibition mechanism is an efficient way to inhibit serine proteases; the majority of these PIs have related broad specificity within sub-classes of serine proteases. The canonical inhibitors are able to

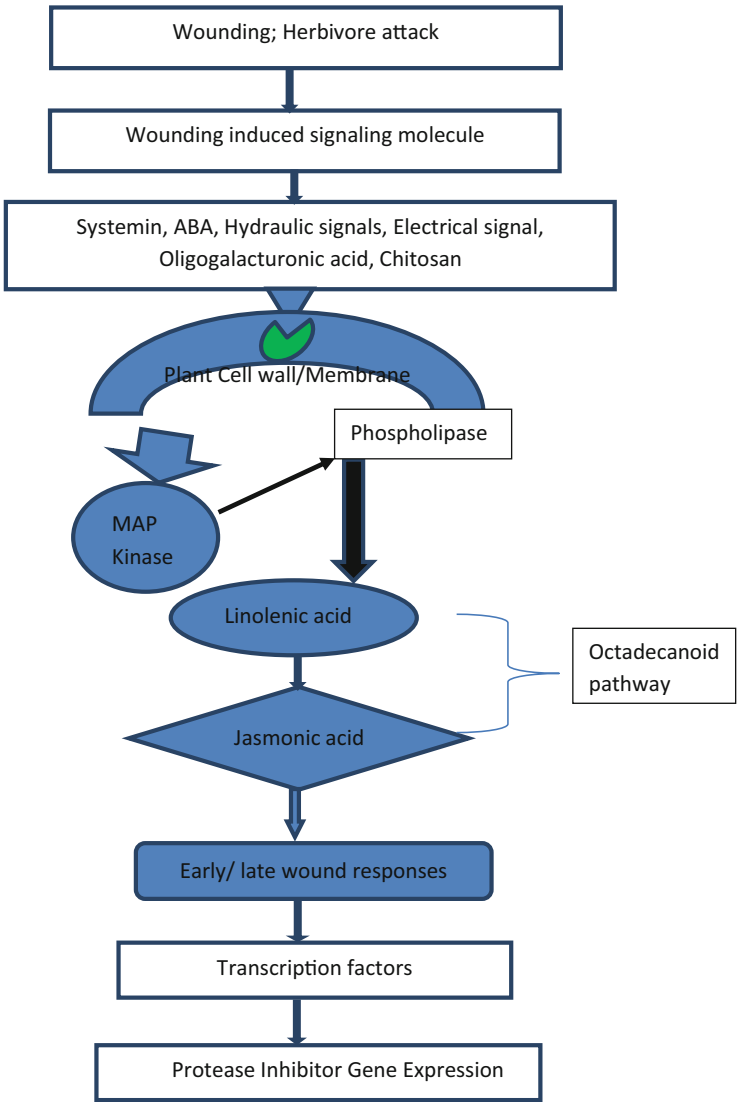

Fig. 12.1 Regulation of plant protease inhibitors

target more than one protease at a time, often with different specificities (Joshi et al. 2013). These inhibitors insert a reactive loop into the active site of the protease and bind in an extended β-sheet with the enzyme in a substrate-like manner in a lock and key fashion. While bound to the protease, "scissile bond" of the canonical inhibitor gets hydrolyzed slowly without the release of products and the amide bond can be reformed (Zakharova et al. 2009; Farady and Craik 2010).

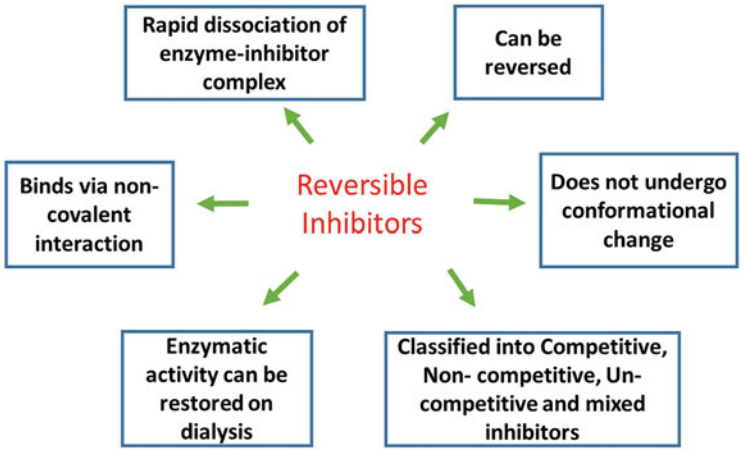

Fig. 12.2 Properties of Reversible Inhibitors

12.5.1.2 Irreversible Inhibition

Serpin PIs, similar to the canonical inhibitors, interact covalently and irreversibly with the serine proteases in a substrate-like manner; but cleavage of a single peptide bond in the reactive center loop (RCL) of serpins leads to conformational change in the structure and the inhibitor never recovers its initial structure, which hinders the catalysis of enzyme. Hence, these inhibitors (**Serpin**, a Serine PI) are also known as suicidal inhibitors. The inhibitor acting as a substrate utilizes the enzymes' catalytic machinery to trap and then inhibit the enzyme. Upon cleavage of the RCL, the N-terminal half of the RCL still attached to the protease as an acyl-enzyme intermediate is inserted into a β-sheet of the inhibitor. The serpin inhibitory mechanism is completely irreversible. Because of the radical nature and irreversibility of this mechanism, serpins serve as protease scavengers, protecting cells and tissues from unwanted proteolytic activity.

12.5.2 Non-standard Mechanism of Inhibition

The plant cysteine PIs inhibit proteases in a non-catalytically competent manner as they do not bind to protease in a substrate-like manner (Farady and Craik 2010). The inhibitory domain of these non-canonical inhibitors contains two hairpin motifs, which interact with the prime side active sites of proteases and the N-terminal region is essential for tight interaction in S3-S1 pockets and for specificity towards cysteine proteases. Hence, both the prime and non-prime sides of the active site are occupied by hairpin motifs and N-terminal segment, respectively, but no interactions are actually made with the catalytic machinery of the enzyme (Bode and Huber 2000).

12.6 Potential of PPIs in Genetic Improvement of Economically Important Crops

To broaden the scope and potential of PPIs in genetic improvement of economically important crops, some possible mechanisms include transgenic plants, gene stacking, transplastomic engineering, recombinant proteases inhibitors, RNAi-based approaches, and CRISPR/Cas9-mediated genome editing. Several PPIs have been well characterized for their potential to control insects and pathogens.

12.6.1 Transgenic Plants

The agriculture based economic development is in consideration all over the world and some of the developed countries have already embraced genetically modified crops for their economic development that helps to reduce investments on pesticides keeping their harmful effects in view. Steadily with time, developing countries are also embracing transgenic crops for development of their agriculture and they are gaining more profits compared to untimely. There has been a large amount of interest in the use of PPIs for the improvement of crop resistance against both biotic and abiotic stresses using transgenic approaches. Many studies have been reported the potential use of PIs to engineer crop for insect resistance since they are potential sources of resistance against pests and pathogens in transgenic plants. The PPIs were potent in inhibiting pathogenic fungi as an antifungal protein and inhibiting digestive proteases of insects as anti-feedent protein. The presence of antifungal and insecticidal activities in a single PI protein has provided an intense challenge in developing transgenic plants against both pests and pathogens resistance (Habib and Fazili 2007).

The application of PIs as candidate genes has three benefits over the other candidate genes. Firstly, transgenic crops expressing PIs are helpful in integrated pest management (IPM) programs for increasing productivity without the use of harmful pesticides (Boulter 1993). Secondly, PPIs improve the nutritional quality of foods as many of them are rich in cysteine and lysine amino acids (Ryan 1989). Thirdly, PIs can protect heterologous proteins when both are expressed together in transgenic crops (Rivard et al. 2006). Two distinct PIs engineered in tomato plants have shown more resistance to both a lepidopteran and a dipteran insect (Abdeen et al. 2005). An unusual SPI identified from *A. thaliana* is involved in the defense against *Botrytis cinerea* and *Alternaria brassicicola* fungi (Laluk and Mengiste 2011). A *Beta vulgaris* serine PI gene (BvSTI) has been reported to be effective for the control of several different lepidopteron insect pests in genetically modified tobacco plants (Smigocki et al. 2013). Trypsin PI gene from ricebean (RbTI) has shown inhibitory activity against the gut proteases of Hessian fly larvae (Katoch et al. 2014).

Key Point
"Cowpea Trypsin Inhibitor (*CpTI*)" the first protease inhibitor gene was successfully transferred to the plant and was highly efficacious for insect resistance against tobacco budworm (*Heliothis virescens*).

12.6.2 Gene Pyramiding

In contrast to mono-trait plant varieties, stacking offers broader agronomic enhancements that let farmers to meet their needs under complex farming conditions. Gene stacks are genetically engineered to have better chances of overcoming the multitude of problems in the field such as insect pests, diseases, and different abiotic stresses so that farmers can increase their productivity. This approach has reduced the potential of resistance breakdown as it is more difficult for the insect to overcome multiple insecticidal proteins. Pyramiding necessitates the stacking of multiple genes leading to the concurrent expression of more than one gene in a plant variety. Several common gene stacking methods can be used to develop the pyramiding crops.

Hybrid Stacking A plant harboring one or more transgenes is cross-hybridized with another plant containing other trait transgenes.

Co-transformation A plant is simultaneously transformed with two or more transgenes. The transgenes are in a distinct construct and delivered to the plant concomitantly.

Multigene Cassette Transformation A plant is transformed with two or more linked transgenes harboring in a single gene construct.

Re-transformation A plant bearing one transgene is transformed with other transgenes.

Some of the technological concerns in molecular stacking of genes include the method of construct delivery into plant cells, design of large size multigene constructs, and the stability of expression of multiple genes. As of now, the most suitably genes stacking can be performed via selected crosses between parents that possess the desired individual genes and identify progeny with the desired combination of traits. The tobacco and potato inhibitors belonging to the same class have been expressed simultaneously in cotton plant and were found effective against non-target proteases of *Helicoverpa* species (Dunse et al. 2010). Overexpression of two different PIs engineered in tomato plants has shown increased resistance to both a lepidopteran and a dipteran insect (Abdeen et al. 2005). The stacking of PI genes Sporamin (trypsin inhibitor) from sweet potato and CeCPI (phytocystatin)

from taro, driven by the wound and pathogen responsive promoter, is an effective strategy for engineering crops against insects and phytopathogens (Senthilkumar et al. 2010). The combination of *Nicotiana alata* PI, pin II inhibitor and *Solanum tuberosum* (StPin1A), pin I inhibitor in artificial diet resulted in transgenic plants that were more effective in reducing the growth and development of *Helicoverpa* spp. than either inhibitor alone (Dunse et al. 2010). The multi-toxin approach has been used to obtain the fusion product of both lentil lectin and chickpea PI gene for improvement of resistance against aphids (Rani et al. 2017).

12.6.3 Protein Engineering

The development of new PIs with increased potency and altered specificity with diverse mechanisms of action can be possible with protein engineering efforts. Research activities are underway to elucidate protein folding and recognize for protein design principles. The comprehensive knowledge of protein structure and its function greatly expanded the abilities of protein engineering in future. Eventually, even unnatural amino acids may be included via new methods, such as expanded genetic code that allow encoding of novel amino acids in genetic code. With recent advancement in protein engineering approach, we can successfully develop recombinant PIs with potent inhibitory activity against herbivore pests. Different inhibitor variants can be generated via site-directed mutagenesis. Using three-dimensional modeling, best inhibitor variant with enhanced potency against target insect and less potency to non-target insects can be screened and selected (Schluter et al. 2010).

12.6.4 Plant Molecular Farming

Plant molecular farming is a new technology that uses plants for large scale production of recombinant proteins and other secondary metabolites (Alireza and Nader 2015). However, one of the most important challenges the science community faces is the use of plants as a productive platform to improve the yields of recombinant proteins expressed within them (Mandal et al. 2016; Tschofen et al. 2016). To prospect new approaches for minimizing the degradation of foreign proteins may increase the commercial potential of industrial interest, pharmaceutical, or vaccines produced in plants (Pillay et al. 2014). The endogenous plant proteases may affect the integrity of recombinant proteins by altering biological activity in the extraction steps and altering the protein production (Castilho et al. 2014). The plant PIs incorporate a new strategy to reduce the degradation of recombinant proteins expressed in plants by protease activity. To control the proteolysis of antibodies throughout the secretory pathway in apoplast, plant Bowman–Birk PIs have been co-expressed during their migration through the cell secretory pathway to protect secreted proteins. The co-secretion of PIs is able to reduce the degradation of immunoglobulin complexes in the secretion pathway leading to increase the

antibody production in the plant roots (Komarnytsky et al. 2006). Similarly, a synthetic trypsin and chymotrypsin inhibitor from *N. alata* engineered to reduce the extracellular protease activity and co-expressed to enhance the accumulation of the recombinant human granulocyte-macrophage (hGM-CSF) in transgenic rice cell suspension culture (Kim et al. 2008).

12.7 Perspectives of Plant PIs in Genetic Improvement of Economically Important Crops

The advances in genetic engineering technique together with gene pyramiding strategy have clearly confirmed the practical potential of these PIs in plant resistance. PPIs are a promising companion to *Bt* toxins for the development of insect-resistant crops. However, in view of ecological and multitrophic perspective, these advances mark the relevance in assessing their inhibitory effects on insect's proteases and proteases of other organisms present in the trophic chain including the host plant's own proteases (Senthilkumar et al. 2010). Future perspective for using PI genes to improve insect, pathogen resistance in transgenic crops will require re-evaluation of their mechanisms, particularly in influencing the processes other than digestion, as epitomized by effects on the sap feeding hemipteran pests. In recent years, major progress has been noticed towards the field deployment of highly potent broad-spectrum PI gene expressing transgenic plants resistant to major herbivore pests. The evaluation of current literature suggests that the non-target inhibitory effects of recombinant PIs in plant could often be insignificant.

It is utmost necessary to develop strategies to fend off proteolytic degradation of recombinant proteins expressed in plants. The recombinant protein targeting to apoplast may help to correct maturation and glycosylation of recombinant proteins (Jha et al. 2012). However, several different studies identified proteases present in the apoplast space of tomato, *A. thaliana*, *N. benthamiana,* and *N. tabacum* (van der Hoorn 2008; Delannoy et al. 2008). So, it is required to confirm the direct involvement of proteases in recombinant protein degradation and develop strategies like co-expression of PI along with the recombinant protein to protect the recombinant protein (Pillay et al. 2016). Although genetically engineered plants expressing Bt toxins from *Bacillus thuringiensis* to protect agricultural crops against lepidopteran and coleopteran pests are available, yet some important agricultural pests, like phloem-feeding insects are not susceptible to *Bt* gene expressing crops; therefore, alternative pest control strategies are necessary to assure that the benefits provided by *Bt*-insect-resistant transgenic plants are not compromised. The cysteine PI has shown enhanced resistance against the aphids and can be used as a candidate gene for developing the transgenic crop plants against the hemipterans. Transgenic plants constitutively expressing phytocystatins have been observed with an enhanced tolerance to drought suggesting that manipulation of cysteine protease domain leads to altered phytocystatin expression in crop plants. These alterations might be used to improve resilience and quality aspects in the face of climate change. The plant transformation studies using PI genes have largely focused on biotic stresses,

specifically against insects and fungal pathogens. However, very few have focused on abiotic stresses. So research in this direction will be more encouraging and may uncover important functions of PI genes in plants in mediating the biotic stresses. In addition, to develop economically adaptable cultivars, more efforts are needed to focus on multiple gene transformation events.

12.8 CRISPR/Cas9-Mediated Genome Editing

It is one of the invaluable and precise contemporary techniques of editing genes among the available genome-editing technologies. It is well-known for its accuracy in diverse species. Genomic changes introduced by CRISPR/Cas9 are both stable and heritable compared to RNAi (RNA interference) technology. In contrary, RNAi-mediated gene silencing is instantaneous.

12.9 Transplastomic Engineering

Several strategies have been used to enhance the pest and disease resistance by nuclear transformation methods in transgenic plants, but a few efforts have been made to use transplastomic engineering technology to increase the resistance in plants (Jin et al. 2012; Chen et al. 2014). The nuclear transformed transgenic plants revealed insecticidal activity against insect pest, but the levels of expression of the transformed genes were low (Singh et al. 2016). The transplastomic technology is advantageous over conventional nuclear transformation techniques with 10–100 times increased expression of transgenes, multigene engineering in single step gene transfer, negligible chances of transgene silencing, and pleiotropic effects. This also prevents the transgene flow by pollen contamination being a maternal inheritance. Chen et al. (2014) showed an enhanced and synergistic effect of pyramiding expression of three genes potato sporamin, taro cystatin and *Paecilomyces javanicus,* chitinase via transplastomic engineering, against a broad range of biotic and abiotic stresses in *Nicotiana benthamiana*. This study involved an independent expression of all the three genes from a monocistronic gene cassette and it differs from usual multigene engineering strategies. It revealed successful expression level of functional proteins from multiple transgenes with a monocistronic gene cassette, instead of a polycistronic gene cassette. The revising research to develop insect pest resistant crop plants using transplastomic approach can be used to boost up plant resistance against a wide range of pests and diseases.

12.10 Current National and International Status

12.10.1 National Status

A protease inhibitor gene from *Vigna mungo* was cloned *in* pET301/CT-DEST vector. The protein was expressed in *E. coli* and the expressed protein was assessed for the insecticidal activity against *Lipaphis erysimi* using diet inclusion bioassay. It was found to be effective and the LC_{50} was obtained as 0.558ug/g of the diet as that *Vigna mungo* protease inhibitor protein had significant levels of resistance against aphids. Saikhedkar et al. (2019) designed bicyclic peptides based on the most potent RCL regions of plant Pin-IIs, mimicking natural Pin-II bi-domain and showed tenfold enhanced protease inhibition compared to their linear RCL peptides. The binding behavior of soybean trypsin inhibitor (STI) with silica nanoparticles (Si20) was evaluated for subsequent delivery to tomato plants with intended focus on controlling the insect pest *H. armigera* (Bapat et al. 2020). Bovine trypsin was inhibited by 80% when the nanoparticle bound STI was used, whereas *Helicoverpa armigera* gut protease (HGP) activity was reduced only by 50%. The Si20 nanoparticles have been recommended to be used for developing plant delivery vehicles in the future for better pest management. Mainkar et al. (2020; data not published) identified 35 members of protease inhibitor from *Cajanus cajan* genome. Based on docking against digestive proteases of *Myzus persicae* and *Helicoverpa armigera*, nine genes were characterized and validated by the artificial diet insect feeding bioassay. Five PI genes were found to be more potent and showed 72% mortality in aphids and *Helicoverpa armigera*.

12.10.2 International Status

The complex nature of plant protease inhibitor was studied (Rustagi et al. 2018) and reported that the activity of a cysteine protease, RD21 is controlled by two different PIs, Kunitz (AtWSCP) and Serpin (AtSerpin1) PIs over plant development and contributed to defenses against herbivorous arthropods and microbial pests. Benbow et al. (2019) identified and annotated the entire "serpinome" of hexaploid wheat and based on RNAseq data showed that Serpin-Z clade is highly expressed during grain development and have important role in response to fungal pathogen. Malefo et al. (2020) studied the maize BBI protease inhibitor and suggested that the overexpression of this inhibitor in Arabidopsis led to drought tolerance associated with reduction in drought-induced oxidative stresses. Studying the genome of *Solanum lycopersicum*, Fan et al. (2020) identified 55 PI genes. The transcriptome study revealed that abiotic and biotic stresses can induce SlPI expression. The protease inhibitor protein has the ability to balance the hormonal signals when subjected to the stresses and this will give an insight in unraveling its potential. These PI genes will be used in development of transgenic crops with increased resistance to various stresses.

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Global Status of Genetically Modified Crops 13

Vipasha Verma, Shivanti Negi, Pankaj Kumar, and Dinesh Kumar Srivastava

Abstract

The biggest problem the world is facing today is hunger, malnutrition, and the rising population. Adopting advanced breeding innovation technologies for enhanced agricultural production is urgently needed to meet the rising global demand for food and nutritional security. Recent advancements in GM technology, genome editing seem promising to accelerate crop improvement by enabling effective targeted modification in most crops/plants. Biosafety regulations for genome-modified plants are essential and often variable in various countries. In India, the GM Crops Release Regulation is one of the most regulated GM technologies in the world. The USA, Brazil, Argentina, Canada, and India planted about 91% of the global GM crop area of 190.4 million hectares. Soybean, maize, cotton, and canola are extensively grown and are of the utmost importance among all GM food crops. On six continents, approximately 32 plants were released for commercial cultivation. In five countries all over the world, 1.95 billion people have benefited from GM food biotechnology. This book chapter highlighted the global and Indian status of research and commercialization of GM crops, regulation of GM, and genome-edited technologies in India.

Keywords

Transgenic · Biotech crops · Hunger · Genetic engineering · Biosafety

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13.1 Introduction

Hunger and malnutrition are the most serious challenges the world is facing today. Food security is therefore crucial to the growing population of the world. The United Nations estimates that the world's population is growing rapidly and increasingly urbanized, leading to continuous degradation of natural resources (Kumar et al. 2020). In additament, the ongoing COVID-19 pandemic will have a severely adverse impact on food security that could exacerbate global hunger and malnutrition problems. To combat malnutrition and food scarcity, provocative and stronger action must be taken, involving collaboration between sectors such as agriculture, food, health, water and sanitation, social protection policy areas, development planning, and economic policy (ISAAA 2019). The adoption of genetically modified crops/biotech crops by several countries can also serve as an important way to meet the growing demand for food and to increase crop yields. Using recombinant DNA technology, various desired traits can be inserted into the genome of plants and genetically modified crops with enhanced resistance and improved yields can be obtained. Despite increasing crop yields and the development of new abiotic and biotic stress-tolerant plant varieties, genetic engineering can be used for a wide range of other purposes, such as generating new animal communities, diagnosing plant or animal diseases, increasing livestock feed and plant-based vaccines (Nalluri and Karri 2020). Various socio-economic benefits of GM crops include global food, feed, and fiber security through increased productivity; nation's arable land independence; prevention of deforestation and conservation of biodiversity; mitigation of climate change-related challenges; and improvement of economic, health, and social benefits. It is necessary to make the socio-economic benefits of GM crops known to the global community so that farmers and consumers remain aware of them and can make informed choices as to which crops to grow and consume. This will also increase awareness among policymakers and regulators of the development of supportive biosafety guidelines for the commercialization and adoption of GM crops and will also be of use to science communicators and media to effectively disseminate the benefits and potentials of this technology (ISAAA 2019).

13.2 Global Status of Research and Commercialization of GM Crops

Globally, after the adoption of two important genetic transformation techniques, such as direct gene transfer and *Agrobacterium tumefaciens*-mediated gene transfer, genetic improvement of major crops has come into existence (Altpeter et al. 2005; Datta et al. 2003; Sahrawat et al. 2003). GM technology emerged in 1983, after the production of transgenic tobacco—the first event of recombinant DNA technology in 1983, but it took 10 years for the commercial release of the first genetically engineered plant named FlavrSavr Tomato (Bruening and Lyons 2000). This crop became the first GM crop approved for commercial cultivation in 1994 (Smith et al. 1988). This event was a milestone and led to the development and

commercialization of various other GM food and non-food crops worldwide. Talking about the current scenario, around 32 crops have been released for commercial cultivation on six continents worldwide, including insect pest-resistant *Bt* cotton, *Bt* maize, *Bt* canola, herbicide-resistant soybean, virus-resistant potatoes, and other crops including papaya, melon, and squash (FAO 2012). Various other important applied research areas involve the use of genetic engineering, including global research on C4 photosynthesis in food crops such as rice and wheat, biological nitrogen fixation, efficient plant solubilization of phosphorus and potassium, and micronutrient-efficient crops, are under consideration. The development of efficient bio-fertilizers and bio-pesticide microbial inoculants through the use of genetic engineering as a tool and the production of biodiesel from genetically engineered plants, bacteria, or algae is of great use in the coming future (NAAS 2020).

With the development of GM crops with biotic stress resistance, including insect pest resistance, disease resistance, and other important features such as herbicide tolerance, improved nutritional quality, etc., the cultivation of GM crops has also increased and ultimately increased GM crop area to a record 191.7 Mha by 2018. However, a slight decrease of 1.3 million hectares (3.2 million acres) in the total GM crop area was reported in 2019, resulting in 190.4 million hectares of GM crops from 191.7 million hectares. A total of 71 countries around the globe have adopted GM crops. Among 71 countries, only 29 countries have grown 190.4 million hectares of GM crops. Out of 29 GM crop-growing countries, 24 developing countries have grown 56% of GM crop area and 5 industrial countries have grown 44% of GM crop area. However, the rest of the 42 countries (including EU countries) imported GM crops for food, feed, and processing. The major producers and exporters of GM crops and their products are the USA, Argentina, and Canada (James 2011). Among developing countries, Argentina, Brazil, China, and India are the largest producers of transgenic crops (James 2014). Around 91% of the global GM crop area of 190.4 million hectares has been planted in USA, Brazil, Argentina, Canada, and India. As a result, 1.95 billion people have benefited from biotechnology in five countries around the globe.

In 2019, the USA planted 71.5 million hectares of GM crops, the majority of which included GM soybean (30.43 million hectares) followed by GM maize (33.17 million hectares), GM cotton (5.31 million hectares), GM canola (800,000 hectares), GM sugar beets (454,100 ha), GM alfalfa (1.28 million hectares), GM potatoes (1780 ha), GM papaya and GM squash (1000 hectares each), and GM apples (265 ha) (Fig. 13.1). Followed by USA, Brazil covers 52.8 million hectares of GM crop area planted in 2019.

This area includes mostly GM soybeans (35.1 million hectares) higher than US GM soybeans, followed by GM maize (16.3 million hectares), GM cotton (1.4 million hectares), and GM sugarcane (16.3 million hectares) (18,000 ha). The third country with the largest GM crop area is Argentina. It covers around 24 million hectares of GM soybeans (17.5 million hectares), maize (5.9 million hectares), cotton (485,000 ha), and more than 1000 ha of GM alfalfa. Argentina showed a rate of adoption close to 100%. Also, the Government of Argentina approved nine applications for biotech in 2019 through the National Advisory Committee on

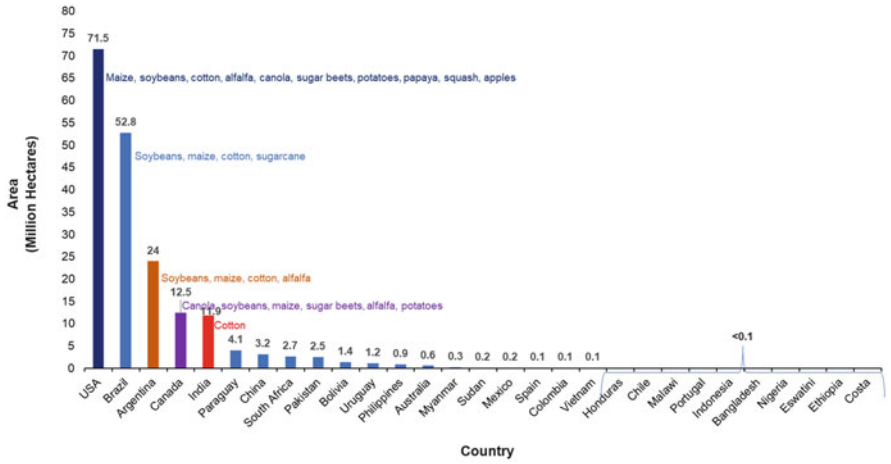

Fig. 13.1 Global area of biotech crops

Agricultural Biotechnology (CONABIA) of Argentina, which includes six maize events, two cotton events, and one soybean event. However, the GM crop area in Canada decreased by 2% in 2019. From 12.75 million hectares in 2018 to 12.46 million hectares in Canada, areas planted with GM maize, canola, and alfalfa have increased marginally, but sugar beet has increased by 23%. In India, the rate of adoption of insect-resistant (*Bt*) cotton has remained stable at more or less than 95% in the last 5 years. In Latin America, 83.9 million hectares of GM crops have been planted in ten countries. However, around nine countries in Asia and the Pacific have grown 19.5 million hectares of GM crops. In the European Union, two countries planted 111,883 ha GM maize. The African continent accounts for 1.54% of the global GM crop area of 190.4 million hectares, doubling the number of countries planting GM crops from 3 in 2018 to 6 in 2019 (ISAAA 2019). It has never been easy for GM technology to establish its place in the global community compared to many other technological advances in many other scientific fields. Although no clear evidence of damage to GM technology has been presented, there is still a wide-ranging debate on its adoption. GM crops have faced serious opposition from various conservative and environmental groups and bans on biosafety from many governments around the world. Various mainstream scientific establishments/institutions and more than 100 Noble Laureates around the world favor the use of GM crops (Roberts 2018). Countries such as the USA, Canada, and Japan have already accepted and marketed a number of food and non-food GMO products with little opposition to certain GM foods. Recently, in the USA, the majority of people strongly support food labeling in order to remain aware of the presence of GMOs in food products, to make their own independent choices for GM or non-GM food (The New York Times 2013). Talking about Europe and the United Kingdom, the status of GM food is still questionable, as the public is more concerned about biosafety and

the environment. Until now, only GM non-food crops are allowed to be used in the UK and Europe. However, the UK Government is making every effort to convince people about the safety of GM food and its dire need to meet the growing demand for food in developing countries (The Guardian 2013). Argentina and Brazil have adopted GM foods with little opposition from the general public. In African countries like Uganda, there is the existence of resistance against GM food (leaf wilt resistant GM banana). According to a few NGOs and local civil society groups in Uganda, transgenic technology is less safe as compared to previously available traditional methods to control banana leaf wilt (The Independent 2015).

A total of 71 countries (29 growing and 42 non-growing) have issued regulatory approvals for GM crops for human food, animal feed, and commercial cultivation. Since 1992, the regulatory authorities have granted 4485 approvals to 403 biotech events from 29 GM crops, except for few floral crops such as carnation, rose, and petunia. Of the 4485 approvals, 2115 were for food, 1514 were for feed use, while 856 were for environmental release or cultivation. Among 71 countries, the USA had the highest number of approved GM events followed by Japan, Canada, Brazil, and South Korea (ISAAA 2019). Taking into account GM crops, maize was found to have the highest number of approved events (146 events in 35 countries), followed by cotton (66 events in 27 countries), potatoes (49 events in 13 countries), soya beans (38 events in 31 countries), and canola (38 events in 15 countries). The highest number of approvals was obtained from herbicide-tolerant maize event NK603 (61 approvals in 29 countries, includes 28 European countries as one). It is followed by GTS 40–3-2 herbicide-tolerant soybean, which has received 57 approvals in 29 countries (Fig. 13.2).

13.3 Status of Research and Commercialization of GM Crops in India

Agricultural biotechnology as the third largest sector in the Indian biotech industry has made a significant contribution to the socio-economic growth of the country. Among Asia and the Pacific region, India is a leading Biotech country constituting around 11.9 million hectares of GM crop area for cotton followed by other Asian countries like China, Pakistan, Myanmar, Vietnam, Indonesia, and Bangladesh. Despite the justification of several researchers for GM food safety, India is still waiting for its first GM food to be commercialized. To date, only *Bt* cotton has been approved as a genetically modified crop for commercial cultivation in India (Choudhary and Gaur 2015; James 2014). In the 10 years since its introduction as a commercial GM crop, India has become the world's largest producer and exporter of *Bt* cotton. The use of *Bt* cotton has doubled its yield and reduced the use of the pesticide to half. The rate of adoption of insect resistant (*Bt*) cotton in India has remained stable (95%) in the last 5 years. Looking at the socio-economic benefits of planting *Bt* cotton, farmers' confidence in the use of GM technology has been increased, which has led them to plant more GM crops that can be profitable in terms of economic gains. Thus, in favor of GM cotton, few groups of framers had

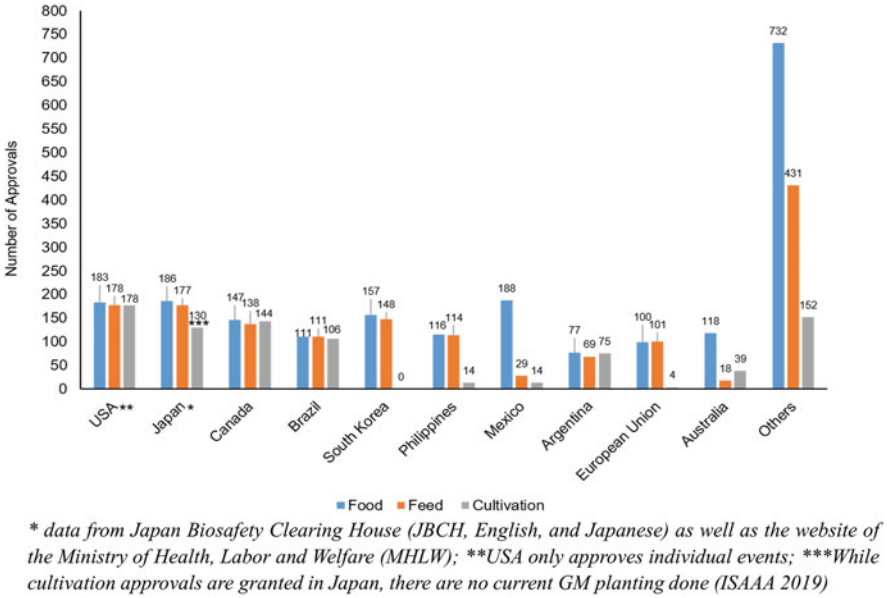

Fig. 13.2 Top ten countries which granted food, feed, and cultivation/environment approvals. * data from Japan Biosafety Clearing House (JBCH, English, and Japanese) as well as the website of the Ministry of Health, Labor and Welfare (MHLW); **USA only approves individual events; ***While cultivation approvals are granted in Japan, there are no current GM planting done (ISAAA 2019)

also planted and pushed for the approval of unauthorized stacked IR(*Bt*)/HT cotton traits. To date, no GM food crop has been commercialized in India. Even after a recommendation from the GEAC, *Bt* brinjal and GM mustard are still awaiting final government approval (Frewer et al. 2013; Kandasamy and Padmavati 2014; San-Epifanio 2017). In 2010, former Environmental Minister, Jairam Ramesh blocked the release of *Bt* brinjal until further notice owing to a lack of consensus among scientists and opposition from brinjal-growing states. No objection certificates from states were made mandatory for field trials. However, in Bangladesh, *Bt* brinjal has got regulatory approval in 2013 and became the first GM food crop released for cultivation in a developing country. In 2016, GEAC gave a green signal to GM mustard for field trial, but SC stayed the order and sought public opinion on the same. Dhara Mustard Hybrid-11 or DMH-11 is a genetically modified hybrid of mustard developed by the Delhi University's Center for Genetic Manipulation of Crop Plants. Researchers at Delhi University have developed hybrid mustard DMH-11 using "barnase/barstar" technology for genetic modification. If approved by the Center, this will be the second GM crop after *Bt* cotton and the first transgenic food crop to be grown in the country. Scientists at leading institutes in India such as MSSRF in Chennai, ICAR-NIPB, ICGEB, DU and JNU in New Delhi, the University of Calcutta, and the Bose Institute in Kolkata have developed GM

crops with a variety of agriculturally important characteristics. One such important research was carried out by Indian Scientists at the MSSRF, Chennai. Transgenic abiotic stress-tolerant crops have been developed by identifying salt and drought-tolerant genes from mangroves and *Prosopis juliflora* (George et al. 2007; Jyothi-Prakash et al. 2014). Genetically engineered rice has been developed with several novel traits such as high iron/zinc/provitamin A, abiotic stress tolerance, and disease and pest resistance (Datta et al. 2000; Bhattacharya et al. 2019). ICAR-NIPB has developed pod borer-resistant pigeon pea and herbicide-tolerant cotton by using its own *Bt* and *CP4* genes, respectively (Ramkumar et al. 2020; Karthik et al. 2020). Numerous other GM crops carrying novel traits such as insect resistance, herbicide tolerance, drought tolerance, salinity tolerance, virus resistance, high yielding, bio-fortified are under different stages of research and field trials in India, namely cotton, rice, wheat, maize, brinjal, potato, sorghum, mustard, groundnut, cauliflower, okra, chickpea, pigeon pea, sugarcane (FAO 2014; Gupta and Ahuja 2016). However, few crops, such as cotton, brinjal, mustard, maize, and chickpea are almost ready for commercial release at the final stage of field trials. Although the Food Safety and Standards Authority of India (FSSAI) issued this order on 1 January 2021, importers of 24 major food crops will have to make a mandatory declaration that the products are not genetically modified and that they are also of non-GM origin. Most of the environmental groups were concerned about the presence of genetically modified organisms (GMOs) in imported food. Those 24 food crops include apple, eggplant, maize, wheat, melon, pineapple, papaya, plum, potato, rice, soybean, sugar beet, sugarcane, tomato, sweet pepper, squash, flaxseed, bean plum, and chicory.

13.4 GM Crops Release Regulations in India

GM technology is one of the world's most regulated technologies. Governmental organizations such as the Food and Agriculture Organization of the United Nations (FAO), the World Health Organization (WHO), and the Organization for Economic Co-operation and Development (OECD) have performed numerous safety studies on biotech food crops and concluded that biotech food crops are as safe and nutritious as traditional and organic food. India has established a well-structured regulatory system for the biosafety assessment of GMOs (Herring 2014). It is one of the most stringent GMO regulatory frameworks in the world. Before granting any regulatory consent, the developer has to perform comprehensive food, feed, and environmental protection studies. India is a signatory to the Cartagena Protocol on Biosafety. The Cartagena Protocol on Biosafety sets out comprehensive biosafety regulations for LMOs (living modified organisms) and provides suggestions on the biosafety criteria for GM crop research and also sets out its role in the process of commercialization and deregulation. In India, the regulation of all activities related to GMOs and products derived from GMOs is governed by "Rules for the Manufacture/Use/Import/Export and Storage of Hazardous Microorganisms, Genetically Engineered Organisms or Cells," (Rules 1989), under the provisions of the Environment

Protection Act (EPA), 1986 through the (MoEF&CC) Ministry of Environment, Forest and Climate Change (Warrier and Pande 2016). The Rules 1989 notified by MoEFCC are very broad in scope essentially covering the entire spectrum of activities involving GMOs and products thereof including the sale, storage, exportation, importation, production, manufacturing, packaging, etc. (MoEF Notification 1989). These rules cover research areas as well as large-scale uses of GMOs and their products and refer to the production, import, and storage of microorganisms and gene technology products; genetically modified organisms/microorganisms and cells and, accordingly, to all substances and products and foodstuffs, etc., of which such cells, organisms, or tissues are part; (James 2014). Gene technology and genetic engineering have been defined as follows in the text of the Rules, 1989.

1. "Gene Technology" means the application of the gene technique called genetic engineering including self-cloning and deletion as well as cell hybridization.
2. "Genetic engineering" means the technique by which heritable material, which does not usually occur or will not occur naturally in the organism or cell concerned, generated outside the organism or the cell is inserted into said cell or organism. It shall also mean the formation of new combinations of genetic material by incorporation of a cell into a host cell, where they occur naturally (self-cloning) as well as modification of an organism or in a cell by deletion and removal of parts of the heritable material. This definition of genetic engineering in the Rules, 1989 implies that new genome engineering technologies including gene editing and gene drives. May be covered under the rules.

Series of biosafety guidelines have been issued under Rules, 1989 to minimize any adverse impact that the GMOs and product thereof would have on the environment as well as human and animal health. There are separate guidelines for various stages of development and use of LMOs, viz. contained use, confined field trials, food safety, environmental release, etc. as indicated:

- Recombinant DNA Safety Guidelines, 1990.
- Revised guidelines for research in transgenic plants, 1998.
- Guidelines for generating preclinical and clinical data for rDNA vaccines, diagnostics, and other biologicals, 1999.
- Guidelines for Conduct of Confined Field Trials (CFTs) of Regulated, Genetically Engineered (GE) Plants, 2008.
- Standard Operating Procedures (SOPs) for CFTs of Regulated, GE Plants, 2008.
- Guidelines for Monitoring of CFTs of Regulated, GE Plants, 2008.
- Guidelines for the Safety Assessment of Foods Derived from Genetically Engineered Plants, 2008.
- Protocols for Food and Feed Safety Assessment of GE crops, 2008.
- Guidelines and Handbook for Institutional Biosafety Committees, 2011.
- Guidelines on Similar Biologics: Regulatory Requirements for Marketing Authorization.

The rules are implemented by the Ministry of Environment and Forests, Department of Biotechnology, and State Governments through the defined competent authorities. There are six competent authorities presently as per the rules, namely:

1. *The Recombinant DNA Advisory Committee (RDAC)*. This committee functions in the Department of Biotechnology and provides reviews about the new developments in biotechnology at national and international levels. This committee is advisory in nature. Thus it recommends suitable and appropriate safety regulations from time to time for research, use, and applications of GMOs. This committee prepared the Recombinant DNA Biosafety Guidelines in 1990, which were adopted by the Government for conducting research and handling of GMOs in India.
2. *Committee on Institutional Biosafety (IBSC)* Committee shall be formed in registered institutions to carry out research activities involving the genetic modification of microorganisms, plants, or animals. The IBSCs include the head of the organization, scientists engaged in DNA research, a medical expert, and a DBT candidate. The IBSC is the nodal point for interaction within the institution for the implementation of the guidelines.
3. *Review Committee on Genetic Modification (RCGM)* committee works within the Department of Biotechnology to track safety-related aspects of ongoing research projects and activities involving genetically modified organisms/hazardous microorganisms. The Review Committee on Genetic Manipulation (RCGM) shall include members of (a) the Department of Biotechnology, (b) the Indian Medical Research Council, (c) the Indian Agricultural Research Council, (d) the Science and Industrial Research Council, (e) other experts in their capacity. RCGM shall draw up manuals and guidelines set out the regulatory protocol for activities involving genetically engineered organisms in research, use, and applications including industry to ensure environmental safety.
4. *The Genetic Engineering Approval Committee (GEAC)* has been renamed the Genetic Engineering Appraisal Committee. GEAC is an apex committee that functions within the MoEFCC and has representatives of the ministries/agencies and experts concerned. GEAC is chaired by a senior MoEFCC officer and co-chaired by an expert appointed by DBT. It is responsible for the approval of activities involving large-scale use of harmful microorganisms and recombinant materials in research and industrial development from an environmental point of view.
5. *The State Biotechnology Coordination Committee (SBCC)*, headed by the Chief Secretary of State, shall be formed in each State where research and applications for GMOs are underway. The Committee shall regularly review safety and control initiatives in the various industries/institutions handling genetically modified organisms/hazardous microorganisms.
6. *District Level Committee (DLC)*, formed at the district level, is considered to be the smallest authority to review safety regulations for installations engaged in the use of GMOs in research and applications. At the district level, each DLC is headed by the District Collector, along with officials concerned with public

health, the environment, pollution control, etc. Interactive processes between committees have also been provided for in the 1989 Rules of Procedure. Both IBSCs are required to review the applications and to send their recommendations and reports to RCGM. RCGM analyses and makes a recommendation on large-scale operations, field trials, and environmental releases to GEAC. DLCs are also required to regularly submit its report to the SBCC/GEAC.

Various sub-committees and Expert committees are set up by RCGM and GEAC on a case-by-case basis and comprise experts from various disciplines drawn from public sector institutions to prepare and review various guidelines and biosafety data. Central Compliance Committees are also set up for monitoring of confined field trials on a case-by-case basis. Also, there are other acts, rules, and policies which are also applicable to these organisms. Some of these are Plant Quarantine Order, 2003; Food Safety and Standards Act, 2006; DGFT Notification Relating to Inclusion of GM Policy in Foreign Trade Policy, 2006–2009, etc.

13.4.1 Genome Editing: A New Era of Precision Plant Breeding

Innovative breeding technology for enhanced agricultural production is urgently needed to meet the growing global demand for food. Recent advances in genome editing seem promising to accelerate crop improvement by enabling effective targeted modification in most crops/plants (Abdallah et al. 2015). Genome Editing (GE) involves the use of site-directed nucleases (SDNs) and sequence-specific nucleases (SSNs) to create useful genetic variations in the target genome region (Langner et al. 2018). Genome editing was initially performed for targeted mutagenesis in the genome by using an array of SDNs such as meganucleases (MN), zinc-finger nucleases (ZFN), and actuator-like transcription nucleases (TALEN). Recently, however, highly versatile clustered regularly interspersed palindromic repeat (CRISPR)-CRISPR-associated protein (Cas)-based genome editing systems have been developed that use RNA-DNA binding for high specificity (Gaj et al. 2016). However, ZFN and TALEN are dependent on the binding specificity of protein–DNA. The basic principle of genome editing is to create targeted double-strand breaks (DSBs) using endonuclease, such DSBs are then repaired using endogenous DNA repair mechanisms, including non-homologous end-joining (NHEJ) or homology-directed repair mechanisms (HDR). Repairing through NHEJ often results in small insertion and deletion (Indels) of the target DNA resulting in point mutations. However, in HDR-based repairs, this leads to highly specific targeting of genes. It involves the use of an exogenously introduced homologous template as a donor DNA, which enables precise modification (insertion, deletion, or substitution) in the targeted genomic region for highly specific targeting of either gene replacement or gene insertion (Bortesi and Fischer 2015; Zhang et al. 2018). The CRISPR-based gene editing tools are highly diversified, sophisticated and are expanding at the fastest pace, including the discovery of various Cas enzymes with unique PAMs and precise/engineered Cas9 variants, cytidine or

adenine base editors, development of new base mutators, and improved HDR mediated gene editing (Molla and Yang 2019).

Among other genome modification techniques, CRISPR-Cas-based genome editing is a simple, efficient, fast, and accurate method for generating desired changes in the plant genome. It is highly versatile with several applications for genome and epigenome modification. It can lead to the creation of Indels in the protein-coding sequence as well as in the promoter region of the gene and is routinely used by researchers for the deletion of whole genes or chromosome fragments, gene replacement, or insertion of a new gene or allele of the same or related species, accurate base editing, gene silencing by RNA processing, and last but not least is epigenome editing. It has many advantages over other genome modification techniques. Traditional plant breeding methods for gene modification such as mutagenesis (natural or induced), recombination (by natural cross-pollination or artificial hybridization), transposon-mediated modification (natural, tissue culture-induced, or by transformation), and transgenic crops generate random changes in the genome and require the selection of the desired genotype using either phenotypic or marker. However, CRISPR-Cas-based genome editing confers target specificity by involving the use of the specific guide RNA which is designed based on the target genes. Genome editing avoids several problems associated with traditional breeding methods and GM crops such as the problem of linkage drag with closely linked useful and deleterious genes, which is quite common in traditional breeding, expression problem occurs due to the random integration of foreign gene into the genome in classical genetic modification (GM crops) and also the integration of selectable markers (antibiotic or herbicide resistance genes) in the genome of GM crops remain. However, precise and stable mutations are created in genome editing, which can be easily segregated from integrated transgenes for the development of transgene-free plants.

In plants, CRISPR-Cas9 based genome editing was first carried out in 2013 in the model plant *Arabidopsis* using reporter genes (Li et al. 2013) and tobacco (Nekrasov et al. 2013). After that, several important gene-edited food crops were developed including rice (Menz et al. 2020), wheat (Wang et al. 2014), maize (Liang et al. 2014), rice (Miao et al. 2013), tomato (Čermák et al. 2015). Using gene editing, several agriculturally important traits have been incorporated into food crops, including (1) herbicide-tolerant maize by targeting acetolactate synthase genes *ALS1* and *ALS2* (Svitashev et al. 2015); (2) targeting of downy mildew resistance gene *SlDMR6–1* tomato resulted in disease-resistant tomato (Thomazella et al. 2016); (3) knock out of polyphenol oxidase (*PPO*) gene using gene editing has led to non-browning in white button mushrooms (Waltz 2016); (4) virus-resistant cucumber by targeting eukaryotic translation initiation factor gene *Cs-eIF4E* (Chandrasekaran et al. 2016); (5) drought-tolerant maize by knocking out a negative regulator of ethylene response (Shi et al. 2017); (6) bacterial leaf blight resistant rice targeting sugar transporter genes OsSWEET11-13 (Oliva et al. 2019). Thus, genome editing technology appears to be promising both in terms of boosting basic science research and in terms of crop improvement.

13.5 Regulatory Framework for Genome-Edited Plants

13.5.1 The Global Regulatory Scenario on Genome-Edited Plants

Gene editing in crops has emerged over a decade ago as the most revolutionary and promising breeding tool designed more precisely to introduce new or modified genes to improve plant characteristics such as better growth, stress tolerance, yield, quality, nutrition, or sustainability. Concerning its several applications for crop improvement, gene editing was referred to as the beginning of the second biotechnological revolution in agriculture. Since most of the products developed by genome editing are not very different from those obtained by natural spontaneous or induced mutations, it is therefore not very easy to monitor and regulate genetically modified crops. Most countries around the world are reconsidering and amending their legislation and biosafety regulations to accept this versatile and highly practical technology. For the regulations on genetically modified crops, the genetically modified crops/products have been grouped into three categories (Friedrichs et al. 2019; Ricroch 2019). Different categories depend on the nature of the combination of the targeted double-strand break (DSB) created by site-directed nuclease (SDN) with or without homology-directed repair. Among the three categories, SDN1 is the first to involve the repair of DSB in target DNA by non-homologous end-joint (NHEJ) through an endogenous repair mechanism without homology-directed repair (HDR). NHEJ-based repairs create small Indels at the junction point, resulting in gene failure or loss of gene function, changes in gene activity. The second category is SDN2, which involves the HDR repair of the targeted DSB using a short single-stranded DNA donor, also known as a repair template. The donor DNA carries one or more mutations flanked by sequences that match one or both ends of the target DSB, allowing the desired alteration of the target gene. The last category of gene-edited plants is known as SDN3, which involves the HDR-guided template of the target DSB using a double-stranded DNA donor containing a complete gene or even a longer genetic element. Both ends of the donor are homologous to the two ends of the DSB (usually over 800 bp each) which allows the gene to be replaced or the new gene to be inserted at the target site. This category of genetically modified products is quite similar to that of genetically modified organisms.

Biosafety regulations for genome-edited plants are variable in different countries. In a few countries, the regulations are the same as those for GM plants, while few countries are less strict concerning GE products than GM plants. Regulations are still under discussion in many countries. However, there are few large countries where there is still no public debate on the regulatory affairs of GE products. In 2018, the Court of Justice of the European Union (ECJ) ruled that genome-based organisms must be regulated in the same way as GMOs, but that chemical and radiation mutagenesis products are exempt from biosafety regulations. New Zealand is the other country that regulates genome-edited organisms (GEO) in the same way as GMOs. The GEO Regulations in both the EU and New Zealand are based on court rulings and have little influence on scientific establishments. Stringency in the regulations on genetically modified crops had a negative impact on the innovations

of this technology, which has led major agricultural companies to move out of Europe. Governments of other countries, such as Norway, Switzerland, and the United Kingdom are considering separate legislation to regulate the GEO (Transkript 2018). In Norway, in particular, the Norwegian Biotechnology Board exempted SDN1 products from biosafety regulations. Certain categories of GEO which do not have foreign DNA have been exempted from regulation by major agricultural countries such as Argentina, Australia, Brazil, Canada, Chile, Israel, Japan, Peru, and the USA. However, countries such as India, Bangladesh, Philippines, Indonesia, Kenya, Nigeria, Paraguay, and Uruguay are actively discussing separate biosafety regulations/exempt for GEO and GMOs. At the same time, there has been no public discussion on this issue in a few major countries such as China, Russia, Mexico, and South Africa.

13.5.2 State of Regulating Genome-Edited Plants in India

Several initiatives have been taken in India by leading research institutions, recognizing the potential of genome engineering technology in both healthcare and agriculture across the globe (Barrows et al. 2014; McHughen and Smyth 2008; Sinebo and Maredia 2016). Despite the availability of trained personnel, India is still a long way behind countries like China and the USA in terms of research and use of this technology. This is due to a lack of policy directions or decisions that allow it to be used. To foster innovation and promote the development of Genome-wide Analysis and Engineering Technologies, DBT formed a dedicated Task Force on "Genome Engineering Technologies and their Applications" in 2014 with a vision to make them accessible and affordable for wider use in life sciences. DBT has proposed to strengthen the infrastructure and facilities for innovative technologies such as gene editing and has also extended support for gene editing research projects. However, more effort is needed to develop human resources to catch up with the growth in genome editing and to make use of its benefits for basic or applied research. Regulation of genome engineering technologies is still the subject of debate in India. India recently released a draft document on Genome Edited Organisms: Regulatory Framework and Risk Assessment Guidelines circulated by the Indian Ministry of Biotechnology, inviting comments from researchers, institutions, and other stakeholders. This draft suggested a risk approach for the regulation of genome editing products (Indian Ministry of Science and Technology 2020). The definition of GE technology as set out in the 1989 Rules is very broad-based and that all new technologies will be subjected to regulation in accordance with the 1989 Rules of procedure (Sinebo and Maredia 2016; Raman 2017). As far as the current definition is concerned, the policy considerations for new and evolving technologies must be on a case-by-case basis, depending on the existing framework, i.e. Rules of Procedure, 1989. There are, however, few recommendations for the Regulation of Genome Edited Plants in India, including (NAAS 2020);

1. The internationally accepted and clearly defined classification of genome-edited organisms as SDN1, SDN2, and SDN3 should be done in place of GEd1, GEd2, and GEd3 as outlined in the DBT draft.
2. As final products of the genome-edited category, SDN1 and SDN2 are free from foreign DNA and are indistinguishable from products developed by traditional breeding methods using natural genetic variation or induced mutations. It is therefore not necessary and also not scientifically feasible to regulate these products in accordance with the rules of the 1989 Environmental Protection Act, 1986.
3. As the initial generation of genome-edited plants involves rDNA techniques, their registration with the IBSC with information from the RCGM will continue. However, for the commercial release of the final product, which is devoid of any foreign DNA, guidelines for the assessment of the efficacy and field performance of the traits shall be carried out under the ICAR-AICRP Guidelines or any other existing assessment, release, and notification procedures as laid down in the Seed Quality Regulations (Seeds Act 1966, Seeds (control) order 1983, Seeds Rules, PPVFRA Act 1986, National Seed Policy and the proposed New Seeds Bill).
4. The rules governing the biosafety and release of SDN 3 products should be less stringent than the conventional GMO in terms of the data required for substantial equivalence.
5. Genome-edited plants involving the replacement of an existing gene with superior alleles of the same species or a primary/secondary gene pool with known protein function should be categorized as an SDN 2 type product.
6. Food derived from categories SDN1 and SDN 2 should not be treated as "genetically engineered or genetically modified food" under the Food Safety and Standards Act, 2006.
7. The national regulatory system for genome-edited plants should be in line with other countries such as Australia, Brazil, Canada, China, Japan, and the USA to facilitate smooth international trade in genome-edited products and the effective exchange and sharing of genetic material for research and development.

Therefore, to meet the UN's zero hunger challenge and meet the 2030 Sustainable Development Goals, by eliminating poverty and hunger, we need to significantly increase agricultural productivity and double farmers' incomes, "leaving no one behind." Such a transformation will only be possible through the use of innovative technologies such as genome editing.

13.6 Conclusion

The global population is constantly increasing and is expected to reach 9 billion by 2050, which is very critical in terms of food security and malnutrition. The Global Food Insecurity Report of 2019 also revealed that the United Nations (UN) Millennium Development Goals (MDGs) could not achieve their targets,

making it more difficult to meet the Zero Hunger target by 2030. Moreover, climate change and other pandemics (Covid-19) have a negative impact on food production and the economy around the globe (Tsatsakis et al. 2017). The adoption of new technologies for crop improvement is therefore crucial to both meeting food demand and boosting the economy. In this respect, genetic engineering and the recently developed technology of genome editing can reduce the current challenges in commercial agriculture and can also bring significant profits to farmers. Both GM and GE crops have a wide range of advantages for the maintenance of ample and nutritious food, climate change and biotic and abiotic stress-related agricultural problems can also be mitigated. A number of added benefits, such as high yield crop varieties, bio-fortified crops (higher nutritional value), herbicide tolerance, tolerance to various biotic and abiotic stresses, the increased shelf life of fruit and vegetables can lead to a boost in farmers' incomes by developing a good market. However, biotech/GM crops have the potential to be a universal solution to the problems of malnutrition and hunger, as well as an important aspect of the food safety program. Public acceptance and enabling policies in favor of biotech crops in government are therefore a key to achieving the agricultural, socio-economic, and environmental benefits of the poor and the hungry.

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Organic GMOs: Combining Ancient Wisdom with Modern Biotechnology

14

Amjad M. Husaini

Abstract

Environment-friendly sustainable food production for a growing human population has become challenging. Synthetic fertilizer-driven, intensive agriculture has resulted in substantial environmental damage. Neither this conventional chemical-based agriculture nor organic farming is sustainable in the long run. Thus out-of-the-box thinking is required to tackle these challenges. The chapter discusses why we should start to grow GM crops organically and provide a "new" choice to consumers of "organically-grown GM crops." Many arguments have taken place on "organic vs conventional" and "acceptance vs non-acceptance" of GM crops. Therefore, the debate needs to go beyond this binary and towards "voluntary extension" of organic agricultural farming practices into "high-tech biotech crops". The purpose is to combine "improved-genetics" with "soil-healthy agronomy" for harnessing the full potential benefits of both in a complementary way.

Keywords

Transgenic · GMO · Genetic engineering · Organic agriculture · Precision agriculture · Robotics · Hunger · Sustainability · SDGs · Food

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14.1 Introduction

Feeding the world without further harming the planet is a great challenge for humanity. While the demand for food may increase 70% by 2050, food production worldwide has a high impact on natural resources on which it is fundamentally dependent. By the year 2100, the number of people on earth is expected to increase to more than 11.2 billion. About two billion people, among the current 7.6 billion, constitute small farming households (with less than 2 ha of land each) and a majority (85%) of the poor and food insecure (Husaini and Tuteja 2013). Enhancing the livelihood security of this population as well as increasing productivity of their small farms is central to the United Nation's Sustainable Development Goals, which emphasize that food needs to be grown "sustainably" using fewer resources on the same arable land. These factors coupled with the new challenges associated with climate change, demand alternative and politically acceptable farming strategies and new thinking.

What is the best way to produce enough food to feed all these people? In a recent report on "Food Systems and Natural Resources," the UNEP provides evidence of unsustainable and wasteful methods used globally in the present food systems. The report highlights that 33% of the world's soil is degraded; 20% of aquifers are overexploited; 60% loss in terrestrial biodiversity is connected with food production, and 80% of mineral inputs are lost to the environment (Axelos 2017). In case we want to increase resilience in the food system while conserving ecosystems, we need to reflect upon climate change, habitat loss, water resources, input availability, urbanization, and United Nations Sustainable Development Goals (UN SDGs). Answers to our future food requirements have to be sought in new crop cultivation approaches, such as genetic modification (GM) and aquaculture, combined with conventional and organic farming methods. Genome engineered crops have a significant advantage over traditional crops and have immense potential. These crops are developed by "borrowing" traits from sexually non-compatible individuals who may live poles apart and belong to diverse kingdoms. However, GM crops often face resistance from civil groups (Husaini and Tuteja 2013). But is the fear against the latest farming methods, in particular against GM crops, real? Do we need to rethink organic agriculture (Husaini and Sohail 2018). Putting things in perspective requires some analysis.

14.2 The Controversies

Unfortunately, controversies developed around transgenic technology due to some internal and external influences. In 1993, the blockbuster movie Jurassic Park showed how human intervention could lead to a disaster by creating horrific creatures like dinosaurs, thanks to the creative imagination of Steven Spielberg—the director. This made the atmosphere fertile for horror against DNA-based technologies. In 1994, the World Trade Organization (WTO) made it compulsory for developing as well as developed countries to provide monopoly rights on seeds.

The seed corporations sought to enforce the intellectual property rights on seeds, but it was costly and difficult. This caused them to search for a self-enforcing biological way to protect their intellectual property. As if this was not enough a scare for neo-colonialism, in 1998 US Patent Office granted the USDA and Delta & Pine Land Company (DPL) a patent for genetic engineering that could kill seeds. It was called "technology protection system" by DPL and "terminator technology" by Rural Advancement Foundation International. This technology was used to impose "writ" in the form of a "virtual ban" on seed reuse, actually a substitute for a practically not-so-easily implementable "de facto patent." With all this confusion around, when Green Peace started opposing it, common people saw it as a savior for a just cause, and slowly a campaign against GMOs began to take its roots. Pusztai affair in 1998 added to the confusion by claiming ill effects on the epithelial lining of mouse fed on GNA potatoes. Immediately after (in 1999) was the Monarch butterfly issue, which raised concerns about the off-target effects on beneficial insect populations living on Bt plants. Is this not worth a serious thought that why there is no opposition to "Humulin"—the first genetically engineered insulin, which comprises a significant chunk of insulin used clinically today?

14.3 Time to Cultivate GM Crops Organically for Intensive Sustainable Farming: The "New" Option

Organic crops may have many merits but the major constraints are limited productivity coupled with huge losses due to pests and diseases (Chang and Zepeda 2005; Martin 2009; Treadwell et al. 2010; Seaman and Sideman 2013). One distinct way of dealing with twenty-first-century challenges is exploring the possibility of "introducing organic practices" in modern crops that are being developed through "genetic engineering" or bring together the best of the two opposite camps for the benefit of the consumers. The use of genetically engineered plants in organic agriculture has been advocated from different perspectives (reviewed by Ronald and Adamchak (2008), Ammann and Montagu (2009), de Renobales Scheifler (2009), Husaini and Sohail (2018)).

Traditionally there were only a few heirloom varieties or landraces that were used as cultivars, and later "artificial" crossing was used to create thousands of modern plant varieties that could suit high input agriculture based on the green revolution. However, with the advent of newer challenges in the present century, these varieties shall no longer sustain long enough. The genetic potential of these varieties needs to be enhanced to combat the newer challenges of climate change and sustainability (Table 14.1).

The two primary inputs for farming are a) Good seeds and b) Good farming technology. These need to be discussed in the context of climate change. Climate changes have adverse effects on key factors on which plant growth depends, carbon dioxide, sunlight, water, an endured temperature range, and nutrients. The undesirable variations in these factors can favor the multiplication of plant pathogens and lead to an outbreak of plant diseases. So, we shall need to either develop seeds that

Table 14.1 A summary of the major options available for crop production

Seed type + farming type	Remarks
Heirloom seed + organic farming = very low productivity	Suitable for eighteenth century
HYV seed + chemical-based farming = high productivity	Environmentally unsustainable
GM seed[a, b] + chemical-based farming = higher productivity	Lower environmental footprint
GM seed[a, b, c] + organic farming = moderate to high productivity	Environmentally sustainable and safe

References: FAO (2002), Wang et al. (2003), Deng et al. (2005), Xu et al. (2006), Dahm et al. (2009), Zhang et al. (2010), Stein et al. (2010), Dar et al. (2013), Klumper and Qaim (2014), Ruddock (2016)

[a]Category 1: Stress tolerance traits against drought, flooding, salt, cold, heat, insects, fungi, bacteria, viruses, etc

[b]Category 2 traits: Quality enhancing traits like delayed-ripening, better nutritional profile (vitamin A, iron, omega 3, phytase, etc.)

[c]Category 3: Multi-trait pyramided plants with better quality, productivity, and nutrient use efficiency

are inherently resilient to such biotic and abiotic stresses or develop such environmentally sustainable agro-technologies that are able to protect the crops at the time of stress or a combination of both (Husaini 2014) (Table 14.1). The "new" choice brings together the "positives" of integrating two diverse practices, old-age organic farming (Gold 2016) with new-age GM technology (Husaini et al. 2010a, b, 2011), and converging to address common issues like ecosystem sustainability and human health.

14.4 Farmers and Consumers Have the "Right to Choose"

Organic farming is about practices of agronomy and on-farm cultural practices with no/least chemical input. It should not matter if crop genetics has improved naturally or through conventional plant breeding or more precise recombinant DNA/genome modification techniques. Both farmers and consumers interested in the "new" option have been ignored to date. Those consumers who may be interested in buying products that possess benefits based on both traditional wisdom and modern engineering may likely be the first to buy agricultural products based on the "new" option. The choice is presently absent from the basket. There should have this additional choice like buying Organic Golden Rice. It could have health benefits associated with its organic cultivation as well as value addition due to genetic engineering.

14.5 What Prevents GM Farms from "Voluntary Adoption" of Organic Farming?

The well-being and the common motive of producing organic food are associated mainly with the absence of chemical residues and higher nutritional value (McEachern and McClean 2002; Hughner et al. 2007). Crops engineered for higher productivity, abiotic stress tolerance, enhanced disease, and pest tolerance, better nitrogen fixation ability, higher nutrient use efficiency, and amenable to resource conservation technologies could completely exclude chemical pesticides, fertilizers and hence be environmentally sustainable (Table 14.1). If this is the case, why can GM crops such as Golden rice not qualify to be "organic" when grown using established organic practices? Even if the current legislative system does not allow that (Seufert et al. 2017), GM farmers should adopt organic practices voluntarily and contribute to environmental sustainability!

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Genomics in Crop Improvement: Potential Applications, Challenges and Future Prospects

15

Jeshima Khan Yasin, Masudulla Khan, Shabir H. Wani, M. Arumugam Pillai, Nidhi Verma, P. Pandey, and Kuldeep Singh

Abstract

Until recent past, genomics and bioinformatics were considered as different subjects and now their applications started yielding crop improvement. Though the resources are being added fast in public databases, the effective utilization of the available resources is yet to be attempted. The reasons behind the slow phase

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of genomics applications and generating resources and possible future path are discussed in this chapter.

Keywords

Plant genomics · QTL mapping · Climate change · Abiotic stress · Genome editing

15.1 Introduction

The epoch of genomics evolved into its newer aspect of genome editing as enormous genomic resources are being added day by day. The major data resources of all three genebanks stopped accepting older versions of high-throughput sequence resources. In genomic resources, still there are unexplored data points. Whole-genome sequencing, assembly and its annotations were considered the last station in many instances and it was left untapped for future applications. SNP chips and QTLs were the maximum outputs relished in plant sciences unlike translational research in animal kingdom. With the available genomics data, lot has been achieved in the model organism, *Arabidopsis*. However, not all genes of *Arabidopsis thaliana* were characterized ever since its genome was sequenced. Though understood, the genetic contributions of maximum genes are not translated into crop plants for effective usage in crop improvement. Within a decade the scenario of using markers and mapping them in populations developed over years has changed into genomic selection and genome prediction of yet to be grown individuals. This paved the way through system biology approach and targeted genome editing. Though such kind of major revolutions in crop improvement is yet to happen, we analysed the trend in recent past to direct our expectations towards an objective oriented lucid crop improvement in front of plant breeder's community.

15.2 Crop Genomics and Strategies for Crop Improvement

Plant genomics stated with the first plant sequenced, *A. thaliana* (Jackson et al. 2011; Vats et al. 2019). Whole-genome shotgun sequencing methods accelerated the genome sequencing by eliminating the library preparation steps and physical mapping steps (Batzoglou et al. 2002). Availability of whole-genome sequence assemblies of crops was utilized in crop improvement, such as maize (Schnable et al. 2009) and soybean (Schmutz et al. 2010), cucumber and apple (Meriç et al. 2020; Velasco et al. 2010). Evolving genomics platforms from Sanger sequencing, Pyrosequencing to PacBio and nanopore facilitated efficient arrangements of scaffolds into linkage groups through hybrid sequence assembly of short reads such as data obtained from illumina and long reads like PacBio and nanopore. Algorithms are also developing simultaneously and computing facilities as well. Hence, it is high time to go for effective utilization of genomic resources than just

developing assemblies and maps of markers. From bird's eye viewpoint, on an average, a plant contains a range of 27,200 to 36,000 genes in their genome. Whereas, the genome size is very uncorrelated to the number of genes or chromosomes present in each genome. In Table 15.1, the major genomic resources of selected crop plants were listed of which less than 1% has been effectively utilized.

The best possible usage so far achieved in crops is development of single nucleotide polymorphism (SNP) from genome sequences and its haplotype analyses by resequencing of larger germplasm collections to identify alleles that can be used as markers for crop breeding. Marker-assisted selection (MAS) provides a way by which selection for specific and unique traits was achieved. Many important traits of crop are polygenic, multi gene epistatic and are impacted by environmental interactions (Fleury et al. 2010; Meriç et al. 2020). Marker-assisted selection is successful for simple Mendelian traits (Bouchez et al. 2002). From available genomic resources, we can directly map genes and gene families as depicted in Fig. 15.1.

Plant breeding is a process to select favourable superior agronomic traits of crop plants. Natural variations in genome occur by mutation and these mutations drive evolution. Random mutations increase genetic variation and can facilitate to improve desired trait. However, mutations can decrease yield. Sequence-specific nucleases (SSNs) can introduce specific mutations at target loci. Furthermore, it is now possible to introduce a foreign gene in host plant at a definite location within the genome, which can improve traits (Till et al. 2006).

Plant genomes are 100-fold larger and complex than any other prokaryotes. Though, there are multiple approaches in bringing the whole-genome sequence assembly of crops, a complete genome without any gaps is still a complex region to explore. Presence of repeat regions makes assembly and mapping further a tedious one to achieve. We have identified 7,34,810 genome-wide SNPs in pigeonpea (PRJEB27956 as on 08/03/2018), but when mapping them to get a saturated map, the SNPs were not distributed equally indicating the gaps in final assembly. Effective mapping strategies and approaching hybrid assembly techniques using short and long reads with better depth and coverage of the genome will eliminate these problems. Apart from that, presence of repeats, evolutionary additions, transposons and multiple copies of same genes or gene families make it difficult to assemble them. This can be resolved by chromosome isolation and sequencing individual chromosomes; but this too is not applicable in all crops as chromosome sorting and pulse field also require considerable quantifiable variations. Handling larger genome needs access to better computational facilities and additional manpower which makes even harder to achieve the goals in a short span of time.

15.3 Genomics Assisted Breeding

In crop plants, many important traits like yield are polygenic in nature, inherited quantitatively and interacting each other by modes of additive dominance and epistatic interactions. Each gene contributes a cumulative effect on its phoenotypic

Table 15.1 Major genomic resources of selected crop plants (data points include related pathogens and multiple species with the same common name)

	Bioproject	Nucleotide	Proteins	Genes	Genome	Assembly	Popset
Rice	7725	5,362,855	4,682,110	79,589	51	502	3530
Wheat	2296	4,401,512	2,318,937	68,256	31	160	1830
Arabidopsis	7935	4,273,645	4,487,524	115,058	15	111	3220
Maize	5522	5,288,770	902,355	56,082	16	44	1325
Groundnut	41	7595	29,928	31	0	16	113
Sugarcane	639	454,709	334,752	25,304	7	9	618
Gram	115	1,049,394	5,042,645	41,647	3	8	219
Sorghum	4847	1,461,348	995,976	71,608	4	8	1141
Banana	301	448,157	465,805	38,722	6	7	766
Coffee	195	608,420	260,039	92,865	9	7	446
Mango	94	134,817	79,968	176	3	5	575
Tea	509	794,725	374,390	4239	6	5	465
Pomegranate	61	130,967	121,831	29,289	2	4	140
Avocado	61	27,567	49,759	131	2	3	219
Coconut	34	216,852	26,217	270	1	3	154
Rose	32	62,570	90,877	40,480	1	1	54

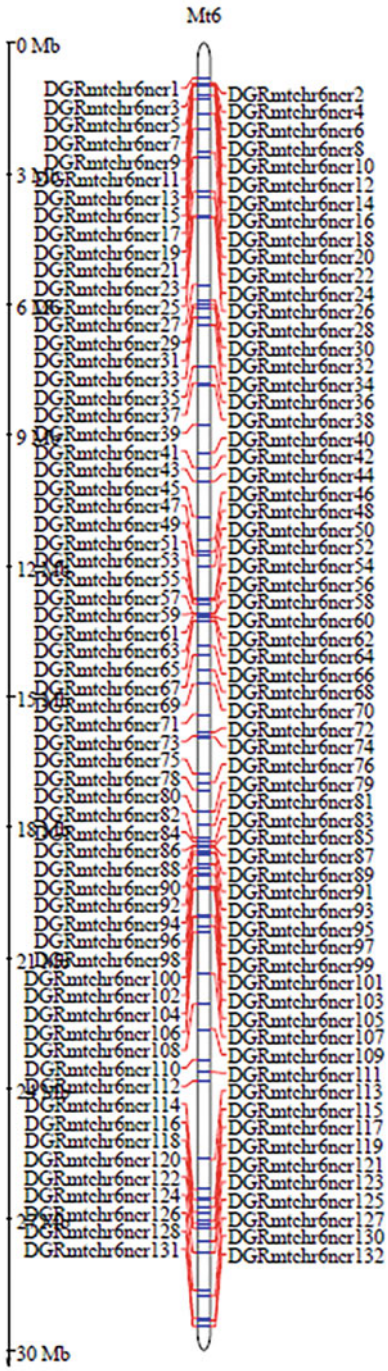

Fig. 15.1 NCR peptide based markers developed at ICAR-NBPGR, mapped on *Mt6 Medicago truncatula* chromosome 6. *DGR* Division of Genomic Resources, *mt M. truncatula*; *chr6* chromosome 6, *ncr* NCR peptide loci

trait. Recent techniques are exploited to map genomic regions that are regulating quantitative traits regulated by quantitative trait loci (QTL) (Kumar et al. 2017). In many instances, QTL analysis revealed the existence of pleiotropic QTL. A list of identified QTLs is presented in Table 15.2. In rice, *SCM2* QTL regulates culm strength and *APO1* gene encodes panicle structure and present on the same genomic region. The pleiotropic effects of *SCM2* QTL, contributing for culm strength was also found to enhance the number spikelets per tiller (Kumar et al. 2017). Only few organisms have saturated or enough genomic resources to proceed. NCBI is having dbGaP for human research with genotype and phenotype data classified with public and private data sets with embargo period mentioned for IPR protection. Such kind of facilities is not made available for any crop species.

In rice, large numbers of QTLs are present, controlling 14 different agronomic traits. The genetic mapping of QTLs has been ongoing for many years. The transcriptome analyses can provide us the information regarding regulation of gene expression. The gene expression data from different developmental stages can provide completely new information of gene interactions with trait expression (Severin et al. 2010). A large number of small-effect quantitative trait loci (QTLs) are present in maize and they regulate flowering period. At present, most comprehensive QTL database Gramene present for rice, contains 8646 QTLs and the most comprehensive QTL database MaizeGDB for maize contains 2294 QTLs (Mace et al. 2019).

Abiotic stresses such as extreme temperature (heat, cold), water stress, submergence, drought salinity and heavy metal stress are a great threat to food production at global level (Batley and Edwards 2016; Wani et al. 2020). Cereal crops are imperative for global food and nutritional security and their production is at stake due to vulnerability to various abiotic stresses under the present changing global climatic scenario. Plant breeders have been working on development of cultivars tolerant to these stresses, but are not able to compete with the growing multiple stresses in the field conditions and alarming increase in the global human population (Gosal and Wani 2020). Therefore, the pace of breeding new cultivars having fair yields and tolerance to abiotic stresses has become a mission of breeders' world over (Bansal et al. 2021). With the advancement in genomic resources and genotyping platforms, it is possible to dissect the complex abiotic stress traits in cereals crops in lesser time and with greater precision (Kumar et al. 2021). Among the various benefits for applying recent state-of-the-art biotechnology tools like high-throughput molecular markers with conventional plant breeding, genomics assisted breeding including QTL mapping has been a success story in dissecting genetic/molecular basis of abiotic stress resilience in crop plants. A few recent accomplishments made in this area on cereal crops are described below.

15.3.1 Rice

Abiotic stresses such as drought and salinity affect rice yields drastically, particularly during reproductive stages under rainfed conditions. The incidence and severity of

Table 15.2 A list of few recent QTLs identified for major abiotic stress related traits at various growth stages in field crops

Crop	Stress	QTL	Growth stage	Chromosome	Mapping population	Reference
Rice	Drought	qRCC1.1	Seedling	1	RIL	Barik et al. (2020)
		qGY8.1	Reproductive stage	8	F2	Baisakh et al. (2020)
		qPH 1. 3	Reproductive stage	1	NIL	Yadav et al. (2019)
	Salinity	*qRSKC1*	Seedling	1	RIL RIL	Chen et al. (2020)
		qDWT8.1	Seedling	8	Introgression lines (ILs)	Puram et al. (2017)
		qRL5	Seedling	5	RIL	Jahan et al. (2020)
		qPn3–2	Grain filling	3	RIL	Sun et al. (2019)
Wheat	Drought	*Q.Rdrw-4A*	*Reproductive*	4A	DH	Salarpour et al. (2020)
		QYld.1A1	*Reproductive*	$1A_1$	RIL	Zandipour et al. 2020
	Heat	*QYld.aww-7A.1*	*Reproductive*	7A	DH	Tura et al. (2020)
		qDH_iari_5A		5A	BIL	Sunil et al. (2020)
	Salinity	*Q.Na2A*	*Seedling*	2A	DH	Genc et al. (2010)
		QK.Asl-5A		5A	DH	Asif et al. (2018)
		QCl.asl-3A		3A	RIL	Asif et al. (2021)
Maize	Drought	*qEL-Ch.4-1*	*Reproductive*	4	F2	Zhao et al. (2018)
		qWS-GY5-1	*Reproductive*	5	DH	Hu et al. (2020)
		RG_CPS		1	DH	Vazquez-Pozos et al. (2020)
	Salinity	*SPH1*	*Reproductive*	1	DH	Luo et al. (2017a, b)
	Heat	*QHSI:PH*	*Seedling*	9	IL	Van Inghelandt et al. (2019)

drought stress is capricious. Most of the modern rice varieties are susceptible to drought (Baisakh et al. 2020). In Asia itself, 42 mha of rainfed rice and 8 mha of upland rice are affected by drought (Venuprasad et al. 2009). The complex nature of drought stress in rice particularly during reproductive stage is very well known. Hence breeding for this trait has been a challenge. Therefore genomics assisted breeding seems to be one of the tools for developing drought and salt tolerance in rice. The novel major QTLs identified can help in better transfer and utilization in rice breeding through Marker-assisted breeding. There are several recent reports on identification and transfer of major QTL for drought (Barik et al. 2020) and salinity stress in rice (Ganie et al. 2021). Recently, QTL mapping study was conducted by using a F7 generation Recombinant Inbred line (RIL) population of 190 plants developed using parents CR 143-2-2 and Krishnahamsa. Inclusive composite interval mapping was carried to perform the QTL analyses that yielded three QTLs for physiological traits, namely relative chlorophyll content (*qRCC*), chlorophyll a (*qCHLa*) and proline content (*qPRO*). The LOD value of QTL (*qPRO*3.1) was 13.93 and phenotypic variance exhibited was 78.19%, hence this QTL was declared as a unique one (Barik et al. 2020). In another greenhouse study on rice with a $F_{2:3}$ population derived from crossing two diverse parents Cocodrie (drought sensitive) x Nagina 22 (N22) (drought tolerant), eight QTLs were identified (Baisakh et al. 2020). The QTL for grain yield was identified on chromosome 1 with a LOD score of 2.78 with phenotypic variance of 11.14%. In most of the QTLs identified in this study, the favourable alleles were contributed by the drought tolerant parent. For salinity stress tolerance, a QTL study was conducted using a RIL population of 148 lines resulting from a cross between two diverse rice cultivars IR29 (salt-sensitive) and Pokkali (salt-tolerant) taking into consideration various physiological and morphological traits under seedling stage (Chen et al. 2020). The above investigation using high-throughput SNP markers created by Rice 56 K SNP array resulted in identification of 23 QTLs for a range of salt tolerance traits. Among the 23 QTLs, a major QTL on chromosome 12 (qSNaC12) elucidated 14.8% of the phenotypic variation. The above QTL identified may be used for breeding rice with improving terminal drought stress and salinity stress tolerance under molecular rice breeding programmes.

15.3.2 Wheat

Wheat growing areas across the globe are prone to drought stress due to erratic weather as a result of climate change (Farhad et al. 2017). To attain the global objective of food and nutritional security, wheat cultivation under rainfed conditions is imperative (Batley and Edwards 2016; Wani et al. 2020). Several physiological and morphological traits such as root traits are a source to study drought stress tolerance mechanism and genetic control. Among various genomic tools, QTL mapping has been considered as a major tool to identify genomic regions associated with the drought related traits. Root traits are one of the most highly studied traits in QTL mapping studies for deciphering drought tolerance in Wheat (Zheng et al.

2019). Recently, a study on wheat QTL mapping for drought stress tolerance was conducted in which a doubled haploid population of 220 lines developed from a cross between two diverse wheat parents between Kukri and RAC875. These lines were studied for grain yield and other morphological traits under various trials including drought stresses experiments. Genotyping was done using SSR, DArT and SNP markers. A total of 11 QTLs were identified for root traits. The QTL *Q. Rdrw-4A* showed a LOD value of 2.9 and phenotypic variance for root dry weight was 10.43 under DrEXP 16 trial (Salarpour et al. 2020).

Another concern for wheat growing areas is soil salinity, which reduces wheat yields significantly (Munns and Gilham 2015). As a result of excessive irrigation, the problem of soil salinity will further worsen in the wheat growing regions particularly under irrigated conditions (Yamaguchi and Blumwald 2005). QTLs for salinity stress tolerance in wheat have been reported in large numbers; however, candidate gene identification is still a challenge in bread wheat (Asif et al. 2019). Recently, a QTL mapping study was conducted in wheat to decipher salinity tolerance using a RIL population derived from a cross between contrasting wheat parents for salinity stress; Excalibur × Kukri. Nine QTLs were identified in wheat using the above study. Among the nine QTLs, six were identified under salinity stress including QTL for maintenance of shoot growth under salinity (QG(1–5).asl-5A, QG(1–5).asl-7B) sodium accumulation (QNa.asl-2A), chloride accumulation (QCl.asl-2A, QCl.asl-3A) and potassium: sodium ratio (QK:Na. asl-2DS2) (Asif et al. 2021). In another study on wheat salinity stress, a panel of 191 wheat accessions was subjected to GWAS to discover SNP markers linked to the traits of interest. From the study it was concluded that 389 SNPs representing 11 QTLs were drastically linked with plant height, spike number, spike length, grain number, thousand kernels weight, yield and biological mass under different salt treatments, with the phenotypic variances ranging from 9.14 to 50.45% (Hu et al. 2021). The above identified QTL will be crucial for identifying genomic regions with the help of linked molecular markers for use in wheat molecular breeding programmes for salinity stress.

15.3.3 Maize

Maize production is hampered by several abiotic stresses among which drought plays a major role, therefore development of abiotic stress tolerant maize is prerequisite to maintain world maize production (Xue et al. 2013). Similarly, salinity is one among the major abiotic stresses that is reducing yields in agricultural crops like maize. The complexity of drought tolerance in maize has been a barrier for breeding new cultivars resilient to drought and water stressed environments. Classification of functional genes or markers strongly associated to genes allied to drought tolerance is a way forward toward genomics assisted plant breeding in maize (Zhao et al. 2018). In addition to various physiological and morphological traits, grain yield stands at top for assessing drought tolerance in maize.

In a study on maize QTL mapping, F_2 population derived from contrasting parents was investigated and phenotyped followed by genotyping using SSR

markers. Composite interval mapping resulted in identification of 69 QTLs for various drought stress indices under four experimental trial sites and different watering conditions. These QTLs exhibited phenotypic variance for drought tolerance traits ranging from 4 to 1.2%. Among these identified QTLs, 52 (75.4%) were detected under water stressed conditions (Zhao et al. 2018). In another recent study on molecular breeding in maize for water stress conditions, a doubled haploid population consisting of 217 maize lines derived from a cross between two diverse maize inbred lines—Han 21 (drought tolerant) and Ye 478 (drought sensitive). Genotyping was carried out using 6 K SNP assay and 756 SNP (single nucleotide polymorphism) markers were used to develop a linkage map with a length of 1344 cM. QTL mapping resulted in detection of 18 QTLs for grain yield and other related traits, out of which 9 were identified in water stressed conditions (Hu et al. 2020). Similarly, for maize salinity tolerance, QTL mapping was attempted using a doubled haploid population of 240 maize lines using high-throughput SNP markers**.** In this study, a major QTL for plant height of mature maize grown under saline soil conditions (SPH) was identified on chromosome 1. This QTL showed LOD score of 22.4 and explained the phenotypic variation of 31.2% (Luo et al. 2017a, b). Therefore, this major QTL detected in mature maize plants may serve as a foundation for candidate gene identification and possible targets for marker-assisted selection for cultivar development in maize for abiotic stress conditions such as drought and salinity.

MAS has achieved in bringing some successful varieties throughout the globe. The achievements can be rapidly increased by using germplasm resources with genomic resources. Recently, ICAR has reported development of resistant/tolerant tomato lines for tomato leaf curl New Delhi virus (Hussain et al. 2018). Still lot can be achieved by exploring the germplasm resources and widening the genetic base of cultivated varieties. Using cultivated varieties for developing and breeding new varieties is the major constrain in many of the breeding programmes.

15.4 Genome-Wide Association Studies (GWAS)

Lot more has been reported and reviewed for GWAS in crop plants. Unlike genomes with sophisticated genomic resources, crops that are poor in genomic resources were also tried with gene identification and association studies. Recently 15 miRNA genes were reported and identified from *Macrotyloma uniflorum* (Yasin et al. 2020). Similarly, in its related species Kerstings's groundnut (*M. geocarpum*) using Diversity Arrays Technology and Sequencing (DArTSeq) and mapping with related *Vigna radiata* and *Vigna angularis* genome, genome-wide associations and genomic predictions were attempted with limited germplasm resources (Akohoue et al. 2020). These two reports exemplify the need for effective usage of available resources instead of just creating an addition to the available one. Genome-wide associations can be performed for SNPs; it can be either integrated with any other molecular data starting from transcriptome, proteome or metabolome-wide associations either individually or with genotyping data to enrich the selection

precision. A recent report emphasize the usage of metabolomics data in genomic selections and predictions where it is possible to identify the genome-wide associations of genes, markers or proteins with phenotypes (Tong et al. 2020).

15.5 Genomic Selection and Genome Predictions

The boom and boon of developments in bioinformatics and initial developments paved way for implementing rapid breeding techniques in animal population, which was further carried forward for crop improvement. Though much is not yet reported, machine learning and simulations can be applied to an extent to predict the phenotype of a developing population. Breeding perennial grains is a toughest crop improvement as evidenced from perennial wheatgrass (*Thinopyrum intermedium);* the inefficient efforts continued since 1980 were turned upside down by genomic selection recently (Crain et al. 2020). Genomic selection is possible through Estimated Genomic Breeding Value (EGBV) where, the precision in selection could be achieved by adjusting the selection models. The look ahead selection (LAS) and Look ahead mate selection (LAMS) are improved methods of selection replacing the genome prediction through an improved algorithm following simulated population values over a future period of time and a terminal future population using EGBVs. As these two selection methods are based on single trait based selections, an improved multi-trait genomic selection (MT-GS) has been proposed using the predefined tandem selection, independent culling, index selection and mate selection strategies. MT-LAS with a heuristic algorithm was used to select from a NAM population of 5022 individuals and along with an inter-mated population of maize simulated for ten generations (Moeinizade et al. 2020).

15.6 Genome Editing

Transgenic crops are genetically modified crops that are produced by the transfer of specific genes. By inserting target genes in a crop plant, we can produce elite crop varieties. Many elite varieties are developed that show resistance against abiotic stresses (Table 15.3). Despite the promising results, transgenic crops hold for health and environmental safety concerns. As a result, they are restricted and have certain limitations due to which they are less demanding nowadays. Apart from this, most of the validations of transgenic lines of altered expressions are proved in model organisms (Table 15.4) and the levels of its functions vary with the crops and generations due to multiple level gene expression regulations in-vivo.

Genome editing may be defined as targeted trait specific modifications at specific genomic region using molecular biology techniques. The targeted regions can be identified by system biology approach and identifying genes and regions through gene and gene network dynamics under treatment conditions (Figs. 15.2 and 15.3). Techniques like transcription activator-like effector nucleases (TALENs), zinc-finger nucleases (ZFNs) and clustered regularly interspaced short palindromic

Table 15.3 Few selected examples of CRISPR approach for abiotic stress tolerance in plants

S. no.	Trait	Gene and promoters	Plant	References
1.	Osmotic salinity stress	SlARF4 gene	Tomato	Bouzroud et al. (2020)
2.	Drought	AREB1 promoter	Arabidopsis	Paixao et al. (2019)
3.	Salinity	*OsRR22* gene	Rice	Zhang et al. (2019)
4.	Drought salinity stresses	*OsNCEB3 gene*	Rice	Huang et al. (2018)
5.	Drought	*ARGOS8* gene	Maize	Shi et al. (2017)
6.	Abiotic stress	OST2/AHA1 genes	Arabidopsis	Osakabe and Osakabe (2017)

repeats (CRISPR)/Cas systems, usually generate double-stranded breaks by recognizing specific DNA sequences. Many gene replacements, knockout mutants and gene replacement and insertion done through the use of genome-editing technologies and these changes may be useful for crop improvement. Zinc-finger nucleases used to modify Nicotiana, Arabidopsis, petunia, maize, soybean, rice, rapeseed and apple. By using ZFN in maize, ZmIPK1 gene sequence was changed by insertion of PAT gene, and it was reported that maize seeds showed herbicide tolerance (Zhang et al. 2018).

Challenges to agricultural practices are increasing and food demand is also increasing with time; so its need for the introduction of newer techniques for breeding the new cultivars. Abiotic stresses are very serious constraint in agriculture production and becoming to worsen with expected climate change (Pereira 2016). Abiotic stress is complex and difficult to manage. It affects multiple genes in plants, and is often difficult to set down (Bhat et al. 2016). Among the new techniques, clustered regularly interspaced short palindromic repeat (CRISPR) is a current new technique employed to develop desirable traits in plant for high production (Zaidi et al. 2019). CRISPR is a simple, highly specific, efficient technique and has paved the way for next generation plant breeding. CRISPR based gene editing in plants can be done through different ways, i.e. Cas9 to plant cells, protoplast transformation, direct bombardment of guide RNA (gRNA), transgene killer CRISPR (TKC) technology and CRISPR/Cas9-derived cytidine base editors technology (Ahmad et al. 2020). It relies on simple RNA/DNA hybrids and can manipulate nearly any sequence in the genome to reveal its function (Sander and Joung 2014a, b; Fauser et al. 2014). It is highly efficient and easy for genome editing (Sasano et al. 2016). The CRISPR induced gene editing in plants was first reported in rice in 2013 (Shan et al. 2013a, b), Arabidopsis (Li et al. 2013a, b) and wheat (Shan et al. 2013a, b).

Bouzroud et al. (2020) reported that CRISPR system can regulate the function of gene *SlARF4*. This gene is effective in osmotic and salinity stress tolerance in tomato plant. Abscisic acid (ABA) plays important role in abiotic stress tolerance. 9-cis-epoxycarotenoid dioxygenase (NCED) enzyme regulates ABA in rice and important regulator of abiotic stress tolerance including drought and salinity. NCED mutant rice has low level of ABA and very susceptible to the stresses. By

Table 15.4 Transgenic approach for abiotic stress tolerance

S. no.	Trait	Source of gene	Gene	Transformed plant	References
1.	Drought salinity	*E. coli*	TPSP	*Solanum lycopersicum*	Ahmad et al. (2019)
2.	Drought salinity	*Methanohalophilus portucalensis*	Mpgsmt and Mpsdmt	*Arabidopsis thaliana*	Wei et al. (2017)
3.	Heat	*Spinacia oleracea*	BADH	*Lycopersicon esculentum*	Luo et al. (2017a, b)
4.	Salinity	*Arachis diogoi*	AdLEA	*Nicotiana tabacum*	Sharma et al. (2016)
5.	Cold	*Stipa purpurea*	SpCBL6	*Arabidopsis thaliana*	Zhou et al. (2016)
6.	Drought Salinity	*Vigna radiata*	VrDREB2A	*Arabidopsis thaliana*	Chen et al. (2016)
7.	Salt Cold Drought	*Triticum aestivum*	TaNAC47	*Arabidopsis thaliana*	Zhang et al. (2016)
8.	Drought Salinity	*Medicago truncatula*	MtWRKY76	*Medicago truncatula*	Liu et al. (2016)
9.	Heat Drought Salt Cold	*Oryza sativa*	sHSP18.6	*Oryza sativa*	Wang et al. (2015)
10.	Drought Cold	*Miscanthus lutarioriparius*	MLNAC5	*Arabidopsis thaliana*	Yang et al. (2015)
11.	Salinity	*Arthrobacter globiformis*	Coda	Solanum *lycopersicum*	Dong et al. (2015)
12.	Salinity	*Citrus tristeza* virus (CTV)	HSP70	*Oryza sativa*	Hoang et al. (2015)
13.	Drought Salinity	*Triticum aestivum*	TaMYB3R1	*Arabidopsis thaliana*	Cai et al. (2015)
14.	Drought	*Capsicum annuum*	CaBZ1	*Solanum tuberosum*	Moon et al. (2015)
15.	Drought	*Arabidopsis thaliana*	AtTGA4	*Arabidopsis thaliana*	Zhong et al. (2015)
16.	Salt Drought	*Suaeda salsa*	SsDREB	*Nicotiana tabacum*	Zhang et al. (2015a, b)
17.	Drought Salinity Freezing	*Triticum aestivum*	TabZIP60	*Arabidopsis thaliana*	Zhang et al. (2015a, b)
18.	Cold Osmatic Stress	*Zea mays*	ZmLEA5C	*Nicotiana benthamiana*	Liu et al. (2014)
19.	Salinity	*Arabidopsis thaliana*	P5CS	*Solanum tuberosum*	Kim et al. (2013)

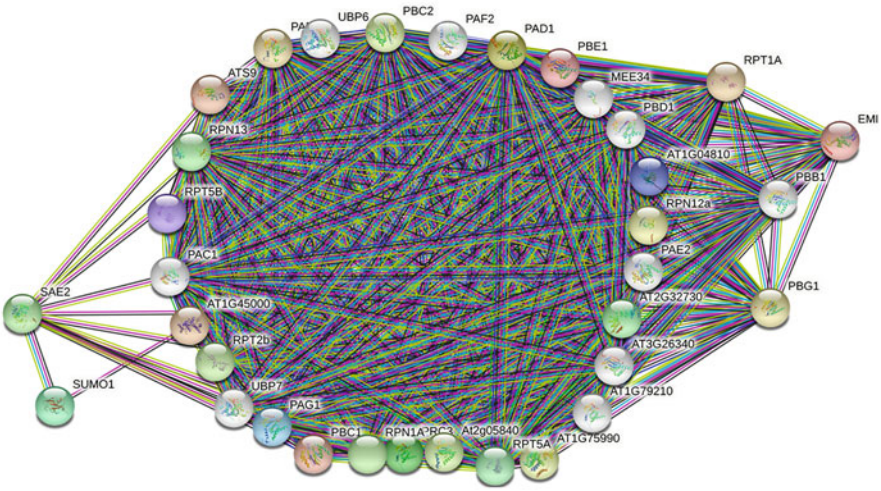

Fig. 15.2 Co-expression network of protein metabolic pathway influencing genes in plant architecture of Soybean with identified nodes for genome editing

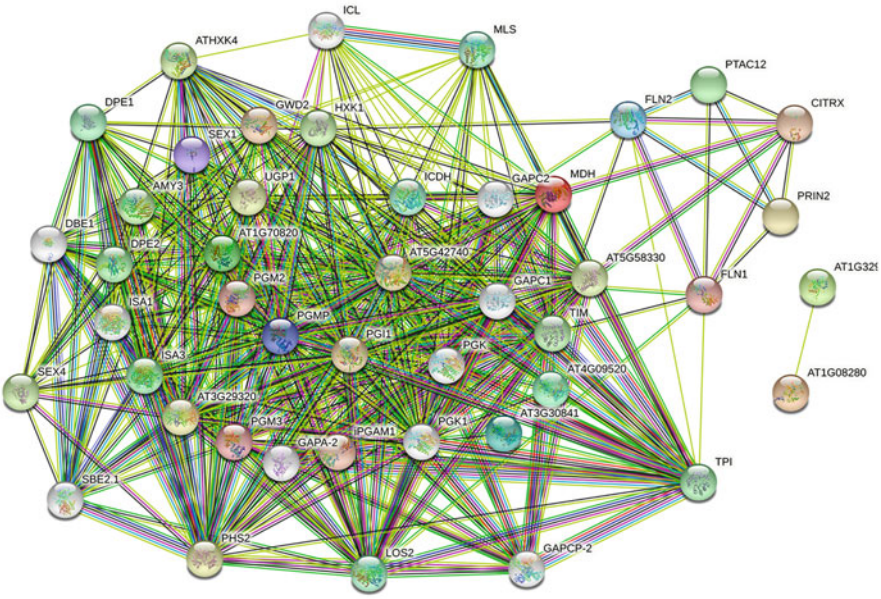

Fig. 15.3 Co-expression network of carbohydrate metabolic pathway influencing genes in plant architecture of Soybean with identified nodes for genome editing

the help of CRISPR system overexpression of *OsNCEB3* occurs in rice, which increased ABA and showed tolerance to drought and salinity stresses (Huang et al. 2018). Paixao et al. in 2019 observed that CRISPR system can induce drought

resistance in Arabidopsis plants by the activation of AREB1 promoter. Promoters regulate the gene expression. Therefore, they are important in genome editing. OsRR22 gene in rice plays an important role during salinity stress. CRISPR system regulates transcription factor that encodes *OsRR22* gene and its overexpression improved salinity tolerance of rice plants and it is also involved in cytokinin signalling and metabolism (Zhang et al. 2019). Shi et al. have reported development of drought resistant maize by CRISPR based genome editing (Shi et al. 2017). They observed that CRISPR induced variations in GOS2 promoter showed improved grain yield in dry field conditions. These results showed that CRISPR can be used in the development of drought resistant varieties. Similarly, Osakabe and Osakabe in 2017 modified the functioning of abiotic responsive genes (OST2/AHA1) in Arabidopsis by using CRISPR.

At present, only few studies have been carried out on CRISPR based abiotic stress tolerance. But it has been proven that it has high potential and this system in future will be used in plant breeding for inducing abiotic stress tolerance. Furthermore, genome editing via CRISPR for targeted mutagenesis can develop elite crop cultivars, which can combat with changing environment. Therefore, CRISPR technique will be the future of plant breeding and can be used to induce resistance during abiotic stress and boost plant growth in agricultural crops.

Editing specific genes or loci in high yielding consumer preferred varieties could benefit the mankind. Recently reported allelic variations in *OsPLD α-1* gene (Kaur et al. 2020) can be exploited in genome editing with novel nucleases for precision editing. Though lot can be achieved by genome editing, the policy implications of genome-edited crops will be major problem. As recent regulations of conventional mutation breeding also instructed to treat the mutants as modified organisms, the genome-edited crops stands in row without raising further debate as LMOs. Hence, there should be no questions on whether to treat them as modified or regular varieties. The marker free edited crops will come with handy other problems as proving the modifications whether it is natural or a manmade. Further to note the off target effects and its segregation screen or a larger scale will be tedious in type I and type II genome editing. Though *in-silico* predicted off targets will be less, in reality it could be further complicated and unproved.

15.7 National and International Status

Throughout this chapter we have discussed the international status of genomic resources. Internationally there are three major gene banks which are united under International Nucleotide Sequence Database Collaboration [NCBI's GenBank (https://www.ncbi.nlm.nih.gov/genbank/), EMBL Nucleotide Sequence Database (EMBL) (https://www.ebi.ac.uk/) and the DNA Data Bank of Japan (DDBJ) (https://www.ddbj.nig.ac.jp/index-e.html)] with many other data banks housing enormous data generated globally managed by dedicated data curators and servers dealing with each type of data sets. They offer 24 hour service to scientists in submitting resources from all corners of the globe. All these three genebanks

repatriate data periodically. Apart from these major genebanks, TAIR (https://www.arabidopsis.org/) is offering end to end services stating from genetic stocks to genes and constructs with all genomic resources. Legume information system (https://www.legumeinfo.org/) housing legume genomic resources and selected genomic resources in phytozome (https://genome.jgi.doe.gov/portal/).

The data resources submitted by large international institutes are in large represent above 95% of the total data volume. Whereas, genomic resources generated and submitted from indigenous resources are less and discontinuous. Many genomic resources developed are in discontinuous nature or incomplete. Most of the indigenous resources are developed and submitted by institutions abroad. This indicates lack of importance provided in generation and utilization of genomic resources nationally.

Apart from crop plants genomic resources of all agriculturally important organisms generated nationally are less from India. Hence, more importance should be given in generation of genomic resources from all national institutes and National Bureaus for crop plants, animals, fish, microbes and agriculturally important insects.

15.8 Conclusions

With the advent of ever-increasing algorithms and precision in estimating the EGBV, using simulated data sets, it will be possible to achieve better crop improvement with shortened breeding cycles. Instead of creating variations or searching them in narrow genetic base of only the cultivated varieties, it will be more suitable to start from germplasm resources in identifying favourable allelic combinations. The system biology approach may help in integrating multi-omics data in targeted marker free genome editing as the latest technology provides editing even a single base in a genome. Integrating the available tools, resources towards bridging the gaps between germplasm resources, genomics and crop improvement will facilitate continued green revolution.

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Proteomic Approaches to Understand Plant Response to Abiotic Stresses

16

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Abstract

Food security is becoming a global issue and demands increasing focus on developing and improving approaches for crop protection. Plant proteomics can serve as one of the most promising approaches for crop improvement. The role of protein in plant sustainability is crucial as they are directly involved in managing physiological traits so as to adapt the phenotype according to the environment. The present chapter discusses the importance of proteomic studies under various abiotic stresses in plants and briefly the novel tools and techniques to analyse high-throughput proteome data. In recent years, much advancement has been made in proteome technologies for improvement of agricultural crops, which have been presented in a broader context in this chapter. The major factors determining protein biological functions include protein cellular localization (Descriptive proteomics), protein post-transcriptional and post-translational modifications (PTMs) (Comparative proteomics) and protein interactions with other protein and non-protein compounds (Interactomics). The proteomics workflow together with state-of-the-art mass spectrometers, generate huge amount of data which needs to be handled precisely. Thus, statistical algorithm and bioinformatics tool implied for data analysis and functional interpretation of proteomic studies is equally important and elaborated in the chapter.

Keywords

Abiotic stress · Bioinformatics · Post-translational modification · Protein–protein interaction · Mass spectroscopy · Interactomics

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16.1 Plant Proteomics: Introduction

The term proteome was first used by Marc Wilkins in 1994 to represent the protein complement of the genome of an organism in any given time period. Unlike genome, proteome is dynamic, any changes in plant proteome along with its transcriptome, epigenome and metabolome control plants phenotype. Owing to the advancements in genome sequencing techniques, mass spectrometry (MS) equipment and bioinformatics tools, plant proteomics has evolved rapidly for deciphering biological interpretation of gene sequences in the last two decades. In the present context, proteomics is explored to understand physiological processes of gene regulation from transcription to metabolite expression via translation and post-translational modifications (PTMs); wherein each of the processes are systematically coordinated so as to allow the organism to develop/adapt to the change in the environment.

As plants are sessile organisms, proteomics analysis plays a major role in revealing the plant response towards various developmental processes and environmental cues. However, plant proteomics is challenging due to the presence of secondary metabolites and other interfering biological contaminants like nucleic acid, carbohydrates and others. The gradual development of novel techniques and protocols have now enabled us to screen large-scale proteome profiling (Song et al. 2018; Gupta et al. 2015). Owing to the improved techniques, it became possible to identify the low abundant proteins involved mainly in signalling processes. This advancement is mainly due to high-throughput instrumentation techniques enabling separation of complex mixtures, identification of protein species, as well as attributed to development in genomic studies (reviewed by Kosová et al. 2015). With the onset of whole genome sequencing and EST database, plant proteome identification started, particularly in *Arabidopsis* in 2000s, and gradually extended to monocots, dicots, economical plants, cereals, other crop plants, etc. As of September, 2020, NCBI PubMed showed 34,715, entries related to 'plant proteomics' keywords. Along with plants with whole genome sequenced, proteomics can also be applied to the plants without whole genome sequenced, with the help of heterologous data. Thus, combined application of proteome and MS data and subsequent downstream analysis using computational tools has allowed understanding the holistic mechanism of plant protein responses and regulation under various physiological and environmental conditions (Agrawal et al. 2012). The chapter further discusses diverse application of proteomics to understand the plant response under various abiotic stress conditions.

Depending on the approach, proteomics discipline is categorized into five subtypes:

1. Descriptive proteomics (total or sub-cellular proteomics).
2. Comparative proteomics.
3. Post-translational modifications (PTMs).
4. Interactomics.
5. Translational proteomics.

Since the advent of the proteome profile technique in early 1990s, up to six consecutive generations of MS proteomics platforms have been developed and employed—first, gel based: 2-D PAGE (two dimensional poly-acrylamide gel electrophoresis); second, multidimensional protein identification technique (MudPIT); differential gel electrophoresis (DiGE), third isotopic and isobaric labelling: ICAT, LOPIT, iTRAQ, fourth: shotgun or gel free, label free; fifth: targeted, mass-western or single/multiple reaction monitoring SRM/MRM and sixth: data-independent acquisition, DIA, and its sequential windowed data-independent acquisition of the total high-resolution mass spectra, SWATH, variant (Jorrin-Novo 2020).

Regardless of the profiling technique, thorough experimental design and high-quality sample preparation are critical for successful identification of proteins (Patole and Bindschedler 2019). Mass spectrometry is a critical component and advances in this technique involving nanospray ionization and nano-chromatography separation have enhanced the detection limit to dynamic protein concentrations. Likewise, protein identification technique has also advanced with respect to mass spectrometry (MS): matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) based peptide mass fingerprinting (PMF), liquid chromatography coupled tandem MS (LC-MS/MS), orbitraps-Q-TOFs (refer review articles by Jorrin Novo et al. 2015; Rey et al. 2019).

PMF is mostly used to identify peptides using MS/MS spectra for candidate peptides in reference protein databases like Ensembl, RefSeq, NCBI, UniProtKB/Swiss-Prot or TrEMBL (Nesvizhskii 2014). Further advancement in this direction is *Proteogenomics*, wherein customized protein sequence databases containing novel predicted protein sequences and sequence variants, transcriptomics sequence information as well as non-annotated genomic sequences are used to identify peptide sequences. This has also been applied to plants like barley and rice (Bindschedler et al. 2009; Helmy et al. 2012).

16.2 International Status

Plant proteomics is a vibrant discipline covering plant protein profiling and their functional elucidation, under varied physiological and developmental conditions. Protein function depends on its molecular structure, as well as cellular localization, PTMs and interacting partners (Jorrín-Novo et al. 2009; Kosová et al. 2011). The main objectives of proteomics experiments are to identify, characterize and quantify as many protein species (proteoforms) as possible. Human proteome research has advanced to six generations of proteomics, as mentioned above; however, plants proteomics, researchers mostly employ DiGE (first and second generation), isobaric or isotopic labeling for relative and absolute quantitation, iTRAQ (third generation) and shotgun approaches (fourth generation) (Jorrin-Novo 2020). Descriptive and comparative proteomics still represent the most widely used approaches in plant proteomics with special emphasis to identify proteins responsible for productivity and agronomic traits, and abiotic and biotic stress tolerance (Katam et al. 2015; Hu

et al. 2015). Its importance can also be determined by several review papers on plant abiotic stress proteomics (Kosová et al. 2011), drought, salinity and extreme temperatures (Ahmad et al. 2016), low temperature stress (Janmohammadi et al. 2015; Johnová et al. 2016), dehydration stress (Johnová et al. 2016) and heavy metal stress (Ahsan et al. 2009; Hossain and Komatsu 2013). Besides this, many specialized review articles, viz. plant PTMs under abiotic stress (Wu et al. 2016), plant phospho-proteomics (Rampitsch and Bykova 2012), S-nitroso-proteomics (Romero-Puertas et al. 2013), crop proteomics (Salekdeh and Komatsu 2007; Kosová et al. 2015), plant proteome responses to salinity (Zhang et al. 2012; Kosová et al. 2013a, b, c), stress responses of major crops (Tan et al. 2017), including rice (Agrawal et al. 2009), wheat (Komatsu et al. 2014), barley (Kosová et al. 2014), soybean (Wang and Komatsu 2016; Yin and Komatsu 2017), common bean (Zargar et al. 2017), solanaceae species (Ghatak et al. 2017), plant root proteome response to abiotic stress (Ghosh and Xu 2014), sub-cellular proteomics under stress and chloroplast proteomics under stress (Ning and Wang 2016) have been published. The list of review articles is the indicative of the abundance of the original research articles in each field. It is expected that these studies could help in identification of protein markers that could be used in plant breeding programs, however, it is still visionary (Ghatak et al. 2017). Elucidation of PTMs and interactomics still remain a challenge but good quality papers are appearing gradually (Hashiguchi and Komatsu 2017).

More than 300 types of protein PTMs have been described, including stress related PTMs, which mainly include phosphorylation, ubiquitination, SUMOylation and modifications by reactive species like: carbonylation, nitrosylation, etc. (Withers and Dong 2017). A survey of NCBI pubmed reflected that the most studied PTM in plants is phosphorylation, yielding 18,579 entries with keywords 'phosphorylation plant', and with stress the number was 3173, followed by other PTMs like redox proteomics (1016, with stress: 556), nitrosylation (184, with stress: 108), glycoproteomics (106, with stress: 9), glutathionylation (55, with stress: 34) and ubiquitination (313, with stress: 114). However, these studies are favoured by computational analysis and in silico predicted PTM motifs and functional association network of genes (Willems et al. 2019). Lately, proteogenomics has also been extended to a number of living organisms, including plants and is reviewed recently (Low et al. 2019). Likewise, importance of plant thiol redox proteomics and redox protein network in understanding plant chloroplast signalling metabolism and stress response has been recently established (Yu et al. 2020a).

16.3 National Status

Several research groups from India are actively involved in research on proteomics of different human cancers and infectious diseases, and are also effectively contributing towards diverse aspects of bacterial, plant and animal proteomics at the global level. Nearly 25 organizations of India are actively involved in proteomics related research with variable interests. Studies related to plant proteomics have been

initiated by Professor A. Grover at University of Delhi, India. They have analysed the proteome profile of rice under flooding conditions and did a comparative profiling between flood resistant and sensitive genotypes, identifying major proteins implicated in rice flooding response (Dubey et al. 2003). Other lead researchers of plant proteomics in India are Dr. N. Chakraborty and Dr. S. Chakraborty at NIPGR, New Delhi, India focussing primarily on chickpea under different abiotic stresses, particularly drought. The study on nuclear proteome in drought-treated chickpea revealed the role of major protein functional groups (Pandey et al. 2008). Proteins involved in chromatin remodelling and DNA methylation, such as cytosine methyltransferase and histone deacetylase (HDAC) revealed alterations under stress (Pandey et al. 2008; Subba et al. 2013). In addition, alterations in proteins involved in nucleo-cytoplasmic transport via nuclear pores such as Ran-binding protein (RanBP) were found under drought stress (Pandey et al. 2008). Likewise, protein profiling of pod wall of soybean under terminal drought stress revealed the upregulation of proteins belonging to stress signalling/regulation, protein folding/stabilization, redox-homeostasis, cellular energy and carbon utilization and downregulation of negative regulators of drought stress and protein degradation related proteins (Sengupta et al. 2019). The effect of water-deficit on mitochondrial function and dehydration response has been studied using iTRAQ based quantitative proteomics in chickpea, which revealed 40 dehydration responsive proteins, that may regulate cell fate decisions (Gayen et al. 2019). Similarly, many labs across India are also contributing towards plant proteomics with diverse objectives, viz. CSIR labs-IHBT, Palampur; IICB, Kolkata and NBRI, Lucknow (Sengupta et al. 2011; Deeba et al. 2012; Sanjeeta et al. 2014; Sinha and Chattopadhyay 2011, Sinha et al. 2013; Bhattacharyya et al. 2012) and other institutes like Bose Institute, Kolkata and BHU, Varanasi (Veeranagamallaiah et al. 2008; Sengupta and Majumder 2009; Kundu et al. 2011, 2013; Rai et al. 2014).

16.4 Application of Proteomics in Studying Stress Responses in Plants

Plants have to continuously adapt to changing climatic and soil conditions. Proteins are the ultimate players in exhibiting physiological processes, thus, it is highly likely that maximum responses that plants are showing can be decoded by performing large-scale proteomic analysis followed by advanced spectrometry techniques. Analysis of the stressed proteins helps in revealing the stress responsive mechanism and key regulatory proteins. This hypothesis may though be restricted by the fact that one protein may exhibit varied functions, depending on its sub-cellular localisation, PTMs and interactions (Jorrín-Novo et al. 2009; Kosová et al. 2011).

Plant productivity is threatened by two types of stresses, viz., biotic (bacteria, virus, fungi and insect pests and other parasites) and abiotic (extremes of temperature, deficiency or excess of water, salinity, ions and metals, etc.). Briefly any stimulus that triggers a plant defence response is considered as stress. Proper sampling of plant at various stages like initial shock phase, an acclimation phase, a

maintenance phase, an exhaustion phase and/or recovery phase after stress treatment followed by large-scale proteomics can interpret the detailed systemic response by plants under the particular stress condition (Kosová et al. 2011).

16.4.1 Plant Proteomics Under Varied Abiotic Stresses

Abiotic stresses like drought, extreme temperature: cold and hot, salinity, UV radiation, heavy metals, anaerobic and toxic conditions on root growth have devastative effects on crop yields. These stresses affect the basic metabolism of the plants like carbon and energy metabolism, basic biosynthetic processes, signalling pathways, stress and defence responses, protein biosynthesis and turnover. In general, the perturbations that the stress stimulates and basic homeostasis that the plant responds to combat the stress, are the decisive fate of the stress. Normally, a stress tolerant plant activates pathways that help it to overcome/modulate/activate the stress, whereas the stress susceptible plants have a different mode of response. Identifying differentially expressing proteins (DEPs) during stress/recovery conditions along with integrated transcriptomics and proteomics patterns help to understand the mechanism of defence evoked by the plant. Comparative proteome profiling of stressed and unstressed plants/tolerant and susceptible genotypes is very informative in revealing stress responses. These responses are dependent on intensity of stress, plant growth stage, plant tissues and their genotypes.

16.4.2 Salinity Stress

Salt stress affects plants in various manners, it causes ion toxicity, induces oxidative stress, causes nutritional disorders along with alterations in metabolic processes, membrane disorganization as well as reduction of cell division and expansion. Hence, a large extent of variation in protein profile is expected. The pioneering work on proteomics during salinity condition was done in salt stressed *Arabidopsis* and was compared with *Thellungiella halophila* (a salt tolerant plant) (Pang et al. 2010). Similar works have been done in many glycophytes and halophytes (as reviewed by Ahmad et al. 2016). Most of the research articles have shown that the general proteins showing differential expression are related to glycolysis and carbohydrate metabolism. Besides, proteins related to ROS detoxification, nucleotide metabolism, fatty acid metabolism and osmolyte biosynthesis are altered and are mostly upregulated. For example, in rice, leucine rich receptor like protein was found to be upregulated (Cheng et al. 2009a). Many other proteins, like LEA proteins (dehydrins), germin like proteins (GLP), universal stress proteins (USP) and proteins involved in cellular detoxification have been identified in salt stressed plant samples (Benešová et al. 2012; Kosova et al. 2013; Jacoby et al. 2013; Maršálová et al. 2016).

Comparative studies between tolerant and susceptible varieties also help in revealing the difference in protein alteration, for example, higher accumulation of

antioxidant enzymes, like APX and MDAR were observed in susceptible barley roots under salt stress (Witzel et al. 2009). Likewise, HSP70 and thioredoxin proteins were observed in susceptible tomato varieties (Manaa et al. 2011). Whereas, tolerant varieties showed up-accumulation of transcription factors like NAC (Maršálová et al. 2016), bHLH (Vincent and Zivy 2007) and MYB-like (Wendelboe-Nelson and Morris 2012) under salt stress.

16.4.3 Drought Stress

Under drought stress, plant restricts general metabolic pathways, like photosynthesis and transpiration to save water, due to limited water availability. Previous reports on proteomics of drought stressed plant samples have shown differential expression of photosynthesis related proteins, like RuBisCo large subunit, FBP and aldolase (Perlikowski et al. 2014). Likewise, enhanced level of redox enzymes APX, Cu/Zn-SOD and Mn-SOD were found in drought tolerant maize, sunflower, oilseed rape and sugar beet (Benešová et al. 2012; Ghaffari et al. 2013; Urban et al. 2017), suggesting the perturbations in redox-homeostasis. In some cases, tolerant lines have shown downregulation of glycolytic enzymes, which may be due to accumulated sugar for attaining enhanced growth in recovery phase. Besides, drought also affects proteins associated with carbohydrate and nitrogen metabolism, cell wall modification, signal transduction and programmed cell death as observed in some recent reports on barley, wheat, millet and other plants (Rodziewicz et al. 2019; Michaletti et al. 2018; Pan et al. 2018).

16.4.4 Temperature Stress

As mentioned, extreme temperatures both heat and cold affect photosynthetic processes, particularly electron transport chain and carbon assimilation enhancing photo-inhibition and thermal energy dissipation (Kosová et al. 2015). Heat stress affects protein denaturation, aggregation and increases fluidity of membrane lipids as well as specifically causes dehydration stress and oxidative stress altering heat shock factors (HSFs) and associated proteins (HSPs) along with many osmolytes and dehydrins (Wahid et al. 2007; Bokszczanin 2013). Similar observations have been made in many recently published reports in soybean, *Clematis florida*, spinach and maize (Katam et al. 2020; Jiang et al. 2020a; Zhao et al. 2018; Valdés-López et al. 2016; Zhao et al. 2016), etc.

Likewise, low temperature, chilling stress, affects plant growth by causing water reduction and osmotic changes in cells. It has been studied in details in whole leaf tissue, specific organellar parts like chloroplast, extra cellular tissue, plasma membrane and in model as well as crop plants like *Arabidopsis* (Visconti et al. 2019; Takahashi et al. 2019), rice (Jia et al. 2020; Gázquez et al. 2020), wheat (Labuschagne et al. 2020), potato (Lin et al. 2019), *Ipomoea batata* (Cui et al. 2020) and wucai (Xie et al. 2019), are just a few to mention. Comparative

proteomics profiling of various genotypes of plants having differential cold stress tolerance capacity has also been performed, for example, tomato (Khan et al. 2019). Many proteomics studies in response to low temperature stress have been performed and supplemented with transcriptomics data to gain a better understanding of acclimation process of plants at proteome level. For example, Cui et al. (2020) reported significant differences in antioxidant enzyme activities of sweet potato at low temperature. Differential expression of proteins related to phenylpropanoids, starch and sucrose metabolism was also observed.

16.4.5 Heavy Metal Stress

Heavy metal stress can be experienced by agriculturally important crops like rice, wheat, maize as well as by known phyto-remediator plants that have a natural tendency to hyperaccumulate heavy metals owing to soil contaminations. In general, it causes stress response due to metal penetration into the cell resulting in ion exclusion or vacuolar compartmentation (Hossain and Komatsu 2013). Cadmium (Cd) accumulation was also noticed in seeds of certain plants, especially tomato; hence proteomics analysis of tomato in presence of Cd was performed to get an insight in to the plant's response mechanism (Borges et al. 2019). The study identified changes in proteins related to diverse functions like redox balance and stress, cell wall and cytoskeleton related proteins, sulphur metabolism and proposed involvement of glyoxalase pathway in Cd tolerance in tomato. Likewise, several proteins related to carbon metabolism, proteolytic enzymes, RNA helicases, DNA replication and transcription factors were found to be specifically phosphorylated and contributing to Cd tolerance in rice (Fang et al. 2019). Similar observation was made on manganese (Mn) tolerant plant, Stylosanthes, wherein alteration in proteins related to defence response and phenylpropanoid pathway, photosynthesis and metabolic processes and protein synthesis and turnover was observed (Liu et al. 2019). Likewise, in potato (Cheng et al. 2019), peanut (Yu et al. 2019), wheat (Jian et al. 2020), many DEPs related to DNA replication and repair, protein metabolism and glutathione metabolism pathway were observed.

16.4.6 Flooding Stress

Flooding or water-logged condition induces hypoxia or anoxia, causing accumulation of organic acids as by-products of fermentation processes and also decreases nutrient absorbing capability of plants (Oh and Komatsu 2015). Similar to other abiotic stresses, it triggers a cascade of proteomic responses in plants. DEPs were identified through iTRAQ in rapeseed root tissue, mostly related to redox processes, signal transduction and carbohydrate metabolism (Xu et al. 2018). In wheat, 34 DEPs were observed, including acid phosphatases, alcohol dehydrogenase and S-adenosyl methyltransferase particularly in tolerant variety, these were postulated as potential marker genes for water-logging conditions (Pan et al. 2019). DEPs like

NADP malic enzyme, glutamate decarboxylase, glutathione synthetase/dehydrogenase/transferase were identified. Proteins related to glycolysis and fermentation like alcohol dehydrogenase and pyruvate decarboxylase were also identified.

Beside these approaches, many studies have reported differential protein profiling during combined abiotic stresses like drought and salinity in wheat (Peng et al. 2009), drought and heat in barley (Rollins et al. 2013) and osmosis and cadmium stresses on *Brachypodium* roots (Chen et al. 2018). Such types of studies have enabled to study synergistic response mechanism of plants during multiple abiotic stresses.

However, despite such vast wealth of information, differential/comparative proteomics alone cannot answer the variations arising from differing protein quality and quantity. Also, it cannot reveal the protein functions, as it depends largely on sub-cellular location, PTMs and interaction. Hence, plant proteomics has expanded to include sub-cellular proteomics, post-translational proteomics and protein–protein interaction.

16.5 Sub-Cellular Proteomics During Abiotic Stress in Plants

Studies of the response and inter-communication of cellular organelles under various physiological conditions are crucial for understanding plants responses. Plant proteomics is advancing progressively, exploring sub-cellular proteomics at various physiological and development conditions so as to understand the mechanism of plant responses and acclimations. The descriptive proteomic analysis of these organelles has been performed in majority of plants like *Arabidopsis*, maize, wheat and Medicago (Dunkley et al. 2006; Komatsu et al. 2014; Lee et al. 2013) for organelles like mitochondria (Huang et al. 2014), chloroplast (Ferro et al. 2010) and vacuole (Carter et al. 2004). These studies have enabled to identify specific proteins, for example, ATPases as specific membrane proteins were later recognized as marker for organelle purification, function and sub-cellular localisation studies (LaMontagne et al. 2016). This approach was also developed for targeted relative organelle abundance profiling, also called as multiple marker abundance profiling (MMAP; Hooper et al. 2017).

Sub-cellular proteomics was also found relevant in understanding stress perceptions at various organellar levels. Effect of water stress (drought and water logging) on soybean root proteome was studied revealing alteration in proteins related to glycolysis, thus activating carbohydrate catabolism and energy release, along with induction of fermentation enzymes, some redox enzymes and alleviation of oxidative stress (Oh and Komatsu 2015).

Classical approaches to cellular differentiation based on differential centrifugation results in six major cell fractions—cell wall, plasma membrane, nuclear, plastidial, mitochondrial and endoplasmic reticulum. In subsequent sections, we have discussed organellar specific proteomics profiling in plants during various developmental and experimental conditions, which will highlight the significance of such studies beyond total protein profiling.

Cell Wall The plant cell wall imparts structural integrity, which is of particular importance during stress conditions. The major components of primary cell wall include pectins, cellulose and hemicellulose. Whereas, in secondary cell wall pectin is replaced by lignin (Albenne et al. 2013). Cell wall proteins (CWPs) constitute only 5–10% of primary cell wall mass, yet contribute significantly in cell wall integrity, signalling and innate immunity (Vaahtera et al. 2019). Most of the CWPs are basic proteins having signal peptides and readily undergo PTMs like hydroxylation, N-glucosylation and O-glucosylation (Albenne et al. 2013).

CWP modifications have been studied in maize and chickpea during drought stress (Zhu et al. 2007; Bhushan et al. 2007). Beside other proteins, specific cell wall lignification enzymes like β-D-glucosidase were found to be up-accumulated under dehydration stress (Bhushan et al. 2007). In another report, CWPs were studied simultaneously under drought and flooding conditions and it was found that specific stress signalling proteins like nucleoside diphosphate kinase (NDPK) and G protein signalling, chaperones, methyltransferases and phenylpropanoid biosynthesis related proteins were modified (Pandey et al. 2010).

Plasma membrane (PM): It is the second layer after cell wall and hosts many membrane proteins as receptors and transporters, thereby acting as the primary site of stress sensing and its transformation into signalling. It is involved in diverse physiological processes, viz., maintenance of electrochemical gradient required for membrane transport and hence osmoregulation of the cell. Initial studies on PM proteins were performed under salt stress in rice roots (Nohzadeh Malakshah et al. 2007; Cheng et al. 2009b). Thereafter, similar studies were performed under osmotic and flooding stresses in soybean (Komatsu et al. 2009; Nouri and Komatsu 2010). Likewise, cold treatment has dramatic effect on PM causing decline of membrane fluidity, transport and metabolic activity, ion homeostasis maintenance, perturbed signalling processes. However, PM has the adaptive ability and does so with the help of PM protein modifications. This has been reviewed by Takahashi et al. (2013). Plants innate immune receptors and defence response regulators are integrally or peripherally associated with PM (Monaghan and Zipfel 2012). In eukaryotes, nearly 30% of the genome encodes PM proteins (Tan et al. 2008); however, in descriptive proteomics they are often masked by high abundant proteins due to their low abundance, physiochemical heterogeneity and hydrophobicity. Many technological advances like gel based or gel free approaches have been adopted to overcome these limitations (reviewed by Yadeta et al. 2013). For example, iTRAQ quantitative proteomics is exploited to study PM related proteins in mature and germinated rice pollens. The differentially expressed proteins among two pollen cell types included protein related to signal transduction, transporters, cell wall remodelling/metabolism and membrane trafficking. Moreover, receptor like kinases (RLKs) and transporters involved in flux of diversified ions and metabolites were identified (Yang and Wang 2017). Modifications of plasma membrane proteins was analysed initially in soybean during osmotic stress using gel based (2-DE) and gel free approaches (LC-MS/MS) (Nouri and Komatsu 2010).

Nucleus It acts as a site of gene expression, thus causing alteration in plant phenotypes in response to stress. The *Arabidopsis* nucleus exhibits cell type specific morphology, and undergoes morphological changes during long distance movement (Tamura et al. 2013). Hence, it is postulated that the nuclear envelop shows structural dynamics being coordinated by its protein constituents (reviewed by Tamura et al. 2015). The nucleus is a double membrane structure, which consists of proteins differing in nature in outer nuclear membrane (ONM) and inner nuclear membrane (INM; Boruc et al. 2012). Comparative nuclear proteomics under stress conditions like cold, drought and MAMP triggered immunity was performed in plants (Bae et al. 2003; Pandey et al. 2008; Subba et al. 2013; Fakih et al. 2016). These studies identified many nucleus specific proteins involved in chromatin remodelling (e.g., cytosine methyltransferase, histone deacetylase), nucleo-cytoplasmic transport, Ran-binding protein and transcription factors like bZIP, bHLH, MYB, MYC and NAC. In soybean also, differentially expressed nuclear proteins included: histone isoforms like H2A, H1 and H3 (Yin and Komatsu 2016). Besides comparative profiling, specific analysis of nuclear proteins in plants was first attempted in *Arabidopsis*, which identified an unknown nuclear specific protein SUN, with varying number of homologs (Graumann et al. 2010). Since then, many studies on plant nuclear proteome were reported in plants like rice, maize and soybean (Agrawal et al. 2011; Casati 2012; Yin and Komatsu 2016). Besides these approaches, much more needs to be done to extract complete, high-quality plant nuclear proteomes. Major achievements were attained for plants like barley, rice and *Arabidopsis* (Blavet et al. 2017; Tan et al. 2007; Aki and Yanagisawa 2009; Bigeard et al. 2014; Goto et al. 2019, Tang et al. 2020); which suggest alteration in proteins associated with chromatin remodelling and transcription factors.

Chloroplast Chloroplast hosts the unique function exclusive to plants, i.e., photosynthesis. It is liable to be affected by any physiological or environmental factors by modifications in its proteomic profile. However, study of chloroplast is quite complex, owing to its regulation, communication and compartmentalisation with other organelles and plant development (reviewed by Jan van Wijk 2000; Moreno et al. 2018). The initial step is isolation of purified chromoplast fraction using sucrose, percoll gradient, etc. The most commonly used method is sucrose gradient (Tetlow et al. 2003; Barsan et al. 2012). The fractionated isolate is screened through various methods like shotgun proteomics, using enzymatic markers (glyceraldehyde 3-phosphate dehydrogenase for plastids, cytochrome-c oxidase for mitochondria, catalase for peroxisomes and lactate dehydrogenase for cytoplasm), Western blot techniques for estimating its purity. The highly pure chromoplast fraction can be used for various gel based and gel free proteomics techniques. Many studies using either of these techniques have been performed to understand plastid differentiation (Barsan et al. 2012; Suzuki et al. 2015). It was observed that the structural differentiation is marked by decrease in thylakoid protein (Barsan et al. 2012). Likewise, chromoplast protein of six different crops, viz., carrot, orange, cauliflower, tomato, red papaya, watermelon and red bell pepper was compared (Wang et al. 2013). Chloroplast proteomics study has also been performed under salinity stress during

tomato fruit development (Manaa et al. 2013). In another study, alterations in electron transport chain components, like ferredoxin—NADPH oxidoreductase and CF_0-CF_1 ATP synthase were reported in maize under salt stress (Zörb et al. 2009). Likewise, GAPDH plastidial isoforms, translation elongation factor in wheat chloroplast and retrograde/anterograde signalling in tomato chloroplast were studied (Kamal et al. 2013; Tamburino et al. 2017). It was observed that starch and sucrose levels were related to ROS scavenging enzyme activities under stress conditions (Hüner et al. 2012). Similarly, chloroplastic proteases were also evaluated revealing more novel proteases and protease interactors (Kim et al. 2009). Overall, chloroplast proteomics revealed that processes like photosynthesis, protein import, proteolysis, chaperon networks are highly connected to each other (Moreno et al. 2018).

Mitochondria Mitochondria, the powerhouse of the cell, is a platform for various metabolic activities like energy metabolism involving tricarboxylic acid cycle and oxidative phosphorylation; along with biosynthesis of coenzymes, amino acids, fatty acids and lipids. It may also export peptides to nucleus as retrograde signalling (as reviewed by Møller et al. 2020). *Arabidopsis* mitochondrial proteome analysis was performed by large-scale LC-MS, revealing new insights of non-abundant proteins involved in DNA synthesis, transcriptional regulation, protein complex assembly and cellular signalling (Heazlewood et al. 2004). Many of these proteins are a part of protein complexes, like respiratory chain complexes, however, mostly high abundant proteins could be characterized, while low abundant could not. Hence, technical advancements were adopted to overcome this limitation like application of blue native gel to identify complex proteins, particularly hydrophobic ones (Klodmann et al. 2011; Senkler et al. 2017). Yet these studies have enriched our understanding of mitochondrial functions particularly in biotic and abiotic stresses. Similar studies were reported under stress conditions also, revealing up-accumulation of ROS scavenging enzymes, enzymes related to Krebs cycle: malate dehydrogenase, F_1F_0 ATP synthase and alternative oxidase (Taylor et al. 2005; Jacoby et al. 2010, 2013). Likewise, in anoxic conditions under flooding, γ-aminobutyrate and TCA cycle intermediates were observed (Komatsu et al. 2011). Further studies identified >2000 mitochondrial proteins showing spatio-temporal specific expression, representing a wide range of proteins like energy-related, membrane transporters, fatty acid synthesis, although low abundant and highly hydrophobic proteins like RNA metabolism are poorly covered (Rao et al. 2017). An exclusive study of purified mitochondria from *Arabidopsis* cell culture revealed that approximately 1.4 million protein molecules make up a single *Arabidopsis* mitochondrion, while some proteins have more than 40,000 copy number, for example, voltage dependent anion channels, others may have as less as one copy number, for example, pentatricopeptide repeat proteins (Fuchs et al. 2020). Lastly, homeostasis of mitochondrial proteins is controlled by protein synthesis and degradation, being coordinated tightly for proper function and regulation of mitochondrial machinery.

The above studies have mostly focussed on description of quantitative changes in plant proteome or sub-cellular proteome, based on gel based or gel free techniques.

However, gradual methodological advancements intended towards PTMs and protein–protein interactions as next level in the field of plant proteomics. These approaches further contributed in detailed functional characterisation that helps to get better understanding of the plant development and stress tolerance and acclimation.

16.5.1 Post-Translational Modifications (PTMs)

PTMs are important determinant of protein functions. These are chemical modifications of amino acids ranging from small molecules like nitrous oxide (NO) to whole peptides like ubiquitin (SUMO). PTMs are reversible and can alter throughout protein life cycle like reversible phosphorylation of protein kinases or protein degradation by ubiquitination. In general, PTM proteins vary in molecular weight and isoelectric point, which can be identified by 1-DE or 2-DE gels.

Phosphorylation Protein phosphorylation is a reversible and covalent binding of phosphate group to hydroxyl group of amino acids like serine, threonine and tyrosine (Reinders and Sickmann 2005). In *Arabidopsis*, nearly 1003 types of kinases and 200 phosphatases have been reported. It is one of the most studied PTMs to understand plant development and stress acclimation. For example, to study the protective role of exogenous nitrogen under cadmium toxicity in poplar (Huang et al. 2019). Likewise, protein PTMs involved in aluminium tolerance in soybean (Han et al. 2020) and to understand the alkali-salinity responsive mechanisms underlying photosynthetic modulation and reactive oxygen species (ROS) homeostasis, physiological and diverse quantitative proteomics analyses of alkali grass (*Puccinellia tenuiflora*) under Na_2CO_3 stress have been reported (Suo et al. 2020). Phosphorylation of proteins during organogenesis has been recently studied in six different stages/organs of *Arabidopsis*, identifying 2187 non-redundant proteins and 1194 phosphoproteins (Lu et al. 2020). Another important aspect depicting role of phosphorylation in stomatal movement during pathogen invasion, phytohormonal and ROS signalling, that is stomatal immune response has also been reported (Pang et al. 2020).

Glycosylation Glucosylation is a highly diverse set of co-translational and PTMs of proteins. It is often site, tissue and species specific. Though, compared to phosphoproteomics, it has been less studied, it has dynamic importance in both plant development as well as stress adaptation. For example, glycoprotein of *Arabidopsis* cell wall undergoes modification in the absence of ascorbate (Sultana et al. 2015). Similarly, glycoproteome of barley aleurone layer was studied in the presence of gibberellic acid (Barba-Espín et al. 2014). Likewise, stress responsive degradation of high mannose type N-glycans in endoplasmic reticulum of *Arabidopsis* during chilling stress was reported (Ma et al. 2016). Maize N-glycoproteomics database has been developed, that covers modification of glycoproteins during de-etiolation, emphasizing potential roles of N-glycoproteins during greening of maize leaves

(Bu et al. 2017). Similar work was reported for tomato ripening (Zhang et al. 2020) and rubber latex (Yu et al. 2020b).

SUMOylation It is a covalent attachment of small ubiquitin related modifier (SUMO) protein to lysine of target proteins. It is mediated by heterodimeric SUMO-activating enzyme (SAE1and SAE2), SUMO-conjugating enzyme (SCE1) and SUMO E3 and E4 ligases, in *Arabidopsis*. SAE 1/2 forms thioester with SUMO, which is then activated and transferred to SCE followed by linking with lysine residue of targeted amino acid (Nukarinen et al. 2017). Many studies have reported combined effect of different PTMs like phosphorylation and sumoylation on functional aspects of plants like signalling mechanism (Nukarinen et al. 2017). These may have common predicted targets, which may be converging points of different signalling paths. In some cases, negative feedback loop of this interaction is also reported like E3 ubiquitin ligase may dampen the immune response triggered by mitogen activated protein kinases (Furlan et al. 2017). Moreover, it was shown that many components of SUMO conjugation system are phosphoproteins, and some regulators and enzymes of protein phosphorylation are sumoylated (Tomanov et al. 2018). *Arabidopsis* ubiquitin ligases (E3s) have been found to be involved in drought responses (Adler et al. 2017). Likewise, SUMOylome profiling during heat stress revealed a diverse array of nuclear targets modified by SUMO ligases (Rytz et al. 2018). Accumulation of reactive oxygen species (ROS) as a consequence of cAMP dependent heat stress response against heat stress has been reported. Modifications in ubiquitin proteasome system (UPS) proteins were identified through large-scale proteomics approach (Paradiso et al. 2020).

Nitrosylation Nitric oxide (NO) along with ROS are produced in cells during normal growth conditions and have enhanced levels during stress conditions, which causes various biochemical aberrations. It has been reported under various environmental stress conditions, viz. cadmium stress in rice plasma membrane (Yang et al. 2016). NO along with S-nitroso-glutathione reductase (GSNOR: a key regulator of S-nitrosothiol metabolism), play important role in plants adaptive response during plant development (Tichá et al. 2018) and against biotic and abiotic stresses like salt (Chen et al. 2016; Jain et al. 2018).

16.6 Protein–Protein Interaction

The proteomics approaches have identified a large number of proteins for different experimental conditions. Nearly, an average of 36,795 proteins are estimated in a plant proteome (Morsy et al. 2008). However, most of these proteins do not function alone, rather in complexes; which are sometimes constitutive, and sometimes regulative (Fujikawa et al. 2014). Constitutive ones are permanent structures like replisomes, ribosomes and proteasomes. While, regulative complexes are specifically formed under particular conditions in response to regulatory stimuli and signalling events. Approximately 75,000–150,000 interaction pairs have been

predicted in plants (Ramírez-Sánchez et al. 2016). PPI have been predicted under various physiological and development conditions in many plants like *Arabidopsis* (Zhang et al. 2016) and apple (Li et al. 2019). Hence, in order to understand protein functions, detailed information about their biochemical activity, cellular localisation and abundance as well as interacting partners is a pre-requisite (reviewed by Struk et al. 2019). However, large-scale PPI was identified for few organisms using experimental techniques, which is laborious, time-consuming and expensive. Recently, computational aided PPI prediction approach was employed on three plants *Arabidopsis*, *Glycine max* and *Zea mays* (Ding and Kihara 2019). The prediction was very accurate up to 90%, with precision as close to 1.0. The PPI predicted ranged from 50,220 in *Arabidopsis*, 13,175,414 in maize and 13,527,834 in soybean. Large-scale proteomic data are often used to get system-level view of metabolic functioning using PPI networks (Macalino et al. 2018). Abscisic acid responsive protein interaction network or MAPK signalling networks are such examples of such PPI networks (Carianopol et al. 2020) that have key roles in abiotic stress resistance.

16.7 Bioinformatics Analysis of Identified Proteins

Experimental wet-lab techniques in plant proteomics are often painstaking, time-consuming and expensive. With ever-expanding biological databases and development of efficient algorithms, it is now feasible to address scientific questions related to plant proteomics using reductionist as well as system approaches. Nowadays, use of computational tools has been a routine process in proteomic studies in plants. With growing amount of data from high-throughput next-generation sequencing and shotgun proteomics, automated annotation pipelines using bioinformatics are faster and convenient method for filling the huge knowledge gap between sequence data and functional information than the conventional annotation method of manual curation and experimental validation. So far, genome assemblies of plant species account for more than 1400 entries in NCBI Genome database. Another major plant genome database Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html) hosts 93 annotated genomes of various plant species including major crop plants such as *Glycine max*, *Sorghum bicolor*, *Zea mays*, *Oryza sativa*, *Triticum aestivum*, *Solanum tuberosum,* etc. While, Protein Data Bank (PDB; https://www.rcsb.org/), the major repository of protein 3D structures derived from major experimental methods such as X-ray crystallography and Nuclear magnetic resonance (NMR), harbours 8352 entries belonging to the *Viridiplantae* (November, 2020).

Large-Scale Protein Identification Large-scale identification and characterization of peptides and proteins of differently treated biological samples from spectra generated by high-throughput MS involves analytical steps of bioinformatics, which generally include in silico digestion of protein sequence database of target species, computation of theoretical masses of the resulting peptides, generation of theoretical MS spectrum and comparison of peaks in observed spectrum with the

theoretical spectrum of candidate protein fragments of databases and assign a similarity scores (Ryu 2014). Differing in the similarity score calculation, the commonly used software for peptide identification in MS analysis are Sequest (Eng et al. 1994), Mascot (Perkins et al. 1999), X!Tandem (Craig and Beavis 2004) and MAxQuant (Tyanova et al. 2016). Based on the assumption that the sequences evolve from a common ancestor and their structures and function are conserved during the course of evolution (Gabaldon 2007), the common approach to identify complete or partial protein sequence derived from automated pipelines of genome or transcriptome annotation is to align the sequences of proteins or peptide fragments using BLAST (Basic Local Alignment Search Tool) to the already annotated proteins in NCBI's Non-Redundant protein database or in the central repository of protein sequences - UniProt Knowledgebase database (UniProtKB; https://www.uniprot.org/) which has two parts: (i) SwissProt containing manually annotated entries and (ii) TrEMBL containing computationally analysed.

Physiochemical Properties ExPASyweb server (the Expert Protein Analysis System; https://www.expasy.org/) hosts several databases and tools to characterize protein structure, function and physiochemical properties. Using the sequence information and Expasy's sequence analysis tools such as PROTPARAM, the novel proteins can be characterized by various physiochemical parameters like molecular weight, theoretical pI, amino acid composition, atomic composition, extinction coefficient, estimated half-life, aliphatic index, grand average of hydropathicity (GRAVY) index and instability index (Gasteiger et al. 2003).

Domain and Motifs Identification of conserved domains in protein sequences is a common approach to get functional insights of proteins. Some protein domains such as domains of specific transcription factors which play major role in plant stress tolerance by controlling gene expression pattern inside the cell. Based on sequence homology, HMM profiles or consensus sequences, the proteome of stress related plant samples can be characterized based on number and family of conserved domains identified. The major protein family and domain databases used to identify functional domains are PROSITE (https://prosite.expasy.org/), InterPro (https://www.ebi.ac.uk/interpro/), Pfam (https://pfam.xfam.org/), SMART (http://smart.embl-heidelberg.de/) and CDD (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The most widely used programs to identify protein profiles and domains are PSI-BLAST (Altschul et al. 1997), HMMER (Finn et al. 2011) and InterProScan (Jones et al. 2014).

Post-Translational Modification Prediction PTMs in proteins can also be predicted using computational algorithms and machine learning. Depending on the types of PTMs, several predictor tools for specific type of PTMs are developed which are reviewed in detail by Audagnotto and Dal Peraro (2017).

Protein Sub-Cellular Localization Prediction Many sub-cellular localization prediction tools solely rely on amino acid composition of protein sequences for

Table 16.1 Sub-cellular localization prediction tools

S. No.	Protein localization server	Method	Weblink	Reference
1	Plant-mSubP	Support vector machine (SVM)	http://bioinfo.usu.edu/Plant-mSubP/	Sahu et al. (2019)
2	LOCALIZER	SVM	http://localizer.csiro.au/ Localization of plant proteins as well as effector protein	Sperschneider et al. (2017)
4	TargetP2.0	Bidirectional Recurrent neural network	http://www.cbs.dtu.dk/services/TargetP/	Armenteros et al. (2019)
5	SignalP5.0	Deep learning	http://www.cbs.dtu.dk/services/SignalP/	Armenteros et al. (2019b)
6	WolfPSORT	k-nearest neighbour classifier	https://wolfpsort.hgc.jp/	Horton et al. (2007)
7	MULocDeep	Deep neural network (DNN) and SVM	Plant mitochondrial protein http://mu-loc.org/	Jiang et al. (2020b)
8	YLoc	Naïve Bayes learning	https://abi-services.informatik.uni-tuebingen.de/yloc/webloc.cgi	Briesemeister et al. (2010)
9	MitoFates	SVM	http://mitf.cbrc.jp/MitoFates/cgi-bin/top.cgi	Fukasawa et al. (2015)
10	DeepLoc	Recurrent neural network	Uses only sequence information http://www.cbs.dtu.dk/services/DeepLoc/	Armenteros et al. (2017)
11	LocTree3	SVM	Ensemble of many programs https://rostlab.org/services/loctree3/	Goldberg et al. (2014)

detection of sorting signals while others look for annotated homologs in databases or co-occurrence of certain domain with known sub-cellular localization (Armenteros et al. 2019) such as TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) and TMPred (https://embnet.vital-it.ch/software/TMPRED_form.html) tools which detect transmembrane helices. Some of the other commonly used tools for sub-cellular localization prediction are compiled in Table 16.1.

Protein Structure Prediction To determine the molecular mechanism behind functioning of the proteins involved in stress tolerance in plants, the knowledge of their native structure can be extremely helpful and supplement the PPI prediction. As the structural information of proteins are accumulating in Protein Data Bank (PDB) by the experimental methods, such as X-ray crystallography and Nuclear Magnetic Resonance (NMR), it can be further deployed to predict the structure of the proteins of which only primary structures are available. Although computational structure

prediction methods are not as accurate as the experimental methods, these can be the starting points to determine intra-molecular details of the proteins that can further complement the experimental methods. Three main categories of such computational methods include i) homology modelling, ii) Fold recognition (Threading) and iii) ab initio method (Template free method). The detailed methodologies of protein structure prediction and related tools have been reviewed by Dhingra et al. (2020) and Kuhlman and Bradley (2019). Studies related to structural aspects of intrinsically disordered proteins whose upregulated expression results into stress tolerance, such as late embryogenesis abundant (LEA) proteins, heat shock proteins (HSPs), etc.

16.8 Protein–Protein Interaction

Using experimental methods, data of PPIs has accumulated through past several years. Combining those evidences with protein sequence data, PPIs can be predicted from orthologs of interacting proteins. Recently, a large-scale plant interactome was derived using interolog mapping in *Arabidopsis thaliana* in which protein interactions were predicted from known interacting protein homolog in yeast, nematode worm, fruit fly and human and the knowledge was integrated with the gene co-expression data and protein sub-cellular localization data to judge the confidence of the PPI predictions (Geisler-Lee et al. 2007) (Table 16.2).

Applying the advanced algorithms and machine learning methods to the available PPI data and associated Gene Ontology annotation, several PPIs were predicted with high accuracy and precision as done with *Arabidopsis thaliana*, Maize and Soybean sequence data (Ding and Kihara 2019). To predict PPIs in silico, the common inference-based technique makes use of knowledge on PPIs in well studied organisms while prediction by domain and interlog-based method rely on the presence of interacting domains in PPIs and the evidence of PPIs in orthologous proteins (Ding and Kihara 2019; Thanasomboon et al. 2020). Some of the PPIs predictors developed in recent years are mentioned in Table 16.3.

16.9 Summary

Analysis of proteome component of plant offers structural, functional and network scale insights into plants metabolism. Proteomics has been a major area of research of several research groups worldwide working in plant system. With the advancement in technical aspects like sample preparation, protein separation, protein profiling and peptide identification, together with bioinformatics methods, understanding of proteins and their expression patterns in plants under various physiological and development conditions have been augmented. The present chapter is an attempt to cover various aspects of proteomics so as to deliver an in-depth analysis of significant achievements in plant proteomics, particularly under abiotic and biotic stress conditions. Profiling and characterization of proteomes of plant tissue samples

Table 16.2 Protein interaction databases

Sl. No	Database	Description	Weblink	References
1	DIP	Experimentally determined protein interaction database	https://dip.doe-mbi.ucla.edu/dip/Main.cgi	Xenarios et al. (2000)
3	IntAct	Curated molecular interactions including PPIs	https://www.ebi.ac.uk/intact/	Hermjakob et al. (2004)
4	MINT	Database of manually curated PPIs	http://mint.bio.uniroma2.it/mint/	Licata et al. (2012)
5	HitPredict	PPIs from IntAct, BioGRID, HPRD, MINT and DIP with reliability score	http://www.hitpredict.org/	Patil et al. (2011)
6	PRIN	Predicted Rice interactome database	http://bis.zju.edu.cn/prin/	Gu et al. (2011)
7	PPIM	Predicted maize interactome database	http://comp-sysbio.org/ppim/	Zhu et al. (2016)
8	AtPID	Protein interactome of *A. thaliana*	http://www.megabionet.org/atpid	Lv et al. (2017)
9	STRING	PPI database with confidence scores	http://string-db.org	Szklarczyk et al. (2017)
10	BioGRID	Curated database of PPIs	https://thebiogrid.org/	Oughtred et al. (2019)
11	AraPPINet	PPI database of *A. thaliana*	http://netbio.sjtu.edu.cn/arappinet/	Zhao et al. (2019)
12	ProtCID	Structural information on PPIs	http://dunbrack2.fccc.edu/ProtCiD/	Xu and Dunbrack Jr. (2020)
13	PlaPPISite	Database of experimentally verified and predicted PPI of 13 plant species	http://zzdlab.com/plappisite/index.php	Yang et al. (2020)

Table 16.3 Protein–Protein interaction Prediction Methods

Sl. No.	Database	Method	Weblink	Reference
1	Go2ppi	Machine learning and semantic similarity measures using ontology annotation	http://bioinformatics.org.au/tools/go2ppi/	Maetschke et al. (2012)
2	MaxEnt-ppi	Maximum entropy model using ontology annotation	https://github.com/ima23/maxent-ppi	Armean et al. (2018)
3	DeepSequencePPI	Deep RNN-based neural network	https://github.com/FGonzalezLopez/DeepSequencePPI	Gonzalez-Lopez et al. (2018)
4	PPI-MetaGO	Ensemble of multiple machine learning algorithms	https://github.com/mlbioinfolab/ppi-metago	Chen et al. (2019)

in response of different abiotic stress reveals the role of a set of proteins inducing stress tolerance while proteomes of sub-cellular organelles or cell parts further narrow down the focus to localized functions of proteins. Post-translational modification and protein–protein interaction studies further complement our knowledge of changes at molecular level. Covering all the aspects of proteomic studies, bioinformatics tools and techniques empowers the analytical step and helps in synthesizing enriched system-level view with subtle details of the reductionist studies.

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Plant Metabolomics for Crop Improvement 17

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Abstract

The recent advances in plant biotechnology tools have considerably increased deeper insights on the metabolic regulations and associated developmental programs in individual plants. The last two decades have witnessed massive implementation of modern molecular omics tools which involved integration of high throughput technologies using LC-MS and GC-MS approach to identify new metabolic regulations in existing pathways which influences the cellular physiology, ultimately the plant phenotype. In addition, emergence of the genome editing tools have enabled plant biologists to perform precise and efficient targeted modification in a wide variety of plant species to identify gene functions and manipulate metabolic pathways. Notably, the application of these modern tools has flourished the crop improvement program by enhancing the quality traits including the flavonoids, folate, protein composition, etc. Here, we

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describe the application and potential of metabolomics in the crop improvement program. In addition, we review the novel breakthroughs which extended the potential of metabolomics for strengthening the crop quality and food security.

Keywords

Metabolomics · LC-MS · NMR · GC/MS · Abiotic stress · Improvement · Omics

17.1 Introduction

Genomics, transcriptomics, epigenomics, proteomics, metabolomics, and phenomics all have played very important role in the scientific field. In the area of plant based research, these omics tools helped in dissecting several traits and developing the molecular concepts. Moreover, integrated approaches which involved multilayers of these omics techniques have played very important roles (Arbona et al. 2013). The process of gene expression is a complicated process and simply evaluation of the transcriptome and corresponding proteomics study often does not correlate with a particular expression of gene with the encodes protein abundance due to several regulations including epigenetic and/or post-translational modification (Kumar et al. 2017, 2018, 2020). The biochemical physiology of the cell driven by the metabolites often controls the tissue or plant phenotype driven by the expression of pathways specific genes (Kumar et al. 2020). Metabolites are the lower molecular weight compounds, which represent both intermediate as well as the end product of the metabolic pathways. Therefore, to completely understand a biological mechanism, integration of metabolomics studies is very critical (Nakabayashi and Saito 2015).

From the past decade, the metabolite profiling platforms have been significantly improved due to advancement in the both column chromatography and analytical tools such as mass spectrometry methods. This has provided an opportunity to identify hundreds of metabolic compounds from a single sample (Kumar et al. 2020). For instance, collection of primary metabolic profile from fleshy fruits such as apple (Cuthbertson et al. 2012), orange (Rangel-Huerta et al. 2017), strawberry (Antunes et al. 2019), tomato (Eloh et al. 2016), mango (Wattanakul et al. 2020), kiwi (Yu et al. 2020), melon (Moing et al. 2020), and cucumber (Zhang et al. 2018); from leaf of spinach (Zhao et al. 2017), tomato (Eloh et al. 2016), tobacco (Li et al. 2016), groundnut (Raval et al. 2018), mustard (Papazian et al. 2019), and rice (Deng et al. 2020); and volatiles compound associated with leaf (Chen et al. 2020), fruit (Der Agopian et al. 2020), and flowers (Dhandapani et al. 2017). Primary metabolites are the major components of a cell required by plant for their normal growth and reproduction. However, change in the external clues could lead to dramatic change in the metabolic profile in a particular tissue in response to stress or the stimuli. These adverse conditions are known to lead differential accumulation

of variety of compounds including secondary metabolites such as alkaloids, cyanogenic glycosides, glucosinolates, flavonoids, organic acids, steroids, diterpenes, non-protein amino acids, and polyphenols (Bowne et al. 2011). It is estimated that plant produces more than 200,000 secondary metabolites, while the total number is estimated to exceed over 500,000. Now several plant based metabolic database including Golm Metabolome Database (GMD) and The Arabidopsis Information Resource (TAIR) are available which act as metabolic data reservoir in the field of plant metabolomics. These libraries are created by collecting the spectral data of various metabolites identified by analyzing various plant tissues collected from normal and treated conditions. Apart from GMD and TAIR, National Institute of Standards and Technologies (NIST) library is one of the most important database for accurate prediction of metabolites.

Recent metabolomics studies have involved characterization of differential expression of metabolites in model crop plants to identify stress responsive or developmental related markers (Kumar et al. 2020). In rice, 170 differentially expressed compounds have been identified which can act as potential markers for bacterial blight resistance (Sana et al. 2010). In addition to proline as metabolic marker, several compounds have been identified to contribute during biotic and abiotic stress (Kumar et al. 2017). Presently, metabolomics has been explored to improve all different categories of crop plants such as cereals, legumes, oil seed crops, fruits, and vegetables. In this chapter, we discuss several important metabolomics studies which have significantly contributed to this filed.

17.2 Metabolic Platforms

Modern metabolomics platforms have the potential to sustenance large-scale metabolite studies capturing both known and unknown metabolites. They are mostly involved in metabolome data generation either using NMR or MS. NMR is used to identify low molecular weight metabolites (<50 kDa), however, its application is diverse like metabolic profiling and fingerprinting, atomic structure information extraction, etc. (Winning et al. 2009). The major drawback of NMR includes less sensitivity and its limited coverage with respect to low abundance molecules. In case of MS, it allows a wide range of metabolites coverage with greater sensitivity. It was very helpful in identifying novel biomarkers and reconstruction of metabolic pathways. Day by day, ionization method is improving such as MALDI-TOF, atmospheric pressure chemical ionization (APCI), and electrospray ionization (ESI) which led to enhance the accuracy of MS. In general, MS is combined with chromatography like liquid chromatography (LC), Fourier transform ion cyclotron resonance (FT-ICR), capillary electrophoresis (CE), gas chromatography (GC), etc. to ameliorate the performance of MS (Issaq et al. 2009). The distinctive feature of NMR to identify the physical properties of ligands, its binding sites and protein ligand complex structure has made it important from decades.

GC-MS platform is mostly employed for non-targeted volatile compounds, whereas LC-MS uses both targeted and non-targeted method to identify both

primary and secondary metabolites. Similarly, combination of Ultra-Performance Liquid Chromatography (UPLC) with Quadrupole Time-of-Flight Mass Spectrometry (QTOF-MS) magnify the peak resolution with better mass accuracy in less time. There are few other platforms such as CE-MS and FT-ICR-MS, which provides high resolution profiles of both targeted and non-targeted metabolites (Ramautar and De Jong 2014; Brown et al. 2005). Humongous data produced from these cutting-edge tools are conceded through data processing platforms like Chromate, MET-COFEA, and MET-X-Align. These platforms basically support in baseline correction, alignment, separation of co-eluting peaks, and normalization. Lastly, identified metabolites are analyzed statistically through heatmap, boxplot, partial least square (PLS), etc. to redesign metabolic pathways using web tool like MetaboAnalyst (Xie et al. 2015). These all exercises help in the identification of metabolic markers of agronomic traits.

17.3 Database and Software for Analyzing Crop Metabolome Data

In the past two decades, metabolomics has expanded greatly as emerging and fascinating approaches for experimental science with application in many areas. Metabolomics researchers needs improved and advanced database and software to command and facilitates the variety of instrument for accurate data analysis and integration of sample (Table 17.1); some important ones as follows:

17.3.1 BioCyc

BioCyc is a group of Pathway or Genome DataBase (PGDBs) developed by Karp et al. 2005 which presents the information on cellular network and genome to fascinate computational analysis. This software is built to query and edit the information of particular pathway at the level of protein, enzymes, substrate, gene and their transcription (Jing et al. 2014). Gramene, PlantCyc, and SoyCyc are the curated pathway/Genome Database that especially provide information related to plants including crop and others. These all pathways are created by different organizations using Pathway Tools of BioCyc. Gramene database provide information about metabolic and regulatory pathway, DNA or protein sequence alignment through BLAST as well as comparative analysis tools for genome to predict major or minor variation in crop and model plants. It includes approximately 1.7 million genes from 44 reference genomes which are organized into 62,367 gene families (Tello-Ruiz et al. 2018). Another metabolic pathway database is PlantCyc containing more than 1000 pathways with their catalytic enzymes, genes, and compounds from over 350 plant species. This database is based on hypothetical pathway from already published peer-reviewed journals and manually validated by Plant Metabolic Network (PMN) curators (Schläpfer et al. 2017) (https://plantcyc.org/about/citing-pmn).

Table 17.1 Selected examples of metabolite mass spectra database and services

S. No.	Database Name	Plant species/Purpose/ technologies	Uniform resource locator
1	GC-MS Website	Explain gas chromatography and mass spectrometry	http://www.scientific.org/tutorials/articles/gcms.html
2	FTIR analysis	Industrial application and compound identity	http://www.semlab.com/ma/ftir/ftir.html
3	NMR analysis	NMR analysis, methodology and equipment requirements	http://www.intertek.com/analysis/nmr/
4	NMR spectroscopy	Explain proton NMR spectroscopy and multi users	https://www2.chemistry.msu.edu/faculty/reusch/virttxtjml/spectrpy/nmr/nmr1.htm
5	Metabolome analysis service by CE-MS	Various applications for CE-MS in drugs, toxicology, disease, blood, cells, and tissues, including some use in plants also	http://www.infinitebio.com/technology/HMT_Metaborolomics_Analysis/index.html
6	Lipid analysis by GC-MS, LC-MS and FT-MS	Identification of Lipid metabolite	http://lab.ucdavis.edu/staff/kind/Metabolomics/LipidAnalysis
7	Pesticide Residue analysis by LC-MS/MS using a C18 column	Agricultural, food, flavor, and fragrance identification	http://www.restek.com/Technical-Resources/Technical-Library/Foods-Flavors-Fragrances/fff_A020
8	NMR analysis, Processing and Prediction	Explanation of NMR analysis	http://www.nmr-analysis.blogspot.com.au/
9	QMS403 C *Aëolos*® Quadrupole Mass Spectrometer NETZSCH	Gas Analysis	https://www.netzsch-thermal-analysis.com/en/
10	Klotho	Classification and collection of biological compounds	http://www.biocheminfo.org/klotho/
11	COLMAR Metabolomics web portal	Web server to generate a covariance of NMR spectrum and NMR data of individual component in mixtures	http://spinportal.magnet.fsu.edu/
12	METLIN metabolite database	Repository of metabolic information and tandem mass spectrometry data	http://metlin.scripps.edu
13	METAGENE	Define the error in metabolism	http://www.metagene.de/appl/index.html

(continued)

Table 17.1 (continued)

S. No.	Database Name	Plant species/Purpose/ technologies	Uniform resource locator
14	Fiehn GC-MS database	It contains records of 713 compounds for which GC/MS data are provided	http://fiehnlab.ucdavis.edu/Metabolite-Library-2007
15	Madison-Qingdao Metabolomics Consortium Database (MMCD)	Database of small molecules of biological significance	http://mmcd.nmrfam.wisc.edu/
16	High quality mass spectral database (MassBank)	It contains high resolution MS spectra of metabolites	http://www.massbank.jp/?lang=en
17	BioCyc (Pathway/Genome Database and pathway tool software)	Provides a reference on the genomes and metabolic pathways of thousands of sequenced organisms	http://metacyc.org/intro.shtml
18	BinBase	It is GC-TOF metabolomic database, employed for automated metabolite annotation	http://fiehnlab.ucdavis.edu/projects/binbase_setupx#binbase
19	PRIMe	Genomics and Metabolomics data /NMR spectroscopy/GC-MS, LC-MS, and CE-MS	http://prime.psc.riken.jp/
20	Golm metabolome database	Exclusive for plant based metabolites: Search and dissemination of reference mass spectra from biologically active metabolites quantified using GC-MS	http://gmd.mpimp-golm.mpg.de/
21	*MMCD (Madison Metabolomics Consortium Database)*	A resource for metabolomics research based on NMR spectroscopy and MS	http://mmcd.nmrfam.wisc.edu/
22	NIST Standard Reference Database	Library for EI and MS/MS spectra obtained exclusively from GC-MS. It provides structures, derivative precursors, etc. with additional features like GC/MS deconvolution and interpretation of mass spectra.	http://www.nist.gov/srd/nist1a.cfm http://webbook.nist.gov/chemistry
23	Plant metabolomics	Arabidopsis and other plants	http://plantmetabolomics.vrac.iastate.edu/ver2/
24	MeT-RO (*Metabolomics* at *Rothamsted*)	Plants and microbial metabolites	http://www.metabolomics.bbsrc.ac.uk/MeT-RO.htm

(continued)

Table 17.1 (continued)

S. No.	Database Name	Plant species/Purpose/ technologies	Uniform resource locator
25	MoTo DB	Tomato metabolite database identified by LC-MS	http://www.transplantdb.eu/node/1843
26	TERPMED	Plant terpenoids, natural products, secondary metabolites, and therapeutic drugs	http://www.terpmed.eu/databases.html
27	SetupX	Web based metabolomics, the capture and display of GC-MS metabolomics data	http://fiehnlab.ucdavis.edu/projects/binbase_setupx/
28	KEGG(Kyoto Encyclopedia of Genes and Genomes)	A metabolite database for metabolomics suing Q-TOF-MS technology	http://www.genome.jp/kegg/
29	MeRy-B	Database of plant primary metabolites	http://services.cbib.u-bordeaux2.fr/MERYB/
30	RIKEN MSn spectral database for phytochemicals (ReSpect) MS/MS spectral tag photochemical library	Data derived from multiple sources including crops using GC-MS and LC-MS technology	http://spectra.psc.riken.jp/
31	Oliver Fiehn Lab	Metabolic and targeted analysis generated from Arabidopsis mutant lines using GC-TOF-MS, LC-Q-MS, CE-MS, and LC-MS	http://fiehnlab.ucdavis.edu/staff/fiehn

17.3.2 KEGG

It is one of the first pathway database originally developed for gene based pathway catalogue by Ogata et al. 1999. Database has collection of manually drawn pathway that represents the information for metabolism of all the biomolecules with consideration of their biosynthesis. Database also facilitates the information about cellular process (Transport and catabolism, cell growth, and dead) as well as disease related entity. KEGG plant serve as an interface to plant related datasets which is categorized in various facilities such as Pathway, genomes, genes, compound, disease drug, and BRITE (Kanehisa 2016). BRITE is a hierarchical classification system consisting functional hierarchies of biological entities by incorporating different types of relationship including genes and protein, drugs, organisms and cells, compounds and their reactions (Kanehisa 2016).

17.3.3 KaPPA-View4

This database is developed by Sakurai et al. (2011) to provide overview of all correlation of metabolic pathway map. Their ability to overlay correlations in the form of curve on either single metabolic pathway or combination of four pathway map at a time for discovery of gene make it a unique database among others. Due to their correlation based pathway map on approximately 150 leaves of Arabidopsis is highly recommended by plant scientists (Tokimatsu et al. 2005). KaPPA-View4 has implemented with Affymetrix GeneChips and correlated data containing genome-sequenced plant species such as Arabidopsis, *Lotus japonicas*, rice, wheat tomato, barley, and maize as well as omics information on Jatropha *curcas* (Sakurai et al. 2012).

17.3.4 Plant CAZyme

Database built upon dbCAN (database for automated carbohydrate active enzyme annotation) for the purpose of pre-computed sequence and data of carbohydrate active enzymes (CAZymes) to plant carbohydrate (Ekstrom et al. 2014). Database is implemented with approximately 43,790 CAZymes of 159 protein families from 35 plant crop and model plants such as *Glycine max, Sorghum bicolor, Zea mays, and Arabidopsis thaliana*. Various facilities such as BLAST (Blastp and Blastx), functional, structural, and phylogenetic annotation as well as downloading of plant CAZymes protein sequences at the level of species, CAZyme domain sequences and their families are incorporated inside it.

17.3.5 Software for Crop Improvement

Based on the identification of particular class of compound, different types of analytical techniques require which is not restricted to a single one. Each technique requires a particular software which commands to analyze the metabolites or compound, some most frequently techniques are liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS), and nuclear magnetic resonance (NMR). Some commonly used software for these instruments are the following.

17.3.6 PyMS

PyMS is an open source Python based software for GC-MS data processing (O'Callaghan et al. 2012). Software gives complete guidance for base line correction, peak integration and their alignment. PyMS containing many parameters for processing of metabolite mix. It supports two standard formats for data input, JCAMP-DX (http://www.jcamp-dx.org/) and ANDI-MS (Erickson 2000). Automated sample processing with PyMS provide substantial saving of times and

are suitable for quantification of both routine and exploratory data (O'Callaghan et al. 2012).

17.3.7 MAIMS

MAIMS is an open source python tool suitable for isotopologue profile deconvolution of large metabolites of LC-MS (Misra 2018). Phyton 2.7 and 3.5+ compliant provide compatibility for proper run and was originally tested in Linux (Ubuntu 16.04) system (Verdegem et al. 2017). Although, it also works on different systems such as macOS, windows, ARM, and containers as well as supported by Java, Ruby, PHP, Go, Rust, and.Net.

17.3.8 NMRSpec

NMRSpec is a suitable software implemented with MATLAB and particularly for pre-processing and functional analysis of 1D NMR originated spectrum (Liu et al. 2017). Baseline correction, peak extraction, spectrum alignment, and peak integration are four major functional blocks which make easy accessible for spectrum analysis.

17.3.9 Mass Spectral Feature List Optimizer (MS-FLO)

MS-FLO is an open source software originally built to minimize unnecessary positive peaks with their identification in non-targeted LC-MS data for overall improvement in numbers of metabolites (DeFelice et al. 2017). Software performance is based on some utility such as accurate mass tolerances, peak height similarity for ion adducts identifications, and duplicate peak along with some statistical method like Pearson's correlation analysis.

17.3.10 KPIC2

KPIC2 is an LC-MS based effective software tool for metabolomics study using pure ion chromatograms (PICs) (Ji et al. 2017). Besides pure ion detection, KPIC2 has ability to align PICs and their isotope annotations. Software has implemented with functions that fill the missing peak and can recognize pattern of peaks generates during analysis.

17.3.11 MS-DIAL

Universal program for untargeted metabolomics that supports several instruments (GC-MS, GC-MS/MS, LC-MS, LC-MS/MS) and their vendors (Tsugawa et al. 2015). Software contains feature for spectral deconvolution for both GC-MS and MS-MS with suitable criteria for peak identification as well as interpretation of imported raw data with their statistical analysis. Recently MS-DIAL version 4 has developed for lipidomics data and lipid pathway discovery (Tsugawa et al. 2020).

17.4 Role of Metabolomics Towards Abiotic Stress Responses

In Indian agriculture system, horticulture sector, with diverse crops, has been a driving force for nutritive diet and its enhanced commercial perspective. Notably, fruits due to their nutritional benefits, and along with vegetables, they are part of everyday meals of a society. Therefore, fruits and vegetables accounts approximately 90% to the total horticulture production in India, ranks second globally in the production of fruits and vegetables (Laxman and Bhatt 2017).

Overall, understanding of abiotic stresses is required in fruit crops in order to develop better adaptation and controlling measures. In the field, plants are regularly expose to an unpredictable and harsh combination of stresses (Slama et al. 2015; Wani et al. 2016), may even worse in the context of climate change, soil salinization and environmental pollution. Particularly, water stress in fruit crops, generally grown under irrigation conditions because of high water requirement, in oppose water-scarcity adversely affect these crops, lead to reduction in number of leaves in a flush, the flush length, and leaf water contents.

Predominantly, in mango water stress also plays a significant role in induction of flowering by influencing floral stimulus produced by mature leaves (Scholefield et al. 1986). Grapes also encounter frequent moisture stress conditions and undergo several morphological and physiological changes under water stress in contrast, grapevines are considered as relatively tolerant to water stress due to large xylem vessels in comparison to other crops (Serra et al. 2014) and also by enhancing root length in oppose reducing the shoot growth (Hardie and Martin 2000). In banana, flowering stage is reported to be the most sensitive, reportedly in cultivar "Elakki," the lowest yield was obtained due to water stress at flower differentiation stage (Murali et al. 2005). Apparently, in papaya under field conditions, water-deficit stress treatment caused 50% reduction in leaves and significantly reduced number of flowers by 86% and fruit by 58% resulted in the retarded development of papaya fruit (Masri et al. 1990).

High temperature stress causes several damages like sunburns on leaves, branches, and stems, leaf senescence and abscission, shoot and root growth inhibition, and lately fruit discoloration and damage (de Almeida and Valle 2007; Wahid et al. 2007). Usually, floral induction in mango is temperature dependent (Davenport 2007) and is triggered by temperatures below 16 °C (Schaffer and Andersen 2018). Thus, adverse temperature conditions due to climate variability collectively

influence vegetative and reproductive cycles but also proportion of female flowers in the panicle, hampering productivity.

In response to that, several metabolic pathways become operative as part of the mango fruit response molecular mechanism to extreme heatwaves and temperature. It seems like heatwaves induce the synthesis of reactive oxygen species (ROS) and increase the rate of the ripening phenomena in mango fruits, subsequently activates antioxidant defense mechanism and to neutralize the heat stress in fruits. In addition, an enhanced expression of glutamate decarboxylase, wound-induced protein, nucleoredoxin, gigantea, and fructose bisphosphate aldolase genes were detected. These genes regulate gamma aminobutyric acid metabolism, cellular senescence, reactive oxygen species homeostasis, circadian rhythm control, carbon and nitrogen flux, respectively (Khanum et al. 2020).

The crops are affected by salinity stress due to high salt deposition leading to high evaporation. The elevated levels of chlorides and calcium sulfate, magnesium, and sodium present in the soils adversely cause considerable damage to many crops and cause toxicity to plants. The salinity stress causes reduction in pseudostem thickness, delayed flowering, reduced finger size, and low-quality bunches (Ravi and Vaganan 2016). In grapes, many physiological parameters, growth, and nutrient uptake are affected considerably by salinity stress (Bybordi 2012).

The part of physical response to stress, cuticle layer acts as a universal outmost shield (Fich et al. 2016). Interestingly, recretohalophytes even evolved a specialized organ to excrete salt, as represented by the epidermal salt gland of Limonium bicolor (Yuan et al. 2013, 2016). Hence, its multirole function such as lipids desaturation, reactive species (RS) scavenger's activation, stimulation of molecular chaperones, and accretion of compatible solutes as a part of collective response to membrane injury, protein denaturation, and osmotic stress (Yeats and Rose 2013; Fich et al. 2016).

Physiological and morphological defenses are orchestrated by a complex regulatory network comprises signaling molecules including stress hormones [e.g., abscisic acid (ABA)], reactive oxygen species (ROS), hydrogen sulfide (H_2S), nitric oxide (NO), polyamines (PAs), phytochromes, and calcium (Ca2+), and transcription factors (TFs). In addition, antioxidant property possessed by plants is a kind of abiotic stress management in plant, with production of numerous antioxidants such as betaines, carotenoids, flavonoids, and vitamin E (Gechev et al. 2006; Zhao et al. 2011). Also, some notable enzymes comprise superoxide dismutase's (SODs), catalases (CATs), and various peroxidases (PODs). Besides, ascorbate peroxidase (APX) involves dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), and glutathione reductase (GR). Other enzymes, such as glutathione S-transferase (GST) and ferritins, participate in detoxification (Gechev et al. 2006; Sharma et al. 2012).

Osmotic stress generally afflicts grapevines; an approach has made in a larger picture of grapevine responses to abiotic stress. Generally, much of the grapevine response to osmotic stress appear to be transcriptionally regulated, but reported proteomic studies have showed that there are post-translational controls. Notably, ABA acts to reduce water loss and increase stress tolerance in grapevines and also

acts as a central regulator of many osmotic responses, NCED1 appears to be the rate-limiting enzyme for the majority of ABA biosynthesis, because of its high proportionate with ABA concentrations in both shoots and berries.

In MeJA fruits, particularly, linolenic acid (18,3) yield into jasmonic acid and its derivatives, namely jasmonates (JAs), a group of stress hormones with a better role in wound response. There is concrete evidence that JA is also employed in defense against other stresses, like salt (Ryu and Cho 2015; Yang et al. 2017) and Ultraviolet radiation (Conconi et al. 1996).

Compatible solutes are tiny organic compounds with electrically neutral with high solubility and low toxicity that can even build up in high concentrations inside the cells with few perturbations. Basically, qualified molecules are sugars, amino acids and their derivatives such as raffinose, trehalose, inositol, mannitol, proline (Pro), and glycine betaine (GB). Under stressful conditions, these metabolites may amass to act as osmo-protectants against dehydration, scavengers of RS, and/or stabilizers of proteins and membranes. Pro, a widely present one, is also able to buffer cellular redox potential and induce gene expression (Slama et al. 2015).

To combat against abiotic stresses, some general defenses are connected by a complex regulatory network composed of several signaling molecules and gene regulation factors. Once triggered, stress hormones (ABA), ROS (H2O2), H2S, NO, PAs, phytochrome B (PHYB), and Ca2+, extensively interplay with others at various levels, synergistically or antagonistically, to establish a specific directive for downstream effectors, TFs in particular, to alter gene expression and protein/enzyme activities in a specific pattern, thereby launching a proper response. Particularly, all biochemical defenses mentioned above said to be mobilized by ABA, including Pro (Ashraf and Foolad 2007), antioxidants (Liu et al. 2018), cuticular waxes (Lee and Suh 2015), 18:3 (Yin et al. 2018), RS detoxifying enzymes (Chen et al. 2013; Hoque et al. 2016), and HSPs (Huang et al. 2016).

Gene regulation factors active at different levels of stress management, like histone acetyltransferases (HATs) (Stockinger et al. 2001), TFs, alternative splicing factors (Laloum et al. 2018), microRNAs (Zhang et al. 2013), and ubiquitination enzymes (Lee and Suh 2015) are commanding the defense systems. However, transcriptional level is still the key regulatory node as many of the TFs have been recognized as stress responsive, mostly ABA-responsive element (ABRE) binding proteins/factors (AREBs/ABFs) (Sah et al. 2016), DELLAs (An et al. 2015), NACs (Tang et al. 2017), WRKYs (Bai et al. 2018), zinc finger proteins (Han et al. 2011; Wang et al. 2018), and the APETALA2/ETHYLENE response factor (AP2/ERF) (Mizoi et al. 2012).

Abiotic stress ultimately causes protein misfolding or the accumulation of unfolded proteins, called as Endoplasmic Reticulum (ER) stress by specific sensor proteins in the ER membrane. This sensing subsequently leads to the activation of the genes encoding molecular chaperones and other related proteins enhances protein-folding capacity, ER-associated degradation (ERAD), or protein translation suppression to lessen the built-up proteins in ER via the PKR-like ER eIF2a kinase (Walter and Ron 2011), helping to restore ER homeostasis, to maintain the

equilibrium between protein-folding demands and folding capacity, and are known as the unfolded protein response (UPR).

17.5 Role of Metabolomics in Biotic Stress Responses

Majority of the world's population primarily depends upon cereal crops such as rice, barley, maize, wheat, oat, sorghum, and millet for food and nutrition. One of the major problems associated with these crops are their biotic factors such as fungi, bacteria, viruses, and herbivores which leads to disease associated severe yield loss (Balmer et al. 2013). While, plants have the ability to counter pathogens through pre-formed barriers, constitutive and inducible immune system (Pieterse et al. 2009). Pre-formed structural and chemical defense includes cell wall and phytoanticipins, respectively (González-Lamothe et al. 2009). Besides pre-existing defense, plants also produce antimicrobial compounds and phytoalexins during pathogen attack in order to suppress pathogen entry and their multiplication (Balmer et al. 2013). Three major groups that constitute chemical defense arsenal of plants are alkaloids, isoprenoids, and shikimates which synthesized via citric acid cycle, acetate-mevalonate or methylerythritol phosphate pathway and shikimate pathway, respectively (Großkinsky et al. 2012). These all-inclusive of metabolites synthesized via variety of pathway are termed as metabolome that have been received inadequate attention in crop development with metabolomics approaches (Kumar et al. 2017). Techniques for metabolomics involve generation of metabolome data of identified analytes. Some modern techniques used for metabolomics profiling for crop improvement related to biotic stress include FT-ICR-MS (Seybold et al. 2019), NMR (Cuperlovic-Culf et al. 2016), UPLC-QTOF/MS (Farahbakhsh et al. 2019), CE/TOF-MS (Suharti et al. 2016), GC-MS (Agarwal et al. 2014), and LC-MS (Jones et al. 2011).

NMR is extensively used for detection of smaller molecular weight metabolites coupled with structural information of compound at the level of an atom. Cuperlovic-Culf et al. (2019) have discovered the disease resistant-biomarkers such as asparagine, phenylalanine, L-alanine, and myo-inositol against *Fusarium graminearum*. Likewise, they also performed metabolomics analysis via the NMR and determined the differential response of inositol, lactic acid, galactose, putrescine, and spermine against biotic stress of *Fusarium* head blight infection in different wheat varieties, namely FL62R1, Stettler, Muchmore, and Sumai3 (Cuperlovic-Culf et al. 2016). Recently, a potential biomarker of fungal associated disease in grape wine have been discovered that explains the abundant expression of leucocyanidin, caffeic acid, catechin or epicatechin, dodecanoic acid, and hexadecanoid acid derivatives in susceptible *Vitis vinifera* while dihydroquercetin and quercetin 3-O-glucoside (isoquercitrin) were found to be expressed in resistant/partial resistant plants using techniques of FT-ICR-MS (Maia et al. 2020). Another study reported a greater increase in phenylalanine and glutamine along with accumulation of linoleic acid in rice against gall midge through GC-MS tool (Agarwal et al. 2014) as well as accumulation of lipid, carbohydrate, xanthophyll, alkaloids in *Xanthomonas oryzae*

pv. *oryzae* (*Xoo*) resistant variety of rice (Sana et al. 2010). Metabolomic study of rice has been also determined in different varieties of germinated rice for their nutritional value and health benefits using NMR techniques with significant increase in fatty acid, linoleic acid, tryptophan, vanillic acid, α-tocopherol, 3-hydroxybutyric acid, and fumaric acid (Pramai et al. 2018). Furthermore, the role of annual legume produced flavonoids in weed suppression through chemometric analysis by UHPLC QTOF-MS (Latif et al. 2019). Some of the lipids like *inositol phosphorylceramide synthase* coordinate the program cell death that plants require to limit pathogen as its self-defense (Wang et al. 2008). As several merits of metabolomics in crop improvement, it has attained prominent focus by researchers and exciting way towards metabolites profile at the level of gene regulatory pathway.

17.6 Improvement of Some Vegetables: Some Examples

Worldwide, vegetables are considered as essential sources for the micronutrients included in healthier diet practices. The World Health Organization (WHO) recommended a 400 g/day intake of vegetables to prevent chronic diseases like heart diseases, cancers, and diabetes and also to compensate much needed micronutrients such as calcium, iron, iodine, vitamin A, and zinc to the consumer (WHO 2015, WHO/FAO 2003). Some of the contents available in vegetables safeguards health, to mention few Potassium in vegetables helps to maintain healthy blood pressure, their dietary fiber content helps to lower the blood cholesterol minimizing the risk of heart disease, folate (folic acid) lessens the risk of birth defects, vitamin A helps to keep eyes and skin healthy, whereas vitamin C keeps teeth and gums healthy but also helps in iron absorption. Understanding the important nutritional and health benefits of vegetables, increased and improved production aids to counter rural poverty and unemployment in developing and in underdeveloped countries. Vegetables are termed as mankind's most affordable source of vitamins and minerals needed for good health, but till today, neither the economic nor nutritional power of vegetables is sufficiently realized (Schreinemachers et al. 2018). However, scope on vegetable farm sector is relatively low, as national and international governments have got limited financial policies to encourage the growers, negatively influencing the economic growth and nutritional demands. On the other hand, public and private investments in agriculture are still mainly focused on staple crops and oil crops, but not on commodities enriched with micronutrients (Haddad et al. 2016; Pingali 2015). To achieve the increasing market needs for vegetables at reasonable prices, apparently production has to increase. This could be materialized via intensification of existing specialized vegetable systems, particularly in semi-urban areas (Beed and Dubois 2015). This chapter primarily focuses on the improving on-farm productivity, reducing postharvest losses, and improving market access of vegetables (Schreinemachers et al. 2018).

Currently, the most consumable vegetables are tomatoes, cucurbits, alliums, and chilies. These vegetables are consumed globally, available in various shapes, sizes, colors, and tastes. Reportedly, tomatoes alone are considered as the fourth most

economically-valuable food crop produced predominantly in low- and middle-income countries, after rice, sugarcane, and wheat (Schreinemachers et al. 2018). Improvement of vegetable crops are managed at different levels to maintain the nutritional value of particular crop. To achieve that following major practices have to be employed by farmers:

- Ensuring that vegetables are safe to eat.
- Improving on-farm productivity.
- Improved vegetable varieties.
- Safe and sustainable pest management.
- Improving market access.
- Reducing postharvest losses.
- Increasing vegetable consumption to improve nutrition.
- Home gardens and rural vegetable consumption.
- Modifying food systems for better nutrition.

To mention, tomato (*Lycopersicum esculentum*) is the self-pollinated, generally considered as the most important cultivation crop rich in vitamins and minerals. Improvement of this particular plant is much needed, due to its demanding market value, by enhancing the self-life, improving the taste, and also by maintaining essential nutrients proportion. Accordingly, breeding is a type of improvement strategy yields resistance to disease and pests. For the last two decades, remarkable progress is made in molecular genetics precisely in gene cloning and DNA sequencing, gene mapping, QTL analysis, and markers development. QTL analysis would be important tool for crop improvement in next decades, also has a very significant impact on breeding programs, as many of the complex traits of the fruit can be determined by this particular method. In contrary, conventional breeding methods will not increase the production of tomato will be potentially replaced by marker-assisted selection in coming decades (Fentik 2017).

Potato (*Solanum tuberosum* L.) is a solanaceous food crop having enormous potential to feed the densely populated world, rich in carbohydrates (starch), proteins, minerals, and vitamins in comparison with other potential food crops, and is considered as a major staple food in many developing countries. Worldwide, several attempts have been made to improve the nutrition value of potato via genetic engineering by keeping the yield unaffected. Accordingly, breeders come up with new variety of crops with enhanced nutritional levels in potato tubers, chiefly, increased *Amaranth Albumin*-1 seed protein content, β-carotene level, vitamin C content, triacylglycerol, tuber methionine content, and amylose content, etc. Conversely, breeders emphasized on removal of anti-nutritional compounds such as steroidal glycoalkaloids, acrylamide, and food toxins breeders to improve potato tuber quality (Hameed et al. 2018). The breakthrough is been made by adopting new breeding technologies like TALEN and CRISPR/Cas9 to produce transgene-free products in a more precise, and in effective way. Reportedly, transient expression of TALENs, delivered through using non-viral *Agrobacterium-mediated* transformation, resulted in targeted mutations in two potato cultivars, Russet Burbank and

Shepody (Ma et al. 2017). The penetrated TALEN constructs were subject to induce mutations, precisely in two different host genes, namely (1) *1,4-alpha-glucan branching enzyme* (*SBE1*), (2) *Vacuolar invertase 2* gene (St*vacINV2*). Furthermore, the readily accessible potato genome sequence and efficient potato transformation systems have remarkably facilitated potato genetic engineering (Hameed et al. 2018).

Bulb onion (Allium cepa L.), commonly referred as onion is an ancient crop reportedly originated in Central Asia, termed as a major vegetable crop cultivated worldwide. It has a biennial life cycle with cross-pollinated nature and high inbreeding depression effectively posing challenges for the breeding and characterization of improved traits. Several, classical genetic and plant breeding approaches have been employed to bring the improvement in onion yield, quality, and resistance against various biotic and abiotic stresses. However, germplasm analysis and mapping in onion is readily in progress to speed up onion improvement. In order to attain this, molecular markers are being rapidly developed, simultaneously, next-generation sequencing (Khosa et al. 2016). However, in the breeding program the following methods can be employed to develop more sustainable varieties of onion crop: RNAi method, to silence specific genes for crop improvement most commonly used in genetic engineering (GE) of onion crops (Saurabh et al. 2014). In contrast, TILLING (Targeting Induced Local Lesions IN Genomes) is a non-GE approach where inducing the changes in the DNA sequence of specific genes, created using a mutagen (Chen et al. 2014). At present, F1 hybrids predominantly grown in regions generally long-day onions are grown and in contrast, open-pollinated varieties (OP) predominantly grown in the short-day growing regions most likely in Asia and Africa (Currah and Proctor 1990; Brewster 1994). As of now, improved OP varieties and F1 hybrids doubled the production of onion in the last 50 years (Brewster 1994). Nevertheless, seasonal change and mounting population pressure are posing serious threats to increasing onion production to meet demand (Brewster 1994). Additionally, onion poorly competes with weeds, during the initial stage of plant growth and causes yield losses if not well managed. Till today, onion growers control the weeds by manual practices or applying some chemicals which are usually costly, ineffective, and incur adverse environmental effects (Brewster 1994). Breakthrough was made by using Agrobacterium-mediated transformation, an herbicide resistant gene was used to develop glyphosate resistance onions, but failed to commercialized till today (Eady et al. 2003).

Cabbage is another important vegetable crop belongs to family Brassicaceae and is cultivated in many countries. There are different varieties of cabbages are available depending upon shape, size, and color. The crop possesses strong disease resistance, wide adaptability, good quality, and uniform economic characters and also high yield. Interestingly, the rate of conversion from open-pollinated varieties to hybrid varieties and seed replacement ratio is very high in this crop. The specific features like, the self-incompatibility and male sterility systems are present in the crop, facilitates low economical hybrid seed production. In addition, advance functional genomics studies provide a better knowledge about plant genome helps in genome modification for the crop improvement. Furthermore, RNA interference,

next-generation sequencing, and nanotechnology have become a new promising technique for better serves for improving crop accordingly in the future need (Rathore et al. 2018).

17.7 Metabolomics Studies in Legumes

Legumes are one of the most important crop plants and they play a significant role in human diets. They enhance soil fertility and contribute towards sustainable agriculture through root nodules—a specialized organ which harbors symbiotic associated nitrogen fixing bacteria *Rhizobium* spp. (Sharma et al. 2020). It is a vital constituent of human dietary proteins along with other nutrients but it is highly susceptible to climatic disparity globally that negatively affecting its productivity (Ciura and Kruk 2018). Soybean (*Glycine max*), common bean (*Phaseolus vulgaris*), cowpea (*Vigna unguiculata*), several Lupines (*Lupinus spp. L*), lentils (*Lens culinaris*), chickpeas (*Cicer arietinum*), and fava bean (*Vicia faba*) are some of the leguminous crops essential for nutrition and food security, mitigates poverty as well as indispensable for sustainable agriculture (Mousavi-Derazmahalleh et al. 2019; Foyer et al. 2016). Earlier Kumar et al. (2017) emphasized on the application of metabolomics in association with other technologies to facilitate development of agronomically superior plants to meet the agricultural challenges of twenty-first century.

Metabolomics profiling of Cowpea (*Vigna unguiculata L. Walp.*), a drought resistant crop reveals presence of 41 primary metabolites, viz., 24 amino acids and their derivatives, five sugars, four polyols, and eight organic acids by GC-MS (Goufo et al. 2017). Additionally, 35 secondary metabolites were identified by LC-DAD such as 15 phenolic acids, 17 flavonoids, and three proanthocyanidins. The most significant response to drought was observed for quercetin, galactinol, proline, quercetin 3-O-6″-malonylglucoside, and kaempferol 3-O-diglycoside (Goufo et al. 2017). The study reveals three drought responsive metabolites such as proline, galactinol, and Quercetin 3-O-6″-malonylglucoside formed by interplay between shikimate and arginine/proline pathway. By correlating grain yield with identified metabolite marker, it provides a basis for improvement of drought tolerance for marker-assisted breeding (Goufo et al. 2017). Likewise, Khan et al. (2017) observed differential accumulation of metabolites in the tolerant and sensitive varieties of chickpeas (*Cicer arietimnum L.)* during drought stress conditions. After drought stress, the leaves of tolerant variety showed higher accumulation of proline, arginine, histidine, isoleucine, and tryptophan. However, compounds such as alanine, α-ketoglutaric acid, phenylalanine, tyrosine, aspartic acid, GABA, choline, glucosamine, adenosine, and guanine were decreased in the two varieties (Khan et al. 2017). Furthermore, Coutinho et al. (2018) observed accumulation of alanine, GABA, sucrose, acetate, citrate, and succinate in the roots, when the two different soybean cultivars were subjected to flooding. But interestingly, the level of most of the compound decreased in leaves. In another study, Muscolo et al. (2015) reported reduction in the level of tricarboxylic acid cycle (TCA) intermediates in response to stress tolerance for lentils—an important Mediterranean legume. Stress specific

metabolites indicators such as ornithine and asparagine for drought stress and alanine and homoserine for salinity stress were also identified (Kumar et al. 2017). The exposure of drought to soybean induces de novo biosynthesis of amino acid which indicates the flow of nitrogen into amino acid metabolism (Kumar et al. 2017). Tripathi et al. (2015) reported a novel metabolite marker "coumestrol" as the indicator of drought/water stress tolerant plants.

Abundance of flavonoids, quercetin, and kaempferol in certain annual pasture legumes, including *biserrula cv. Casbah* and yellow *serradella* representing its weed suppressive potential provides strong preliminary evidence for their key role opening the new avenues for future research (Latif et al. 2020a). Multiomics platform along with the use of integrated experimental approach enable researcher to uncover the metabolic pathways and their genetic regulation associated with secondary metabolite production in important legumes. Using these approaches, legume-based forage quality would be improved through recurrent selection, cultivar specific phytochemicals, and species engineering (Latif et al. 2020b; Butkutė et al. 2018).

A different pattern of defense response was revealed through metabolomics and transcriptomics in *Arabidopsis*, a model plant. In response to drought stress, proline was accumulated but was replaced with sucrose (osmoprotectant) during combination of stresses Similar more studies are required in other agronomic valued crops such as chickpeas, pigeon peas, and peanut (Ramalingam et al. 2015). Metabolomics approaches along with other omics technologies are valuable to scientific communities as helps in trait dissection which is prerequisite for development of effective strategy (Sharma et al. 2021). These will further assist in a user-friendly, cost effective, and less time consuming legume improvement program.

17.8 Improvement of Cereals

Cereals are staple food crops and are primary source of nutrition worldwide. Cereals such as wheat, rice, maize, sorghum, millet, barley, and rye in their natural form are rich source of carbohydrates, proteins, fats, oils, vitamins, and several minerals (Sarwar 2013). Several metabolomics studies were carried out by different research groups to identify metabolic biomarker in cereal crops which would be helpful to develop elite cultivar through metabolic assisted breeding (Khan et al. 2019a, b; Chang et al. 2019; Zhou et al. 2019; Seybold et al. 2019; Farahbakhsh et al. 2019).

Drought, as one of the water stresses strongly inhibits growth and development of wheat (Razzaq et al. 2019). Several metabolites such as sugars (glucose, sucrose, fructose), organic acids (oxalic acid, malic acid), amino acids (threonine, proline, γ-aminobutyric acid), sugar alcohol such as myo-inositol were identified in six wheat genotypes emphasizing their importance in drought tolerance (Marček et al. 2019). Similar results were reported by Michaletti et al. (2018) which showed accumulation of tryptophan interconnected to shikimate pathway and reduction in glutamic acid linked to spermine synthesis. In another study, metabolomic profiling in wheat predicted proline, tryptophan, N acetyl glutamic acid, pipecolate, linamarin and DIBOA glucoside as metabolite marker and their level could indicate drought

tolerance in wheat varieties (Rahman et al. 2017). Yadav et al. (2019) demonstrated that tolerance to drought is predicted by amino acid response in glasshouse grown wheat cultivars. Metabolomic profiling to understand the key metabolite and the biochemical pathway in different wheat varieties show alteration of amino acids, organic acids, sugar, and sugar alcohol under drought stress (Borrelli et al. 2018; Kang et al. 2019). The significant observation made on wheat and rice cultivars revealed lysine, proline, methionine, tryptophan, phenylalanine, six phosphogluconate and lactate as important biomarkers (Herzog et al. 2018; Locke et al. 2018). Metabolomics studies based on heat stress tolerance performed on different wheat varieties showing similar accumulation of compounds such as pipecolate, tryptophan, sucrose, and G1p (Xueli Qi et al. 2017; Thamason et al. 2018; Wang et al. 2018). Cuperlovic culf and colleagues indicated disease resistant markers against Fusarium graminearum such as trehalose, asparagine, phenylalanine, myo-inositol, 3-hydroxybutarate, spermine, putrescine, GABA, inositol, galactose, and lactic acid for wheat cultivars. Another metabolomic study reported benzoxazinoids pathway compounds are associated with aphid resistance in durum wheat (Shavit et al. 2018).

Drought tolerance in maize is developed by understanding the metabolic response based on metabolomics analysis in combination with agronomic traits. Maize drought tolerance can be improved through simple sugars, oxypilin, and ROS accumulation (Yang et al. 2018). In addition, accelerated glutathione accumulation and upregulation of carbohydrate and lipid metabolism were also observed (Yang et al. 2018). Accumulation of many amino acids (Glycine, serine, valine, isoleucine, threonine, four amino butanoate, and myo-inositol) were induced in drought tolerant maize cultivars as a response to drought stress (Obata et al. 2015). Maize metabolomics analysis showed lignin, flavonoids, polyphenols as biomarkers providing resistance against pathogens (Vasamtkar et al. 2019).

In rice (*Oryza sativa* L.), 4–hydrocinnamic acid and ferulic acid were considered as key metabolites in response to drought tolerance (Ma et al. 2016). A study on Indian rice varieties tolerant to salinity stress revealed increased levels of serotonin, gentisic acid, ferulic acid, and vanillic acid as biomarker compound (Gupta and De 2017). Furthermore, a study by Chang et al. (2019) suggested malate and sucrose in the leaves and mannitol in the roots as important metabolic markers for salinity tolerance in rice (Chang et al. 2019). Recent study in rice by Kurotani et al. 2015 reported application of jasmonate could effectively reduce the cellular damage caused due to the salt stress. Additionally, Peng et al. 2016 showed GABA and glyoxylate as the important metabolite which offers resistance against pest brown plant hopper in rice varieties.

Osmotic adjustment (OA) for drought avoidance and drought tolerance by ROS scavenging was proposed as the strategies by the spike organs of barley (*Hordeum vulgare L.*) to cope with draught (Hein et al. 2016). Proline, organic acids, sucrose, xylose, and maltose are the key metabolites identified for salt tolerance in barley (Shelden et al. 2016).

Identifying metabolic biomarkers through metabolomics profiling predict the nature and scale of biotic and abiotic stress. In combination with other technologies, metabolomics has uncovered many metabolites which can act as biomarker

including some novel compounds not reported widely earlier. Quality, yield, shelf life, and other attributes of crop plants could be improved through these biomarker studies. Thus, metabolomics assisted breeding helps in crop improvement program for development of stress tolerant and high yielding crop varieties (Kumar et al. 2017; Razzaq et al. 2019).

17.9 International and National Status

Metabolomics studies have become integral part of functional genomics studies. Especially, the LC-MS and GC-MS have become the much preferred platforms. The metabolomics work is leaded by few international labs/institutions such as Wageningen University & Research, Netherland; Weizmann Institute of Science, Israel; Metabolomics Research Group, RIKEN Center for Sustainable Resource Science, Japan; Fiehn Laboratory, UC Davis; Duke molecular physiology Institute, United States America; Max-Planck Institute for Molecular Genetics, Germany; etc. Now, a joint initiative from four leading institutes, viz University of Cologne (UoC), Max-Planck Institute for Plant Breeding Research Cologne (MPIPZ), Heinrich Heine University Düsseldorf (HHU), and Forschungszentrum Jülich (FZJ) have started CEPLAS (Cluster of Excellence on Plant Sciences) Plant Metabolism and Metabolomics Laboratories in Germany. In India, several institutes work in the area of metabolomics, including identifying the important components of the medicinal plants. The Department of Biotechnology, Delhi, India has funded several research grants which have equipped many institutions with LC-MS, GC-MS, NMR-MS, etc. For instance, Repository of Tomato genomics and resources, University of Hyderabad (also recognized as DBT Centre of Excellence and Innovation in Biotechnology) have built sophisticated lab with high sensitive metabolic plant forms including Nano LC-MS and GC-MS.

In the two decades, every year's huge amount of data's is being generated through metabolic profiling. The data generated via LC-MS, usually require more memory than that of GC-MS. Therefore, metabolomics workbench has been developed which is an international as well as national repository for metabolomics metadata, and provide access to the data analysis, and access to the protocols, training, and tutorials. The maintenance of this data base is funded by National Institutes of Health (NIH) Metabolomics Common Fund, US; to also support the development of next-generation technologies, capacity building center for metabolomics studies, generation of high quality reference standards and provide opportunities for collaboration to attract researchers throughout the world.

17.10 Future Perspective

In the current era metabolomics has become indispensable component of plant and animal based research. Metabolomics studies are also crucial for clinical research. In plant, metabolomics driven research has provided researchers to develop approaches

such as mGWAS and mQTL that are key to gene(s) and trait(s) discovery. The research spanning the areas of plant pathology and nutritional profiling is one of the most important areas where metabolomics intervention has made remarkable impact. Availability of genome editing techniques has enabled precise metabolic engineering of multiple genes from more than one pathway in single plants; this is one of the biggest achievements of the twenty-first century. For instance, improvement of rice cultivar for drought tolerance by introducing mutations through CRISPR/Cas editing in the 13 homologs of *Pyrabactin resistance* (*PYR 1–13*), an abscisic acid receptor encoding gene associated with both ABA biosynthesis and signaling (Miao et al. 2018). Now, several such evidences are available like introducing de novo domestication in rice and tomato through CRISPR/Cas. We believe a combined metabolomics research would provide better opportunity for trait dissection and in the next 10 years more products will come.

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New Generation Plant Phenomics Applications for Next Generation Agricultural Practices

18

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Abstract

Changes in the environmental variables such as soil texture, light intensity, or climatic indexes reflect their direct effects on plant phenotype as well as genotype. Along with the noteworthy improvements in genome wide association studies, we need to screen large populations under multi-environmental conditions. To achieve this, more individuals are needed to be escaped from microlevel pots to macrolevel field trials by using extended phenotyping platforms. Additionally, non-invasive and speed selection of a specific phenotype which is carrying out a trait of abiotic or biotic stress resistance can gain momentum for both breeders and producers. In the line with technological improvements, omic-based applications are integrated into the agricultural practices might provide more sustainable genotypes. In this way, monitoring combined effects of external factors on plants might be precisely analyzed on synchronously. So, high-throughput phenotyping platforms can be developed with the capacity for quantitatively assessing from thousands to millions of plant phenotypes in greenhouse and field conditions, respectively. As a result, sorting the phenome specific responses under the pressure of abiotic and biotic stressors and mining their results are requiring both multi-disciplined approach and high-throughput infrastructure. This chapter aims to describe the new generation plant phenotyping tools coupled with advanced screening technics.

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Keywords

Field screening · Image analysis · Phenomics · Remote sensing · 3D screening · Field biotechnology

18.1 Introduction

From the beginning of cereal domestication, there has been a close relationship between quantitative and qualitative crop management systems. Therefore, tracking the physiological, morphological, biochemical, and cellular changes in plants is important to support a new layer for molecular studies. In this context, next generation crop breeding applications such as marker assisted selection (MAS), association mapping, and sequence-based genotyping have gained value day by day and provided a sustainable linkage between laboratory and field (Morgante and Salamini 2003; Reynolds and Borlaug 2006; Rafalski 2002; Walther et al. 2002).

On the other side, however, there are some adapted plants against to the extreme environmental territories, most of them have been taken place in the agriculturally useless group. Also, one of the core components for choosing the right plant genotype passes through multiple selection parameters including phenotyping (Velu and Ravi 2013). Additionally, capturing different traits with nondestructive way and following their dynamic measurements via automated systems are two major concerns during plant development (Fiorani and Schurr 2013). For achieving deep and rapid phenotyping, researchers are needed to interlock results coming from both greenhouse and field screening studies. At this point, advancements in non-invasive methods appear as powerful and user-friendly tools for exact identification of plant phenological responses under different environmental conditions (Faget et al. 2013). By this well-structured screening network, a wide amount of analysis can be enabled to identify the maximum changes between genotypes in minimum time intervals. Under this automated concept, high-throughput measuring is performed in intact tissues of a plant under its natural growing area. As a plus, data collection can be done by smart computational systems which can accurately divide the phenological responses into accompanying panels of interest (Walter et al. 2012). Rapid application of plant phenotyping systems boomed after 2010 by the development of cutting age technologies and helped researchers to track quantitative characteristics of a genotype (Costa et al. 2019).

A good phenotyping starts from the seed and can be carried to the cell, tissue, individual plant, and population level. For example, measuring the light intensity is an important parameter in phenotyping. Because of leaf shape, thickness and photosynthesis rate can be varied by direct and diffused light intensity (Gutschick 1999). In the early plant developmental stage, a snapshot screening of each plant sample without missing any data in the frame of the planned time and environmental conditions requires specific equipment and multifactorial image screening systems. Recently, field plots can be screened phase by phase in different growth stages of plants. Kipp et al. (2014) investigated the early plant vigor in wheat by introducing

high-throughput screening infrastructure such as "GreenSeeker" and "CropCircle" that were combined with novel sensors. Thus, a novel phenotypic prediction was performed on winter wheat cultivars in a wide range of field plot by year comparison. From this point of view, sensor applications in agricultural systems are used to estimate the moisture capacity of soil, weed control, dysfunctional tissue tracking in trees, fungal infection type and intensity in crops, etc. (Kayad et al. 2020). Either in the field or in the glasshouse trials, perception of heat and light intensity for different individuals can be followed by the fluorescent imaging systems and near infrared cameras, respectively. For example, Dowell et al. (2009) have utilized the method for near infrared spectroscopy and waxy wheat selection. Amylase-free starch carrying hexaploid wheat kernels was selected via NIR technology. Also, this system has been calibrated to analyze partly waxy kernel in durum wheat. On the other hand, locus specific new allelic variations have small effects on individual selection and make the measureable dissection difficult without detailed phenotypic screens. For complete annotation of plant phenomes, especially complex traits nested in polyploids, all stages of development should be tracked synchronously. To achieve an optimum genome wide association study for agronomically important traits and go over the phenotyping bottleneck, high-throughput phenotyping has become a must. Also, making a true trait characterization, plant phenotyping can be extended with the automated platforms which run under the control of computational infrastructure. In this way, adequate number of genotypes can be introduced and genomic scripts can be solved via integrated statistical methods (D'Agostino and Tripodi 2017).

In a basic breeding scheme, crossing has been performed for obtaining the homozygous genotypes. During this process, individual's phenotype might be subsequently differed between crossings and selected genotypes. Same situation might be occurred for some agronomical traits due to the expected and observed nature of genetic distribution. Additionally, however, missing phenotypes can be observed at lower levels, phenotype-based screening is still an essential way for breeding studies (Robertsen et al. 2019).

Especially, the effect of environment on genotype is a determinative factor for exact phenotype formation. Either, testing of genotypes or during breeding efforts, climate-based selection emerges as descriptive factor. Mostly, climate might affect the transpiration rate, water use of a plant or cause salinization and drying of soil. So, during plant phenotyping efforts, fluctuations in the environmental parameters should also be taken into consideration. As an example, evapotranspiration at both micro- and macroscales affects the total water content in the atmosphere and plants vice versa. As it is known as remote sensing, geophysical imaging provides gather subsurface variables across time and space (Gorji et al. 2019; Jayawickreme et al. 2014). Blum (2011) underlined this interaction for drought stress and emphasized the construction of certain simulation models for understanding the dominancy effect of environment. From this point of view, biochemical or metabolic phenotyping with high precision can be utilized to promote standard phenotyping approaches (Finkel 2009). As a relevant example for remote sensing, soil moisture estimation for large scaled areas has been done with the help of the cosmic-ray

neutron sensing (CRNS). In this alternative system, a large scale-based monitoring can do with two major advantages that include one probe measurement and necessary depth of root soil penetration (Stevanato et al. 2019). In addition, geophysical imaging can be used for cereal rust epidemiology tracking that is followed by the satellite-based remote sensing. In this system, potential rust outbreaks can be eliminated before spreading. Jinya et al. (2018) have developed a monitoring system which detects the different developmental stages of wheat yellow rust via captured spectroscopic imaging system. Thus, a centimeter resolution can be provided by multispectral Unmanned Aerial Vehicle (UAV) platform with low cost.

Phenotyping can be integrated with metabolome and next generation based transcriptome analysis (Patt et al. 2019). Considering the plant genomes, experimental design for unraveling the reliable phenological alterations is the first important step at the onset of monitoring and acts as a key role at the beginning of monitoring process. Due to the flexible genome structure and environmental effects, it is not easy to detect the defined phenotype in each time. So, in the basis of phenotyping, experimental set up should be designed according to these changeable parameters. However, several modern tools have been set either in controlled or stress-prone conditions, and impact of monitoring can only be achieved by high-throughput identification.

Recent conditions of plant phenotyping systems will be discussed on the basis of their applicability, necessity, and limitations level. However, deep phenotyping analysis is under the pressure of synchronous measurement, which also causes a time limitation for detection of results. It might be foreseen as little bit utopic to select the distinctive phenotype at single nucleotide level due to the multiple gene control and complex nature of genome. From this point of view, combined disciplines may provide promising tools and accurate automatic detection. Under the next headings, high-throughput imaging technics will be reviewed in order to screen facilities and agronomical imaging properties.

18.2 Phenotyping Systems

With the expanded plant breeding efforts, manual plant screening systems have been improved for multiple automated selection. Scanalyzer3D is a fully automated high-throughput screening system and it was presented for plants like maize, rice, *Arabidopsis*, poplar tree, barley, and wheat in greenhouse (http://www.lemnatec.de/scanalyzer_gh.htm). This screening system is able to 3-dimensionally screen up to 4000 plants per day efficiently and precisely. Scanalyzer3D can be sampled for a complete, reproducible, and nondestructive analysis of free of subjective influence. As a nondestructive system, it has the ability to measure the shoot growth and physiology using RGB (red-green-blue) cameras, fluorescence, and hyperspectral imaging by quantifying the basic geometric properties of the plant. With Lemnatec Scanalyzer3D, Mazis et al. (2020) have evaluated the two oak (*Quercus)* seedlings according to their plant height, projected leaf area (LA), plant/canopy width, ConvexHull, and plant aspect ratio and assessed their response to a dry-down period

under controlled environment. Also, same system has been used to screen the different rice (*Oryzae*) ideotypes before genome wide association. This high-throughput platform containing two 5-megapixel RGB/VIS cameras performed individual plant monitoring during 18 days (Avi et al. 2016). In addition to the normal growth conditions, Golzarian et al. (2011) have phenotyped diverse cereals under salinity conditions via Lemnatec Scanalyzer3D. To dissect the drought stressed 16 German two-rowed spring barley cultivars and two parents of a DH-mapping population (cv Morex and cv Barke), Lemnatec Scanalyzer3D has been used in additional image analysis (Chen et al. 2014).

Beside these, Apogee SI is a precision infrared radiometer and it measures the subject's surface temperature without physical contact (http://www.apogeeinstruments.co.uk/). In this way, canopy temperature can be easily detected by infrared sensors of system. Solar radiation, humidity, precipitation, and wind are other measurable parameters and they are transferred to analyze data. Also, one of the improved versions of Apogee WS-100 monitors can detect water usage during agricultural applications. The station can be configured with a cell modem to transmit data wirelessly where cell phone reception is available. Bai et al. (2016) used Apogee sensor in the field for phenotyping soybean and wheat. From another aspect, crop water stress index—CWSI is a means of irrigation scheduling and crop water stress quantification based on canopy temperature measurements and prevailing meteorological conditions (Payero and Irmak 2006). However, infrared sensor technology has appeared in the early 1930s with advancement of sensor systems. Todays' infrared detection systems have upgraded probes which can provide accurate and quick detection of leaf canopy temperature by transmitting the thermal distribution of the surface into a visual colored images. Today, this technology has been applied for large scale thermal imaging of fields. In this context, plant temperature is an important indicator of plant water status because stomata close in response to soil water depletion causing a decrease in water uptake and an increase in leaf temperature. Precise measurements particularly canopy temperature are required. Apogee infrared radiometers have been used by Dr. Suat Irmak from the University of Nebraska, in his CWSI research. As another Apogee application, canopy temperature has been measured in upland cotton genotypes for determination of drought adaptive traits (Thompson et al. 2018). Thereafter, as another approach, 3D scanning has been used for maize leaf samples canopy temperature. In this quantification method, FastSCAN hand scanner measured the 3D maize plant heights, stem diameter and results showed a good correlation with conventional methods (Ma et al. 2019). 3D individual-based model called FlorSys is functional on predicting the weed growth and development under daily weather conditions. Additionally, imaging technics, which run under the automated systems, are given in Table 18.1.

Digital imaging platforms are aimed to improve the detection of different plant parts during early and late plant growing stages. They can track even small changes before they were reflected on whole plant level. In non-invasive phenotyping, laser scanning, terrestrial laser scanning, or structured light technologies have met the gradual measurements of single, multiple, or field-based level and link between the

Table 18.1 Examples of different software and systems used for plant phenotyping

Platform	Web source	Function	References
GiA roots (software)	http://giaroots.biology.gatech.edu/	2D root system architecture analysis	Galkovskyi et al. (2012)
HTPheno (software)	http://htpheno.ipk-gatersleben.de/	Supplies a flexible and adaptable ImageJ plugin	Hartmann et al. (2011)
HYPOTrace (software)	http://brie.cshl.edu/~liyawang/HYPOTrace/	Automatically extracts growth and shape information from electronic gray-scale images	Wang et al. (2009)
IAP (software)	http://iap.ipk-gatersleben.de	Supporting large scale image analysis tool for images obtained in different spectra	Klukas et al. (2012)
KineRoot (software)	http://plantscience.psu.edu/research/labs/roots/methods/computer/analysis-of-real-time-root-growth-with-kineroot-program	MATLAB-based program that tracks root movement and growth from a set of sequential photographs	Basu et al. (2007)
LEAF GUI (software)	http://www.leafgui.org/	Analyzing leaf vein structure	Price et al. (2011)
LEAFPROCESSOR (software)	http://www.iff.fraunhofer.de/de/geschaeftsbereiche/biosystems-engineering/forschung/leafprocessor.html	Analyzing leaf shape parameters	Backhaus et al. (2010)
Lamina2Shape (software)	http://www.sciencedirect.com/science/article/pii/S0168169909002191	Allows an efficient processing of lamina images to analyze their shape	Dornbusch and Andrieu (2010)
LeafAnalyser (software)	http://sourceforge.net/apps/trac/leafanalyser/	Leaf shape variation analysis	Weight et al. (2008)
LeafJ (software)	https://bitbucket.org/jnmaloof/leafj/wiki/home	Measure petiole length and leaf blade parameters	Maloof et al. (2012)
PlaRoM (software)	http://dx.doi.org/10.1071/FP09167	Investigate root extension profiles in various growth conditions	Yazdanbakhsh and Fisahn (2009)
RootReader2D / RootReader3D (software)	http://www.plantmineralnutrition.net/rootreader.htm	Image processing and analysis tools for root system images	Clark et al. (2011)
RootTrace (software)	http://sourceforge.net/projects/roottrace	Measuring primary root lengths across time series and count emerged lateral roots	French et al. (2009); Naeem et al. (2011)

(continued)

Table 18.1 (continued)

Platform	Web source	Function	References
Rosette tracker (software)	http://telin.ugent.be/~jdvylder/RosetteTracker/	Analysis tool for evaluation of plant-shoot phenotypes	De Vylder et al. (2012)
SmartRoot (software)	http://sourceforge.net/projects/smartroot/	Quantitative analysis of root growth and architecture of complex root systems	Lobet et al. (2011)
GROWSCREEN-root (software)	http://www.fz-juelich.de/ibg/ibg-2/DE/Methoden_JPPC/GROWSCREEN_root/_node.html	Automatic analysis of root architecture	
WinRHIZO Tron (software)	http://www.regent.qc.ca/assets/winrhizotron_about.html	Analyze images coming from minirhizotron underground video camera systems	
GROWSCREEN 3D (system)	http://www.fz-juelich.de/ibg/ibg-2/DE/Methoden_JPPC/methoden_node.html	Chlorophyll-fluorescence measuring	
GROWSCREEN-Rhizo (system)	http://www.fz-juelich.de/ibg/ibg-2/DE/Methoden_JPPC/GROWSCREEN_rhizo/Rhizo_node.html	Non-invasive quantification of root and shoot growth	
PHENOPSIS (system)	http://bioweb.supagro.inra.fr/phenopsis/	Acquire the leaf size	Granier et al. (2006)
PlatScan 3D meshes (system)	http://www.plantphenomics.org.au/node/157	Analysis of vegetative growth phenotypic parameters estimation and plant organs tracking	Paproki et al. (2012)
SPICY (system)	http://www.bioss.ac.uk/people/yu/spicy/	3D reconstruction of the plant canopy and statistical features derived directly from RGB images	Gerie van der Heijden et al. (2012)
TraitMill (system)	http://www.cropdesign.com/tech_traitmill.php	Automated high-resolution phenotypic evaluation of crop performance	Reuzeau et al. (2006)
VPhenoDBS	http//medbio.cecs.missouri.edu/VPhenoDBS	Allow simultaneously query phenotype data by image example sequence, ontology, genetic and physical map information, and text annotation	

plant structure with its functions or responses under different growth regimes. Photon System Instruments (www.psi.cz) write protocols specific paths and system can be maintained for true imaging and high-density automatic measurements during experiments. Every samples are tagged with a barcode and proceeded their targets. During this small trip, each plant queried according to the uploaded parameters which were written as scripts on system software.

Beside of all, seeds play a key role during plant development and it has become an expected product of each generation. Moreover, a seed is the core plant part during plant breeding and it determines the progenies. Seed shape is an important parameter that is basically calculated with seed length, width, and area. Tanabata et al. (2012) have developed the SmartGrain software for high-throughput detection of seed shape. Outline of seeds has been automatically recognized from digital images and several shape parameters such as seed length, width, area, and perimeter length are calculated.

18.3 Plant Phenotyping Projects

Phenotyping cannot be defined not only a simple monitoring process of a plant, but also it has a sufficient capacity to clarify the communication signals among different plant parts. By increasing demands on association mapping studies under fast climatic changes and population bottleneck, it is forced to re-understand the architecture of plants much better (Furbank and Tester 2011). Practically, population and individual phenoscreening can be done with high and medium throughput systems. Thus, it is aimed to select the best practical individuals or population for future breeding prospects.

So, there is a need for multi-disciplined infrastructure and funds for measuring, processing, and analyzing the data. European Plant Phenotyping network (EPPN) is one of the organizations which is structured to develop plant phenotyping linkages between experienced institutes and enables a core community for other joint participants (eppn2020.plant-phenotyping.eu/EPPN). With this platform, common phenotyping protocols can be developed and reference methodologies supported with experimental approach will be released for common research attempts.

PhenoCrops project has been funded by EU EFRE (005–1105-0035) for 3 years period and planned to establish the infrastructure for both individual high-throughput and population based medium throughput phenotyping in the greenhouse conditions. Also, large scale plots were screened by flying tools to provide a nondestructive approach. CROP.SENSe.net project supported by German BMBF (0315531C) (http://www.cropsense.uni-bonn.de/Forschung/teilprojekte/d1) has been established to measure the structural and functional traits related to yield and population efficiency under greenhouse and field experiments by applying the sensory techniques for high-resolution temporal and spatial control of plant stocks. For monitoring the up and down parts of sugarbeet, non-invasive imaging techniques applied to understand the root architecture and leaf development status under pathogens conditions. Also, hyperspectral reflection measurement has been

done for modeling the leaf canopy structure under the continuously changing growth conditions which is not quite possible to measure for different time scales.

As another example, field phenomics is the main site for the project "*A field-based high-throughput phenotyping platform for plant genetics*" and it is funded by the National Science Foundation Plant Genome Research Program until the middle of the 2015 year. Another project that is titled with "*High-throughput Phenotypic Characterization and QTL Analysis of Stress Adaptive Traits in Wheat*" got almost two hundred thousand US dollar support from "Monsanto Beachell-Borlaug International Scholars Program." There are several teams coming from different Universities (Kansas State University, University of Arizona) and Research Institute (USDA-ARS). Large scale phenotyping requires both genome wide molecular markers and accurate measurement system for screening of population or individuals (fieldphenomics.org/research/).

There are other funding centers, Biotechnology and Biological Sciences Research Council (BBSRC) is one of them and it is centered in Nottingham University, UK (www.nottingham.ac.uk/research/groups/cvl/projects). The 4D Plant project is supported by a grant from the BBSRC and aimed to model canopy movement and dynamic photosynthesis in rice and wheat populations. Additionally, reconstruction of 3D modeling for root and shoot imaging in rice and wheat plants has been investigated. As a second, LeMuR: Plant Root Phenotyping via Learned Multi-resolution Image Segmentation project is also supported by the same foundation and serves a machine learning platform for root architecture imaging. In the micro-term, an integrable root analyzing investigated with the DEEPER project that was supported by grant from US Department of Energy. This project has been aimed to improve the crop and biofuel productivity under drought stressed environments for maize plants. Moreover, region based classification of soil will be provided to manage nitrogen use efficiency in maize under field conditions. With this point of view, monitoring root architecture and responses via high-throughput tools can give direction to the field and greenhouse based phenotyping studies.

18.4 Application Fields of 3D Plant Phenotyping

Crop water stress is appreciated by analyzing the root zone soil water depletion. Soil water content measurement of the deeper layers (from 40 cm to 2 m at 20 intervals) of soil was determined by using a neutron probe (CNC-503DR, Beijing HeAn Company, China) (Liu et al. 2007). On the other hand, plant water status is a key metabolic indicator before each irrigation step either in the field or in glasshouse and several methods have been routinely used to measure the leaf water potential. In the past, this process was with labor-sensitive methods to see the changes in the leaf tissue water status and it was dependent to the equipment sensitivity. Also, following the movement of water particles, both in xylem and phloem can give clues about the placement and amount of internal water content. The basic methodology has been occurred in a pressure chamber and the critical step was bagging stage of tagged leaf which is taken up with its shoot tissue. Earlier, a simple method was used by

Schaefer et al. (1986) with a Wescor HR-33 T dew point hygrometer in growth chamber experiments. By the nuclear magnetic resonance (NMR) of water protons, quantification of water particles in xylem and phloem vessels achieved by Magnetic Resonance Imaging (MRI) (Windt et al. 2009). In the second method, more than one individual plant can be added to screening by using the power of water proton movements in the cell.

One of the other morphological 3D imaging techniques is high-resolution X-ray computed tomography (HRXCT) and it has been used for ex vivo scanning of plant tissues at cellular level and in vivo scanning of morphological traits. This method is firstly used in 1970s in medical diagnosis. In time, it is well adapted to analyze plant structures such as Arabidopsis hypocotyls and flowers (Dhont et al. 2010) and to follow the potato tuber development (Hagenmüller et al. 2007). In spite of the advantages of the HRXTC, there is a restriction about object focusing and if the distance between the object and X-ray tube increases, magnification and resolution fall down. This is also valid for the extended scanned area. In the field research, it is important to note that this type of problem can be alleviated by individual sample measurement using connected zoom-in scans to see all results in a merged frame. Today, it is performed with terahertz waves in the field-based studies. Water strongly absorbs the terahertz radiation. Breitenstein et al. (2011) set up a method that photomixer converts the optical beat signal with a non-destructive way.

Plant root systems have complex architecture and usually root–soil and root–microbe dynamic interactions could not allow us to visualize a clear 3D image (Dunbabin et al. 2013). In practical, there are some hindering factors in front of the root phenotyping studies and solution priority should be taken to fullfill the phenotyping gap and its further applications for large scale experiments. Topp et al. (2013) have reconstructed a semiautomated 3D phenotyping system and recognized 89 quantitative trait loci (QTLs) and five impacted genome regions in rice biparental mapping population Bala × Azucena. In their estimation system, in silico computer simulations and resin root modeling systems have been validated for both 2D and 3D screens, and relationship calculations between traits and root shape have been accomplished by statistical programs. Root allocation is accepted as a limitation due to the potential differences observed for roots which were grown in the top and deep soil levels (Yazdanbakhsh and Fisahn 2009).

Transition from micro to macro-phenotyping tools for understanding the complexity of phenotype has been accelerated after large scale breeding practices. Comprehensively, microscopic high-throughput imaging has emerged from microCT laser, paraffin sections, or hand cutting fluorescently colored images. Considering the macro-level plant parts, shoot, and root, both anatomical and physiological responses have been important and needed to measure with a serial imaging technique (Zhao et al. 2019).

18.5 Remote Sensing Efforts

Phenological and agronomical crop traits depend on density, plant type, plant growth stage, biomass and these parameters need a large scaled scan with high resolution. Also, detection of flowering time and making leaf surface analysis in field experiments accelerated the remote sensing methods and their subsequent use in agricultural practices. Currently available sensors such as digital red-green-blue (RGB) cameras, near infrared (NIR) sensors combined with satellite based crop monitoring have been used in winter and spring pea breeding in USA (Quirós et al. 2019). For mapping the irrigated wheat fields, combined satellite remote sensing tools were used by Er-Raki et al. (2010). For tracking soil content, in the Southeastern side of Turkey, NIR spectroscopy technique has been utilized to assess the salinity affected areas (Bilgili et al. 2011).

As a nondestructive approach, remote sensing stands on the first row during field-based selection and crop breeding and detection can be done at altitudes of meters to kilometers with high-resolution multispectral imaging infrastructure. However, an airborne derived spectral indices can help to capture the daily/weekly field images, environmental changes such as rain conditions, snowy weather may negatively affect the spectral image resolution (Tattaris et al. 2016). In Australia, a hyperspectral fingerprinting of grapevine field has been performed with a photographic airplane circles. Also, inner qualities of plants are made with a special software developed by Fraunhofer Institute for Factory Operation and Automation IFF in Magdeburg. The data coming from the specific wavelength spectrum via the camera chip system recorded as signals and processed as pixels for transferring the data into a biochemical fingerprint (www.sciencedaily.com/releases/2013/09/130903123141.htm). Before data analysis, reference plants are used for calibration and ordering the true fingerprinted constituents. In addition to the biochemical property detection, this system can be used for disease detection, water use, irrigation times, nutritional deficiencies of crops in the field.

Application of field phenotyping methods may provide specific solution for solving the phenotyping bottlenecks. In this case, remote sensing can serve an early imaging of crops in the field and advanced monitoring for complex vegetation. Using with appropriate temporal and spatial scales, every agronomically relevant trait can be captured with a cost and time effective way (Li et al. 2017). At this point, hyperspectral imaging is one of the sensor based systems which is mostly combined with remote sensing technology and measures the internal content of a plant such as nutrient and grain protein content, weed detection, pathogenic symptoms, and senescence (Roitsch et al. 2019). Both hardware and software combinations can scale up the experimental conditions in remote sensing approach under field conditions. In the core of every sensor system, image data integration into other omic tools can facilitate the reliability and availability of results. Hence, a universal protocol can be maintained for image processing and data analysis. As an example, a combined method was used to detect the plant height QTL/s in diverse maize inbred lines with the help of remote sensing monitoring which used the unmanned aerial vehicles (UAV) for field phenotyping (Wang et al. 2019).

Biomass accumulation in different plant parts is important for functional biology and detecting multiple parameters at synchronous level needs to be an extended sample amount with labor-sensitive work. On the basis of remote sensing, two-dimensional modeling of above ground area used for plant biomass estimation in the field (Lamb and Brown 2001). In remote sensing detection, there are also optical, thermal, microwave-based satellite sensors which were used to collect the data at global term during agricultural production. Beyond of all, remote sensing efforts can be used to achieve the sustainable agricultural production in agronomically important crops such as wheat, barley, maize, lentil, common bean, etc. To support the sustainability in agriculture, smart fertilization management has been applied to avoid the plant nutrient deficiencies under field conditions (Yousfi et al. 2019). Also, the status of soil-water content, monitoring drought prone lands, harvesting dates, estimation of farmland, and quantification of crop yield can be estimated with remote sensing technology in real time.

18.6 National and International Status of Next Generation Agricultural Technologies and their Reflections

The rapidly changing information technologies have caused countries to enter a digital transformation. With the fusion of agriculture and information technologies, many new technical terms have begun to be heard in daily life. Components of this technology include basic systems such as global positioning systems, geographic information systems, remote sensing, etc. Today, the environmental impact of agricultural production inputs and the pressure to reduce input costs are increasing with the advancing technology. In these respects, as the smart agricultural areas, image perception and phytobiological information, remote sensing with satellite and air tools, talking herbs and talking fruit application, machine learning in agriculture, sense and information management in plant protection, drug application techniques in plant protection, information technology in greenhouse based agricultural activities, plant screening under large environments, breeding true genotypes with the support of sensing systems are important subjects. In this frame, both R&D and smart agricultural practices doubled between 2015 and 2020, reaching to 26 billion dollar.

According to the European Agricultural Machine Association, there is an urgent need for planning the agricultural activities under the umbrella of Agriculture 4.0. Thus, the power of competition of EU with other developed countries such as USA, Canada, New Zealand will be strengthened. After 2015, in Turkey, three publicly funded projects were performed for increasing the smart agricultural practices. So, sensor and drone technologies gained momentum both for farmers and researchers closely studying in agricultural biotechnology. In this context, following the drought and yield based studies carried out for different crops. As in the countries which use the cloud system to save the agricultural data via www platforms are also used in Turkey. To provide a sustainable communication network, digital application can be downloaded and used for watering, determination of harvesting times, or following

the climatic conditions by the farmers and research centers. Both public and private sector have R&D laboratories and projects that were related to following, selection and detection of true genotypes are increasing day by day. There is still need to develop cheaper and accurate feasible detection systems in the agricultural biotechnology and farming sector. Due to this, phenological assessment needs to be improved and integrated to agricultural practices. Hence, starting with right genotype may support the traits of interest such as yield and stress resistance and may prevent the time-consuming efforts via smart approach including cheap technologies.

18.7 Conclusion

Typically, root and leaf tissues are the most important and active parts of a plant, and there is a close cooperation between them beyond our visible sights. In the first quarter of twenty-first century, innovations will redesign our demands on plants resources. New plant phenotyping resolutions emerged from the necessities, which were either related to the changes in appearance of a plant or cellular responses of some specific plant parts. To provide a sensitive detection, robots donated with the sensitive sensor features will be used to accelerate the measurement process. Also, deposition of the huge amount of giga base level data which come from each individual sample can be managed within the high-throughput smart computers. Current plant modeling systems will let their seats to a wider field-based experimental set up. Under several selection pressures, genome and phenome bottleneck is still to continue to reflect their effects on plant breeding studies. The most important challenge will be the elimination of genotypes with small phenotypic changes which could not be distinguished in conventional phenotyping efforts. New experimental pipelines will support plant sciences by providing new insights for benchtop and field studies. At this point, either it is established in the stress-prone or in non-stressed growth fields, revealing the internal and external effects on plant development, will help to shade the light on and make more visible our sights for field-based crop management. In the near future, studies on plant biology will be strengthened with these auxiliary tools that is improved to solve the complexity of plant responses. Seemingly, it will be possible to monitor large scale plant populations for ideal traits of interest by means of improvements in plant biology, system engineering, and computational software. Suitability of the measurement techniques at physiological, biochemical, and morphological level has been approved by the ability of sample penetration and volumetric density with the high resolution. Recent improvements in imaging methods effectively enable us to see the big and deep picture of plant. New high-throughput plant phenotype data as well as the increasing volumes of genotypic diversity data has become a pre-request. It will be possible with the association of this diversity data with heritable phenotypes which will likely drive genome database development over the coming years.

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RNA Interference Technology as a Novel and Potential Alternative for Plant Improvement

19

Ranjeet Kaur, Arundhati Ghosh, and Manchikatla V. Rajam

Abstract

RNA interference (RNAi) is a versatile and powerful technique, which is routinely being used to down-regulate specific target genes in different organisms, including plants. It employs small RNA (sRNA) sequences, either short-interfering RNAs (siRNAs) or microRNAs (miRNAs), to recognize the complementary mRNA sequences in the host genome, leading to their targeted silencing via RNA induced silencing complex (RISC). The RNA silencing tool has been highly beneficial in functional validation of genes by reverse genetics approach. It has been extensively used in crop improvement, including biotic and abiotic stress tolerance, yield enhancement and nutrient quality improvement. Apart from these, it has contributed towards development of plants with healthier oils, delayed ripening, male sterility, modification of flowering time and flower colour, enhancement of secondary products, and removal of allergens and toxins. In this chapter, we have provided a detailed literature on the use of RNAi technology as a novel and potential alternative for plant improvement. However, we have not covered the information on the use of RNAi for biotic and abiotic stress tolerance as there are a number of recent and comprehensive reviews on this topic.

Keywords

RNA interference · Small RNAs · Small interfering RNA · MicroRNA · Crop improvement · Gene silencing · Functional genomics

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Abbreviations

amiRNAs	Artificial microRNAs
miRNA	microRNA
RISC	RNA induced silencing complex
RNAi	RNA interference
siRNA	Small interfering RNA
sRNA	Small RNA

19.1 Introduction

Evolution of humans on planet earth has been a dynamic process that has included the domestication of plants and animals for their survival. Over the years, several practices such as breeding have aided in the improvement of the commercial varieties for the purpose of satisfying the ever-increasing requirements of food and nutrition. Green revolution of late 1960s witnessed the mammoth produce of wheat and rice, which were selected for the beneficial traits of high yield and short height, with the supplementation of fertilizers. This sufficed the hunger of several developing countries, including India, and was regarded as a milestone in achieving agricultural sustainability in the past. However, global demand for food has risen sharply with the rapid increase in world population, which is expected to reach the 9.7 billion mark by 2050. This has led to the development of various strategies for crop improvement to feed the surplus and meet the demand for food by the teeming millions. According to the estimates of US FAO of United Nations, nearly 1.3 billion tonnes of food which is about one-third of all food produced for human consumption is lost or wasted annually to natural calamities and man-made disasters. This is equivalent to a loss of 750 billion US dollars each year and threatens the economic as well as food security of the nations. Apart from hunger, several countries face an acute problem of malnutrition, which refers to a lack of consistent access to food that diminishes dietary quality, disrupts normal eating patterns, and can have negative consequences for nutrition, health and well-being (McGuire 2015; Garg et al. 2018).

Genetic engineering has provided promising answers to the problem of crop yield losses due to environmental and biotic factors along with solutions to the nutrient quality improvement. One such novel strategy that has taken the world by storm and also shown pathway to ensure global food and nutritional security is the phenomenon of RNA interference (RNAi). RNAi has emerged as the novel technique for crop improvement (Rajam 2020) and has been found to be successful in targeting specific genes to yield enhanced versions of the existing varieties. New avenues for the improvement of plants have opened up with the implementation of RNAi strategies to crops and they provided better solutions to attain self-sufficiency in the field of agriculture.

Gene silencing is an interesting phenomenon that has intrigued researchers over the decades. It has been reported in a wide variety of living organisms as a part of their defence and regulatory mechanisms that govern the gene expression in a specific pattern. RNAi is a conserved method of gene silencing across the eukaryotic systems and is reported to be an ancient immunity mechanism against virus attack. In this process, small RNA (sRNA) sequences of 21–24 nucleotides bring out the degradation of the cognate mRNA before it can synthesize its target protein (Rajam 2020). These sRNAs are known to control several genetic and epigenetic processes including growth, development, reproduction and defence responses (Borges and Martienssen 2015).

RNAi controls the gene function at post-transcriptional level and plays a key role in numerous vital biological processes like cell growth and proliferation, tissue differentiation, genomic stability, heterochromatin formation, transposon activity and epigenetic modification (Pareek et al. 2015; Li and Zhang 2016; Mamta and Rajam 2018; Wallis et al. 2019). It is an indispensable mechanism for an organism as any alteration in the normal functioning of RNAi activity can lead to several disorders such as neurological, cardiovascular as well as cancer (Wilson and Doudna 2013). RNAi has immense potential for changing the outlook in the field of agriculture and crop production. It has been named as 'the Break-through of the Year' by Science in 2003. The discoverers of RNAi, Andrew Fire and Craig Mello, received Nobel Prize in Physiology or Medicine in 2006 for this important breakthrough discovery. RNAi has been widely used to combat biotic and abiotic stresses, improve yield and nutrition of food crops, increase fruit shelf life and secondary metabolites. Apart from being extensively applied for generating designer crops, RNAi technology has also been used for targeted silencing of desired genes to understand their role in the context of plant growth, development and stress management. Amidst all the talks on the acceptability of genetically modified (GM) crops, the transgenic plants harbouring sRNAs have been considered much safer for human consumption by FDA (Kamthan et al. 2015; Rodrigues and Petrick 2020). Thus, RNAi can be safely declared as a potential alternative to crop improvement with wider applications and enhanced consumer reach.

In this chapter, we have covered the RNAi-mediated gene silencing mechanism and its use in functional genomics and various potential applications in the field of agriculture including yield improvement, nutrient quality improvement and other important applications such as enhancing fruit shelf life, secondary metabolite production, reduced allergenicity, removal of toxic compounds and developing seedless fruits among others. Further, we have also discussed on the prospects of RNAi as a novel and powerful alternative to crop improvement strategies of the future world. However, we have not covered biotic (Chauhan et al. 2017; Basso et al. 2019; Celik et al. 2020) and abiotic (Megha et al. 2018; Xu et al. 2019; Kaur et al. 2019; Dutta et al. 2020) stress tolerance as there are number of recent and comprehensive review articles on these aspects.

19.2 RNAi and Gene Silencing

Silencing of genes has been an important part of research programmes and has captured the attention of the global scientific community to drive their goals. However, homology-dependent gene silencing at post-transcriptional level was first reported in plants about three decades ago. Transgenic petunia plants over-expressing the chalcone synthase (*CHS A*), a key enzyme in anthocyanin biosynthesis pathway showed an unexpected variegation as well as loss of pigmentation in flowers (Napoli et al. 1990). This peculiar down-regulation of the endogenous gene as well as the transgene in the transgenic plants was named as co-suppression. It was later renamed as post-transcriptional gene silencing (PTGS) as it involved events that occurred after the transcripts were formed. A similar phenomenon, referred to as quelling, was reported in the fungus *Neurospora crassa*, and it displayed transgene-induced gene inactivation when it was transformed with homologous sequences of the candidate genes (Romano and Macino 1992; Pandit and Russo 1992). In another report, sense and antisense RNA were found to be equally effective in silencing of targeted gene in *Caenorhabditis elegans* (Guo and Kemphues 1995). The exogenous application of a double-stranded RNA (dsRNA) targeted for the *unc22* gene was found to be at least ten times more effective in shutting off the gene function than the sense or antisense RNA alone in *C. elegans* (Fire et al. 1998). Similar results were also observed in the silencing experiments conducted on trypanosomes (Ngo et al. 1998) and flies (Kennerdell and Carthew 1998), and the associated mechanism was named as RNAi.

RNAi is a conserved and naturally occurring phenomenon of PTGS that involves short dsRNA sequences, which binds to the homologous part of its target gene and degrades its transcripts, rendering it non-functional. The key driving molecules of RNAi are the non-coding dsRNA sequences, which are cleaved by RNAase III enzyme Dicer to produce sRNAs (Rajam 2020). In plants, two major sRNAs are present, viz., microRNAs (miRNAs) and small interfering RNAs (siRNAs) (Pareek et al. 2015; Rajam 2020). One of the two strands of the mature sRNA, known as the guide (antisense) sRNA, is incorporated into special effector proteins called Argonaute (AGO) to form the RNA induced silencing complex (RISC) (Wang et al. 2019). The other strand, known as the passenger (sense) sRNA is degraded in the process. The activated RISC now hunts for its target mRNAs bearing sequence complementarity to the loaded guide sRNA and causes the silencing of the target gene (Borges and Martienssen 2015). A very small region of highly conserved heptametrical sequence bearing complementarity to the target mRNA is known as seed sequence or the seed region of the sRNA. If the sRNA-mRNA binding is perfect, it results in site-specific cleavage of the target transcript, whereas if the binding is near perfect due to a few mismatches, then it results in translational repression of the gene (Tyagi et al. 2019; Kaur et al. 2020).

Although the mode of action remains the same for both, the biogenesis of siRNA and miRNA involves subtle differences (Fig. 19.1). The siRNA is generally formed from the processing of dsRNAs introduced due to the invasion of viruses, transposons, transgenes, aberrant mRNAs, inverted repeats, etc., while the miRNAs

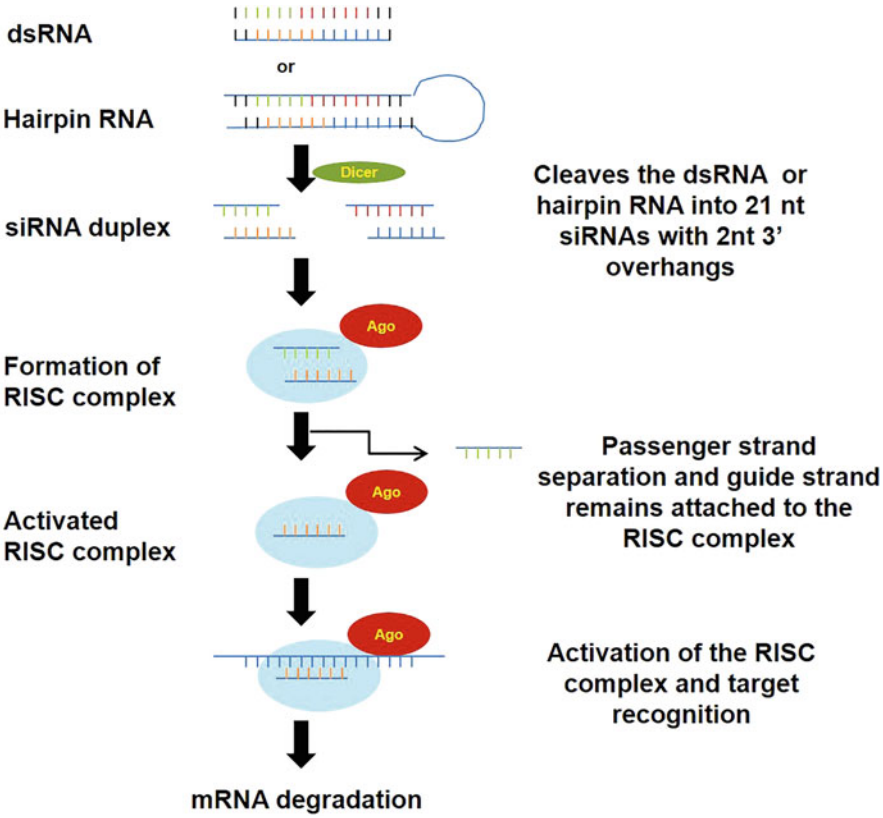

Fig. 19.1 Biogenesis and mechanism of action of small RNAs in plants: (**a**) siRNA biogenesis and mechanism of action in plants (adapted from Yogindran and Rajam 2015); (**b**) miRNA pathway/biogenesis in plants (Adapted from Kaur et al. 2020)

are of endogenous origin, transcribed from nuclear-located *MIR* genes, preferably by RNA polymerase II (Rajam 2020). The primary miRNA (pri-miRNA) transcripts are then processed into stem-loop structured precursor miRNA (pre-miRNA) by the microprocessor enzyme complex comprising of Dicer like-1 (DCL1) and its accessory proteins (Pareek et al. 2015; Wang et al. 2019). The pre-miRNA is further cleaved by DCL1 to form miRNA/miRNA* duplex, which is transported by the exportin-like protein, HASTY, to cytoplasm, where it is loaded onto the RISC for targeted gene silencing (Borges and Martienssen 2015; Kaur et al. 2020).

In order to accomplish stable gene silencing in plants via sRNAs, scientists prepare RNA silencing constructs. These usually comprise partial target gene sequence (200–300 bp) in two orientations, viz., sense and antisense, separated by a small intronic spacer DNA (Rajam 2020). This design enables the formation of a hairpin (hp)-like structure due to the complementary base pairing between the sense and antisense strands. The hp. RNAi constructs are spatio-temporally controlled by an appropriate promoter, either a strong constitutive promoter like CaMV35S or

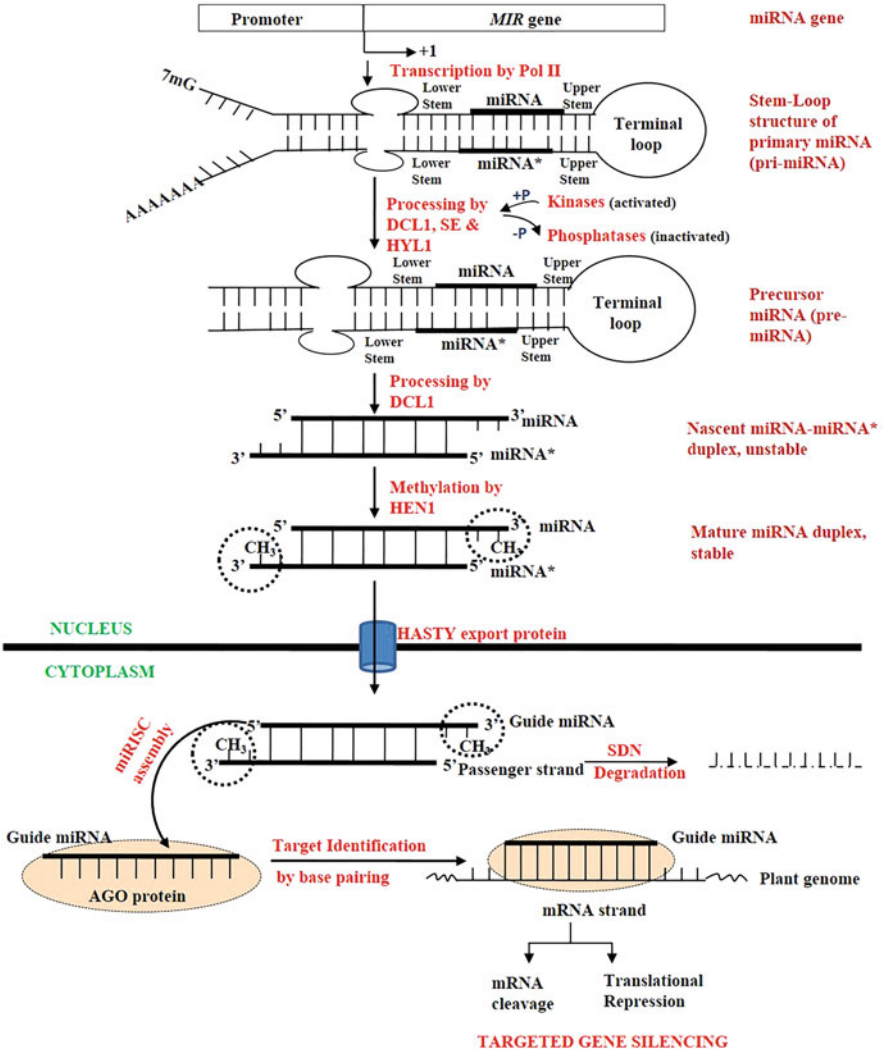

Fig. 19.1 (continued)

inducible promoter, to drive the formation of the dsRNA in vivo (reviewed in Karan et al. 2007). The dsRNAs are then cleaved by Dicer, leading to production of multiple siRNAs, which direct gene silencing in plants (Yogindran and Rajam 2015). On the other hand, miRNAs can be synthesized artificially by over-expressing the endogenous pre-miRNAs in transgenic plants. This artificial miRNA (amiRNA) strategy results in synthesis of short duplex sequences of amiRNA/amiRNA*, which are then processed and recruited by AGO proteins to

execute RISC-mediated targeted gene silencing in plants (Yogindran and Rajam 2016).

19.3 RNAi and Functional Genomics Studies

RNAi is regarded as an important tool to down-regulate the expression of desired gene(s) in different systems, including mammalian and plant systems, often achieving nearly 90% success in most of the cases (Tenea and Burlibasa 2012; Hammond 2005). Targeted suppression of the candidate gene has enabled the researchers to explore the extent of effect that gene has on cellular function. Besides, it also has the potential to be used in other applications, such as in the therapeutic and clinical analysis of the drugs (Tenea and Burlibasa 2012). The study of functional genomics is an integral part of molecular biology as it involves understanding the functions of novel genes.

RNAi has been extensively used to unravel the role of numerous genomic sequences and plays an important part in the area of functional genomics (reviewed in Mustafiz et al. 2016). The study basically involves targeted silencing of the desired gene(s) by use of RNAi technology and then examining the morphological changes in the resulting transgenic lines harbouring the RNAi construct(s). These 'loss of function' mutants provide useful tools to analyse the functional aspects of the gene, rather than the tedious conventional methods for mutant production, such as homologous recombination and random mutagenesis (Karan et al. 2007). In the conventional approach, it takes several generations of crosses to analyse the phenotypic effects of a desired gene in the backdrop of the marker allele. On the other hand, the use of RNAi silencing technique to functionally characterize a gene significantly reduces the time taken for its assessment if its gene sequence is correctly known (Tenea and Burlibasa 2012). The seed sequence has to be carefully chosen to match the desired gene so as to avoid any errors or off-targets during the homology-dependent down-regulation of the target mRNA (Sigoillot and King 2011). For instance, it has been observed that use of 3'UTR region as a part of the seed sequence results in targeted gene silencing and it also produces dominant loss-of-function mutations in polyploidy plants (Ifuku et al. 2003; Miki et al. 2005). Hence, every RNAi experiment calls for strict vigilance in the initial design, independent validation of screening the mutants and proper interpretation of the results (Sigoillot and King 2011).

Generally, there are two major genetic approaches to identify a gene function, viz., forward and reverse genetics. Forward genetics involves identifying gene mutations that disrupt the function or pathway being studied. On the other hand, reverse genetic approaches, such as RNAi, disrupt a gene with an unknown or suspected function to determine the effect on a function or pathway (Dorsett and Tuschl 2004). Functions of several genes were characterized based on RNAi-mediated gene silencing in agriculturally significant plants such as wheat, rice, maize, barley, cotton and tobacco, and studying its effect on phenotype of the transgenic plant (reviewed in McGinnis 2010; Abdurakhmonov et al. 2016; Mustafiz

et al. 2016). For instance, RNAi silencing of gene encoding the NAC transcription factor was shown to regulate senescence and improved grain protein, zinc and iron content in wheat (Uauy et al. 2006). The ornithine decarboxylase (*ODC*), a key gene involved in polyamine biosynthesis was silenced by RNAi in tobacco, and the transgenic plants displayed reduced levels of cellular polyamines, which in turn resulted in significant physiological and morphological abnormalities including small leaf size, delayed flowering, partial male and female sterility, reduced seed setting and viability. Such ODC knockdown lines also showed reduced abiotic stress tolerance, decreased chlorophyll and carotene content and poor in vitro regeneration response from leaf explants. Further, microarray analysis has revealed a genome-wide gene expression changes in response to lowered polyamine titers in an ODC knockdown line (Choubey and Rajam 2017). Down-regulation of *OsBADH2* by RNAi resulted in aromatic transgenic rice plants, validating its role as a negative regulator of aroma production in rice (Niu et al. 2008). Song et al. (2011) generated RNAi transgenic rice against transcription factor *OsNAC5*, which were less tolerant to cold, drought and salinity stress as compared to wild-type plants, indicating the regulatory role of *OsNAC5* in providing enhanced stress tolerance. Thus, the reverse genetics approach of RNAi can be successfully employed for the functional validation of genes with guided design and proper vigilance.

19.4 RNAi and Plant Improvement

RNAi has numerous applications for plant improvement, including yield improvement and nutrient quality improvement (Fig. 19.2). We have reviewed the studies encompassing this vast yet important field, with additional applications that aid in enhancing the overall improvement in the plants for the benefit of humanity.

19.4.1 Yield Improvement

The world today is facing a dearth of food crops in terms of the mouths that are to be fed. This is a direct outcome of a mammoth increase in global population, which has led to a sharp decline in the availability of the food resources. Apart from making stress tolerant crops, there is an urgent need to develop smarter crops with higher yields. In other words, high yielding crops can prove to be a ray of hope in feeding the teeming millions. RNAi has proved to be a potential alternative in producing high yielding crops by increasing the plant biomass and altering the plant architecture for enhanced production.

19.4.1.1 Increased Biomass

Plant biomass is the weight of living plant material that can be effectively used as a renewable source of energy. It is an indirect and efficient method of increasing the plant yield. One of the major ways of utilizing plant biomass is by removing lignin from the cell wall, since it is known that lignin is the major component, which leads

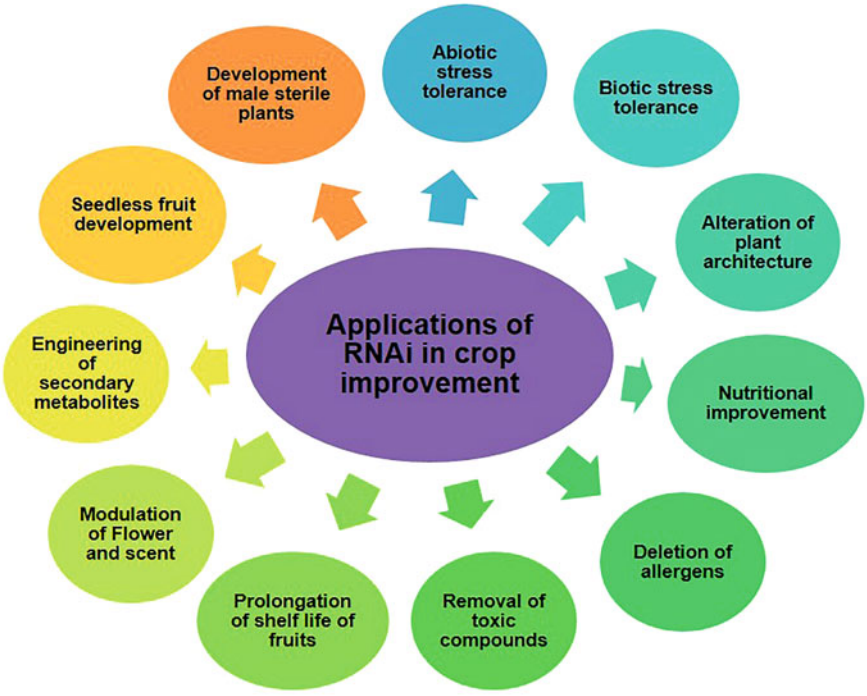

Fig. 19.2 Applications of RNAi in crop improvement

to biomass recalcitrance. The overall lignin content can be reduced by down-regulating the lignin biosynthetic pathway genes, but without compromising the mechanical strength of the plant (Hosseini 2019). Sugarcane is the major source of biofuel in the form of bioethanol. The biomass recalcitrance is a major issue in sugarcane. The down-regulation of the sugarcane caffeic acid *O*-methyltransferase (*COMT*) gene has shown to reduce the lignin content effectively (Jung et al. 2012). A moderate decrease in lignin (3.9–8.4%) can, in the one hand, reduce the recalcitrance of sugarcane biomass, and on the other hand, the plant performance was also not affected in controlled environmental conditions (Jung et al. 2012). Another study on sugarcane targeted the 4-coumarate: coenzymeA ligase (*4CL*) gene, which is the key enzyme for biosynthesis of phenylpropanoid metabolites, such as lignin and flavonoids (Jung et al. 2016). In the study, two full length *4CL* genes (*Sh4CL1* and *Sh4CL2*) were isolated and characterized but only one of these, *Sh4CL1*, confirmed as a major lignin biosynthetic gene. The field grown sugarcane with intragenic RNAi suppression of *Sh4CL1* resulted in reduced total lignin content by 16.5%, without reduction in biomass yield. They demonstrated for the first time that the intragenic precision breeding strategy can be utilized for better biomass production.

Recalcitrance to saccharification is a major problem for the production of biomass in alfalfa (*Medicago sativa* L.). The specific cytochrome P450 enzymes of the lignin

pathway were down-regulated by RNAi in transgenic alfalfa lines, which showed decreased lignin content and increased biomass production (Reddy et al. 2005). The seven enzymes independently down-regulated in alfalfa were cinnamate 4-hydroxylase (C4H); hydroxyl cinnamoyl CoA: shikimate hydroxyl cinnamoyl transferase (HCT); coumaroylshikimate 3-hydroxylase (C3H); caffeoyl CoA 3-O-methyltransferase (CCoAOMT); ferulate 5-hydroxylase (F5H); caffeic acid 3-O-methyltransferase (COMT). The recalcitrance to both acid pre-treatment and enzymatic digestion is directly proportional to lignin content. The total flux of lignin was reduced, with maximum effects from down-regulation of earlier enzymes in the pathway (Chen et al. 2006; Chen and Dixon 2007).

Switchgrass (*Panicum virgatum* L.) is a source for bioethanol and other biofuels, but is not cost effective source due to biomass recalcitrance. The genetic modification of switchgrass by down-regulation of *COMT* gene decreased lignin content, reduced the syringyl:guaiacyl lignin monomer ratio, improved forage quality and also increased the ethanol yield. The transgenic lines required less stringent pre-treatment and lower cellulose dosages for equivalent yield by saccharification and fermentation by yeast (Fu et al. 2011). Another study on switchgrass, where the precursor of *miR156b* was over-expressed and the effects on its target gene, Squamosa Promoter Binding Protein-Like (*SPL*), were analysed by microarray and quantitative RT-PCR. The *miR156* was shown to control the apical dominance and floral transition in switchgrass by suppressing the *SPL* genes. The transgenic plants were characterized on the basis of morphological alterations, biomass yield, saccharification efficiency and forage digestibility. Lower levels of over-expression with normal flowering increased the biomass yield. Moderate levels of over-expression showed improved biomass, but the plants were non-flowering. Higher levels showed severely stunted growth. The degree of morphological alterations of the transgenic switchgrass depended on *miR156* level. Therefore, it was inferred that the over-expression of *miR156* can lead to morphological alteration and improved biomass yield (Fu et al. 2012). DeSouza et al. (2018) demonstrated the role of *BAHD01* gene in arabinoxylan (AX) feruloylation, i.e. addition of esterified ferulic acid in the side chains of arabinoxylans, improvement of grass crops for biofuel, bio-refining and animal nutrition applications. The orthologs were silenced in the model grasses, *Setaria viridis* (*SvBAHD01*) and *Brachypodium distachyon* (*BdBAHD01*), and effects on AX feruloylation were determined. The silencing of *SvBAHD01* in *Setaria* decreased AX feruloylation in stems consistently across four generations. The silencing of *BdBAHD01* in *Brachypodium* stems showed improvement in the feruloylation, due to higher expression of functionally redundant genes.

19.4.1.2 Alterations in Plant Architecture

Plant architecture is the overall three-dimensional make-up of the plant body, which includes shoot branching, plant height, stem elongation and inflorescence morphology. These features are usually under genetic control, but environmental conditions such as light, temperature, humidity, nutrient status have effect on it (Reinhardt and Kuhlemeier 2002). The RNAi strategy has been reported to improve the crop quality and yield by altering the plant architecture.

According to the study by Xu et al. (2005), *OsPIN1* plays an important role in auxin-dependent adventitious root emergence and tillering. *OsPIN1* was expressed in the vascular tissues and root primordial in a manner similar to *AtPIN1*. The RNAi transgenic plants of *OsPIN1* showed inhibited adventitious root emergence and development, a phenotype similar to wild-type plants treated with N-1-naphthylphalamic acid (NPA), an auxin-transport inhibitor. Treatment with α-naphthylacetic acid (α -NAA) rescued the mutated phenotypes in RNAi plants. Over-expression or suppression of the *OsPIN1* expression through a transgenic approach resulted in changes of tiller numbers and shoot/root ratio (Xu et al. 2005). Zhou et al. (2006) demonstrated the RNAi strategy to suppress the expression of *OsGlu1* gene encoding a putative membrane-bound endo-1,4-beta-D-glucanase in rice. The RNAi plants showed alteration and structural changes in cell wall composition due to decrease in cellulose content and increase in pectin content. This caused reduction in cell elongation, affecting the internode elongation and produced the dwarf phenotype.

Gibberellin (GA) 20-oxidase (GA20ox) is a regulatory enzyme for the synthesis of biologically active GAs in plants. The loss-of-function mutation in *OsGA20ox2* of rice results in semi-dwarf phenotype. Qiao et al. (2007) generated semi-dwarf plants from a taller rice variety QX1 by RNAi on the expression of *OsGA20ox2*. In this study, endogenous GA assays revealed that the contents of the GA20ox2-catalysed products GA19, GA20 and the down-stream biologically active GA1 were drastically reduced in the RNAi semi-dwarf lines. The semi-dwarfism of the RNAi lines was associated with the decreased expression of *OsGA20ox2* gene and the reduced content of endogenous biologically active GA1. Also, the analyses of panicle length, seeds per panicle and 1000-grain weight suggested that the RNAi semi-dwarf lines showed stable grain yield compared with the wild-type plants. Hu et al. (2009) demonstrated the effect of RNAi on histone deacetylase (*HDAC*) genes. Down-regulation of *HDA710* affected vegetative growth. *HDA704* RNAi altered plant height and flag leaf morphology. Down-regulation of *HDT702* led to the production of narrowed leaves and stems. The study suggested that rice *HDAC* genes may have divergent developmental functions compared with closely related homologs in *Arabidopsis*. Recent studies have shown that miRNAs play regulatory roles in altering the plant architecture and can be targeted to achieve the ideal plant architecture for enhanced yield in important crops such as rice (Peng et al. 2019; Kaur et al. 2020).

19.4.2 Nutrient Quality Improvement

The common notion of the people is to fill their stomach with food in order to live, i.e. the quantity of the food that is eaten has been our primary focus. We have hardly paid any serious attention to the quality of food that we have on our platter. Food is actually composed of the various nutrients; some are required by our body in large amounts and are known as macro-nutrients, while some are needed in small amounts and are known as the micronutrients. It is the quantity of these micronutrients which

need to be optimum in our daily diet to allow our body to function properly. Majority of the developing nations are currently facing the problem of lack of proper nutrition, which has led to severe deficiency diseases and also affected proper growth and development of the children. According to estimations by UN FAO, around 792.5 million people are malnourished globally, and majority of them belong to the developing countries (McGuire 2015). Additionally, around two billion people across the world suffer from another type of hunger known as 'hidden hunger', which is caused by an inadequate intake of essential micronutrients in the daily diet (Garg et al. 2018). Biofortification of essential crops is a smart and promising way to fight against the problems of malnutrition and hidden hunger. The important aspects that have been considered for enhancing nutrient quality of crops include increasing the levels of antioxidants and essential amino acids, along with improved fatty acid profile of the target plant to improve the quality and stability of the edible oils. Among other genetic engineering approaches, RNAi has proved to be helpful in improving nutrient quality of food crops in a promising way.

19.4.2.1 Increasing the Antioxidant Levels

β-carotene content in potato was increased by RNAi-induced silencing of the gene encoding β-carotene hydroxylase which converts β-carotene to zeaxanthin (Van et al. 2007). RNAi-mediated down-regulation of the lycopene-ε-cyclase (ε-*CYC*) gene helped to enhance the levels of β-carotene as well as xanthophylls and lutein in the mustard seeds (Yu et al. 2008). Carotene as well as flavonoid content was highly enhanced by RNAi-mediated suppression of the *De-Etiolated1* (*DET1*) gene that controlled endogenous photomorphogenesis in tomatoes (Davuluri et al. 2005) as well as in canola (*Brassica napus*) (Wei et al. 2009). A significant point to note here is that by using fruit-specific promoter, the researchers limited the effect of RNAi in a particular organ only, without affecting the other parameters of the fruit quality (Davuluri et al. 2005). In another case, RNAi suppression of ε-*CYC* resulted in simultaneous increase in the levels of β-carotene, xanthophylls and lutein contents in canola (Yu et al. 2008).

19.4.2.2 Improving Stability of Edible Oils

Vegetable oils are an important part of our daily diet and source of essential fatty acids that are highly required to maintain human health (Bhunia et al. 2016). Fatty acid biosynthesis pathway employs several fatty acid desaturase (FAD) enzymes that are involved in catalytic conversion of several key steps in the formation of the major FAs. Presence of high linoleic acid (18,2) and α-linolenic acid (18,3) is highly undesirable in seed oil crops as these are known to reduce the shelf life of the vegetable oil. Nutritional improvement of sesame seed oil is being targeted by diverting the carbon flux from the production of 18:2 to 18:3 and modulation of the fatty acid biosynthetic pathways (reviewed in Bhunia et al. 2016). siRNA induced silencing of *FAD3* gene down-regulated the levels of 18:3 in seed oil content and increased the oil stability in soybean (Flores et al. 2008). Catalytic conversion of oleic acid to linoleic acid was suppressed by RNAi-mediated silencing of *FAD2* gene, thereby resulting in down-regulation of the linoleic as well as

palmitic acids, with a concomitant rise in the levels of the beneficial oleic acid in transgenic rice (Zaplin et al. 2013). Simultaneous silencing of *FAD2* and fatty acid elongase 1 (*FAE1*) genes resulted in high oleic acid content in transgenic *Arabidopsis* as well as in a dedicated oilseed crop called crambe (Li et al. 2016), camelina (Nguyen et al. 2013) and rapeseed (Peng et al. 2010). Rice bran oil is health-beneficial, nutritious oil extracted from the bran tissues of the rice grain, but its longevity is comprised by the hydrolytic and oxidative rancidity due to action of endogenous lipases and lipoxygenases, respectively. Targeted silencing of these enzymes by RNAi has the potential to enhance the self-stability as well as quality of the oil, thereby increasing its consumer reach.

19.4.2.3 Enhancing the Essential Amino Acids Content

In countries where corn is the sole source of food and nutrition, its nutritional quality needs to be enhanced to meet the daily recommended dosage of the essential nutrients. Thus, in order to re-distribute and improve the maize kernel protein profile, there have been several attempts to replace the low quality maize zein proteins with nutritionally rich non-zein proteins carrying the essential amino acids. RNAi suppression of the *opaque 2* gene involved in several endosperm pathways resulted in decreased zein content and 10-20 folds increase in the contents of lysine, tryptophan and free amino acids such as asparagine, aspartate and glutamate, in transgenic maize as compared to its wild-type counterpart (Frizzi et al. 2010). In another similar studies, zein production was suppressed by transforming maize plants with the 22 kDa zein RNAi construct (Segal et al. 2003) and a double RNAi construct against both the zein proteins (22 kDa and 19 kDa), leading to increased accumulation of the lysine and tryptophan in the transgenic maize (Wu and Messing 2011; Huang et al. 2006). Endosperm-specific transgenic expression of the RNAi construct against two genes involved in lysine catabolism, viz., dihydrodipicolinate synthase (*DHPS*) and lysine ketoglutarate reductase/saccharopine dehydrogenase (*LKR*/*SDH*), resulted in enhanced free lysine contents in maize (Frizzi et al. 2008; Houmard et al. 2007) as well in rice (Lee et al. 2001; Yang et al. 2016). In order to produce lysine-rich rice, RNAi suppression of the 13kD prolamins was effected in transgenic rice that resulted in 28% increase in seed lysine content along with a rise in the levels of the other beneficial seed storage proteins such as 10kD prolamins and chaperone proteins, thereby improving the nutritional quality of rice (Kim et al. 2013). Endosperm-specific RNA mediated down-regulation of the lysine catabolic enzyme enhanced lysine content in transgenic maize (Houmard et al. 2007) and rice (Yang et al. 2016). Furthering the goal of maize biofortification, several zein RNAi lines crossed with high methionine containing maize line PE5 obtained from another study (Planta et al. 2017), resulted in production of corn kernels rich in lysine as well as methionine (Planta and Messing 2017).

19.4.2.4 Other Ways of Improving Nutrient Quality in Crops

Alteration of seed storage protein profile by means of RNAi silencing of targeted genes has helped to improve the nutritional quality of crops and also increased their agronomic value. RNAi inhibition of *GluB* gene resulted in low glutelin content in

transgenic rice, which can be beneficial to patients with kidney related disorders and recommended low protein diet (Kusaba et al. 2003). Transgenic rice plants carrying RNAi suppression 13 kD prolamins showed an increase in other storage proteins, including 10 kD prolamin, glutelins and chaperone proteins, thereby improving the nutritional quality of rice (Kim et al. 2013; Kawakatsu et al. 2010).

Phytic acid acts as an ion chelator in crop seeds and reduces the bioavailability of several micronutrients such as Fe and Zn, thereby leading to their deficiencies and malnutrition in people who consume them. Thus, low phytate levels are desirable to alleviate this problem, which was achieved by seed-specific silencing of genes involved in its biosynthesis. RNAi-mediated suppression of myo-inositol-3-phosphate synthase (*MIPS*) gene reduced phytate levels and improved mineral bioavailability in transgenic rice (Ali et al. 2013a) as well as in soybean (Kumar et al. 2019). Similarly, silencing of another such gene, inositol 1,3,4,5,6-pentakisphosphate 2-kinase (*IPK1*), also showed significant decrease in phytate levels in transgenic plants such as rice (Ali et al. 2013b), soybean (Punjabi et al. 2018) and wheat (Aggarwal et al. 2018). In another study, phytate levels were suppressed by RNAi-mediated silencing of *ABCC13 transporter* gene in transgenic wheat, but at the cost of grain yield (Bhati et al. 2016).

Transgenic barley containing high amount of health-beneficial resistant starch was produced by using a chimeric RNAi hp. resulting in the simultaneous suppression of all the genes encoding for starch binding enzymes (SBEs) (Carciofi et al. 2012). The problem of low nutritive value of the sorghum was solved by RNAi-induced suppression of major *kafirin* subclass genes, which resulted in improving the protein digestibility index of the transgenic sorghum (Elkonin et al. 2016; Grootboom et al. 2014; Li et al. 2018). Potatoes being the fourth largest food crop in the world are kept in cold storages for longer duration to suffice the consumption demands throughout the year. However, low temperatures often induce a phenomenon of cold sweetening in potatoes due to breakdown of starch to reducing sugars by amylases, thereby reducing the quality of its processed products. The problem of starch breakdown was tackled by RNAi-mediated silencing of genes encoding for β-amylase (Hou et al. 2017), sucrose phosphatase (Chen et al. 2005) and starch phosphorylase L (Kamrani et al. 2016), that highly curtailed the conversion of starch to reducing sugars. Thus, RNAi was successfully employed in inhibiting the phenomenon of cold-induced sweetening in potatoes and restoring the quality of its processed products.

19.5 Other Applications of RNAi

19.5.1 Reduced Allergenicity

There are several harmless protein components in our dietary items that have the potential to cause hypersensitive responses leading to anaphylaxis in certain patients via immunoglobulin E-mediated allergic reaction (Anvari et al. 2019). RNAi has been exploited to suppress these allergens and produce food, which is suitable for

one and all. RNAi-induced gene silencing for *Ara h* gene and its variants showed substantial reduction in peanut allergy (Dodo et al. 2008; Chu et al. 2008; Ananga et al. 2008). Transgenic soybeans harbouring RNAi constructs for allergen protein Gly m Bd 30 K were found to have reduced allergic effects (Herman et al. 2003). The hp. RNAi construct for targeted silencing of *Mal d 1* helped in reducing IgE antibodies cross-reactivity to the birch pollen allergen *Bet v 1* in transgenic apple plants (Gilissen et al. 2005). Transgenic tomatoes with reduced allergenicity were produced by RNAi silencing of the *lyc e 1* gene (Le et al. 2006). In a remarkable research, multiple potential allergens such as α-amylase/trypsin inhibitors, α-globulin and β-glyoxalase I present in rice seeds were down-regulated by RNAi silencing method without affecting the seed phenotype (Wakasa et al. 2011). As rice is the staple food for majority of the world population, this RNAi generated rice can be safely consumed by the patients with food allergy and exhibiting clinical symptoms such as eczema and dermatitis, thereby increasing the rice agronomic as well as market value.

19.5.2 Enhanced Fruit Shelf Life

Post-harvest ethylene-induced ripening of fruits leads to their spoilage during transportation and incurs loss of billions of dollars globally. Efforts have been made to prolong the fruit shelf life through RNAi-mediated targeted gene silencing approach significantly reduced ethylene production and slowed the fruit ripening process by at least 120 days in transgenic tomato plants (Xiong et al. 2005). Chimeric RNAi construct was used to silence three homologs of 1-aminocyclopropane-1-carboxylate (ACC) synthase gene during the course of ripening, which effectively reduced the ethylene production in tomato fruits. Fruits from such lines showed delayed ripening and extended shelf life for ~45 days, with improved juice quality. The increased polyamine levels and altered levels of various ripening-specific transcripts were also observed in such fruits (Gupta et al. 2013). Down-regulation of tomato *Agamous-Like 1* (*TAGL1*) gene resulted in production of firmer fruits with extended shelf life (Vrebalov et al. 2009). In another study, loss-of-function of genes encoding for the *N*-glycoprotein modifying enzymes such as α-mannosidase and β-D-*N*-acetylhexosaminidase via RNAi resulted in at least 2.5 times firmer fruits and around 30 days of prolonged shelf life of transgenic tomatoes (Meli et al. 2010). Recent studies that employed RNAi-induced silencing of novel genes such as fruit shelf life regulator (*SlFSR*) (Zhang et al. 2018) and pectate lyase (*SlPL*) (Yang et al. 2017) also showed development of firmer fruits and enhanced fruit shelf life in tomato. RNAi silencing of two MADS box genes, *MADS1* and *MADS2*, resulted in delayed ripening in transgenic banana plants (Elitzur et al. 2016).

19.5.3 Removal of Toxic Compounds

Presence of certain toxins and toxic compounds can make the plant less favourable for consumption and also obstruct the extraction of desirable products. For instance, the toxic gossypol within seed glands of the cotton was reduced by RNAi-induced targeted silencing of the δ-cadinene synthase gene, thereby making the seed oil fit for human consumption and also permitting normal synthesis of terpenoids in leaves to enable protection against biotic stress (Sunilkumar et al. 2006). RNAi was used to block the synthesis of unwanted cyanogenic glucosides from cassava, a major staple food in tropical countries (Jørgensen et al. 2005). Decaffeinated coffee beans were produced by RNAi-mediated silencing of *CaMXMT1* gene in transgenic coffee plants that displayed up to 70% reduction in caffeine content (Ogita et al. 2004). Decrease in nornicotine content was obtained by RNAi-induced inhibition of nicotine demethylase gene in transgenic tobacco, thereby reducing the production levels of potential carcinogenic compound (Lewis et al. 2008).

19.5.4 Development of Seedless Fruits

Absence of seeds is considered to be an asset for the fruit and increases its market value for purpose of fresh consumption or processed food industry. Seedless tomato and eggplants are in demand by patients who have tendency of forming kidney stones. RNAi-induced suppression of genes such as *ARF7* (De Jong et al. 2009) and *AUCSIA* (Molesini et al. 2009) that play crucial role in regulating the phenomenon of fruit setting in plants have been employed to achieve seedless fruits, particularly in tomato. Apart from these, targeted down-regulation of chalcone synthase (*CHS*) gene also resulted in production of seedless tomatoes due to change in auxin distribution pattern that led to the onset of parthenocarpy (Schijlen et al. 2007). RNAi strategies can be extended to other plants such as watermelon, custard apple and guava to achieve seedless fruits.

19.5.5 Secondary Metabolite Production

Secondary metabolites are small organic molecules released by plants as a part of their defence mechanism to ward off any kind of possible attack on their integrity either by herbivores or any other pathogens. These are antifungal and antibacterial as well as antiviral in nature and thus prove to be highly beneficial to mankind especially in medicine. These serve as important sources of medicinal components, pesticides, food additives, fragrances and pigments. These are usually organic compounds produced from the subsidiary pathways that are derivatives from the key biochemical pathways involved in primary metabolite production (Hussein and El-Anssary 2018). Some of the examples of plant secondary metabolites include atropine, flavonoids, nicotine, caffeine, waxes, essential oils, phytic acid, lignans, phytoestrogens and carotenoids.

The California poppy (*Eschscholzia californica* Cham.) cell cultures produce several benzophenanthridine alkaloids, such as sanguinarine, chelirubine and macarpine, which have pharmacological implications. The two enzymes involved in benzophenanthridine alkaloid biosynthesis, the berberine bridge enzyme (BBE) and N-methylcoclaurine 3′-hydroxylase (CYP80B1), were silenced, and introduced separately into California poppy cell cultures (Park et al. 2002). The transformed cell lines showed low levels of BBE or CYP80B1 mRNAs, and also reduced accumulation of benzophenanthridine alkaloids compared with control cultures. They also had more concentration of amino acids including alanine, leucine, phenylalanine, threonine and valine as compared with controls. The level of tyrosine, which is an important component of benzophenanthridine alkaloids, was higher in silenced cells as compared to control, which concluded that alterations in the metabolic flux through benzophenanthridine alkaloid biosynthesis could affect the regulation of amino acid pools (Park et al. 2002). In another study by Allen et al. (2004), the codeinone reductase (COR) in poppy *Papaver somniferum* was silenced by RNAi. The transgenic plants accumulated the non-narcotic alkaloid codeinone. After gene silencing, the precursor alkaloid (*S*)-reticuline, seven enzymatic steps up-stream of codeinone, accumulated in transgenic plants at the expense of morphine, codeine, oripavine and thebaine. Methylated derivatives of reticuline also accumulated. Fujii et al. (2007) targeted *BBE* gene in California poppy cells. The *BBE* mRNA accumulation and enzyme activity were effectively suppressed in transgenic cells. The end-products of isoquinoline alkaloid biosynthesis such as sanguinarine were reduced and reticuline was accumulated at a maximum level. Allen et al. (2008) over-expressed the gene encoding the morphinan pathway enzyme salutaridinol 7-O-acetyltransferase (*SalAT*) in opium poppy, which resulted in an increase in capsule morphine, codeine and thebaine on a dry-weight basis. The suppression of *SalAT* in both leaves and latex resulted in novel accumulation of the alkaloid salutaridine. Reverse transcriptase PCR and high-performance liquid chromatography (HPLC) analyses confirmed co-segregation of the expressed transgene with the salutaridine accumulating phenotype.

19.5.6 Production of Male Sterile Lines

Cytoplasmic male sterility is maternally inherited inability of a plant to produce or shed viable pollen. It prevents self-fertilization of flowers and is beneficial to plant breeders, as they need not remove the anthers manually (Altman and Hasegawa 2011). Also, crossing female counterparts with male counterparts having desirable traits can be achieved by male sterile lines. *TA29* is expressed in the tapetum as described by Koltunow et al. (1990). RNAi can be used as a tool for the production of male sterile lines. Nawaz-ul-Rehman et al. (2007) showed that silencing of male-specific genes by RNAi generated male sterile lines for producing hybrid seed. According to their study, 10 out of 13 tobacco lines transformed with a hp. RNAi construct containing *TA29* sequences were male sterile. When the transgenic and non-transgenic plants were compared, the transgenic plants were phenotypically

distinguishable. During the anther formation, pollen grains from transgenic plants were aborted and lysed. Also, the microscopic analysis of anthers showed selective degradation of tapetum, with no microspore development in transgenic plants. Cross fertilization of transgenic plants with pollen from non-transgenic plants showed that the female parts are not affected in transgenic plants. *OsGEN-L*, a new member of the RAD2/XPG nuclease family, was silenced by RNAi in rice (Moritoh et al. 2005). Most of the *OsGEN-L*-RNAi plants were less fertile, while some were even sterile. The plants lacked mature pollen, which showed a defect in early microspore development. The *SAMDC* gene, which is involved in polyamine biosynthesis, was targeted in tapetal tissue of tomato using RNAi to check for its effect on tapetum development and pollen viability (Sinha and Rajam 2013). The RNAi plants showed the aborted and sterile pollen with shrunken and distorted morphology. The same RNAi plants, when cross-pollinated, showed fruit setting, which confirmed that female fertility factors remained unaffected in the RNAi male sterile plants (Sinha and Rajam 2013).

Fujii and Toriyama (2008) investigated the role of *DCW-11* in CW-*Oryza sativa* L. *DCW11* is one of the down-regulated genes in *CW-CMS* encoding a protein phosphatase 2C (PP2C). *DCW11* mRNA was preferentially expressed in anthers with the highest expression in mature pollen. *DCW11*-RNAi plants showed loss of seed set fertility without visible defect in pollen development. This phenotype resembled *CW-CMS*, which implied that *DCW-11* correlated with *CW-CMS*. It was further supported by the up-regulation of alternative oxidase 1a (*AOX1a*) gene, which is known to be regulated by mitochondrial retrograde signalling, in *DCW11* knockdown. These results indicated that *DCW11* could play a role as a mitochondrial signal transduction mediator in pollen germination.

19.5.7 Modification of Flower Colour and Scent

The modification of flower colour is an agro-economic trait and has aesthetic value as well. The suppression of biosynthetic genes of flower colour is an important strategy to modify the flower colour. Fukusaki et al. (2004) reported modification of flower colour of the garden plant *Torenia hybrid* by RNAi against the gene encoding CHS, a key enzyme for anthocyanin and flavonoid biosynthesis. The original flower (blue) was modified to white and pale colours by the targeted down-regulation of the coding region and 3′ untranslated region of the *CHS* mRNA, respectively. In another study on modification of flower colour in *Torenia hybrid*, the anthocyanidin synthase (*ANS*) gene was silenced by RNAi. The transgenic plants showed white flower colour, which remained stable for years (Nakamura et al. 2006). Another enzyme of the flavonoid biosynthesis, chalcone isomerase (CHI), was silenced by RNAi and the effects were studied in *Nicotiana tabacum*. The transgenic plants showed reduced pigmentation, change of flavonoid components in flower petals and accumulation of chalcone in pollen (Nishihara et al. 2005).

The flowers of gentian plants have ornamental value. The three anthocyanin biosynthetic genes; *CHS*, *ANS* and flavonoid-3′,5′-hydroxylase (F3′5′H) of gentian

plants were targeted by RNAi. The transgenic gentian plants with suppressed CHS activity in petals emerged pure white to pale blue colour, and those with suppressed ANS activity in petals showed pale blue colour. The suppression of the *F3′5′H* gene decreased delphinidin derivatives and increased cyanidin derivatives, leading to the development of magenta flower colour (Nakatsuka et al. 2010). The flower colour of gentian plants is due to the accumulation of a polyacylated delphinidin 'gentiodelphin' in their petals. The essential enzymes of the gentiodelphin biosynthesis pathway, 5,3′-aromatic acyltransferase (5/3′AT) and F3′5′H, were silenced by RNAi. The transgenic gentian plants showed lilac coloured flowers in one and pale blue in the other. Northern analysis confirmed that both the transgenic lines had suppressed *5/3′AT* transcripts. Also, the HPLC analysis of anthocyanin composition showed down-regulation of the *5/3′AT* gene, leading to increased accumulation of non-acylated anthocyanins (Nakatsuka et al. 2010).

The flower scent is also another agro-economic trait and has aesthetic value. It is an important component for reproduction as it attracts many pollinators. The floral scent is a mixture of volatile compounds belonging to the mono-terpenoids or phenylpropanoid/benzenoid classes of compounds. RNAi-mediated silencing was used to target benzoic acid/salicylic acid carboxyl methyltransferase (*PhBSMT1* and *2*) in petunia. The transgenic *PhBSMT* RNAi flowers with reduced *PhBSMT* mRNA levels showed a decrease in methylbenzoate emission, with minimal changes in other petunia VOCs (Underwood et al. 2005). The *Odorant1* (*ODO1*) gene, a member of the R2R3-type *MYB* family is involved in regulation of volatile benzenoids in *Petunia hybrid*, cv. W115 (Mitchell) flowers. RNAi-induced silencing of *ODO1* led to the reduced levels of volatile benzenoid through decreased synthesis of precursors from the shikimate pathway in the transgenic plants (Verdonk et al. 2005). Several genes involved in this pathway showed reduced expression by suppression of *ODO1* expression. However, flower pigmentation, which is due to the shikimate precursor, remained unaffected, since colour and scent biosynthesis occurred at different developmental stages (Verdonk et al. 2005). Orlova et al. (2006) generated transgenic petunia in which the expression of benzoyl-CoA:benzyl alcohol/2-phenylethanol benzoyl transferase (*BPBT*), a gene involved in the production of benzyl benzoate was silenced. The transgenic plants had decreased endogenous pool of benzoic acid (BA) and methylbenzoate emission, and increased emission of benzyl alcohol and benzaldehyde, which confirmed the contribution of benzyl benzoate to BA formation. The morphology of petunia plants was also affected, the plants had larger flowers and leaves, thicker stems, and longer internodes, along with the increased auxin transport. Another study by Maeda et al. (2010) involved the silencing of arogenate dehydratase (*ADT*), which is an important component in synthesis of L-Phe from prephenate via an arogenate and/or phenylpyruvate route. RNAi-mediated suppression of *ADT1* in petunia petals reduced ADT activity, levels of Phe, and down-stream phenylpropanoid/benzenoid volatile compounds. Thus, RNAi has proved of high commercial value in the modification of flower colour and scent.

19.6 International Status

The popular gene silencing technique of RNAi has an illustrious history of research, leading to its discovery by Fire and Mellow in the nematode *C. elegans* (Fire et al. 1998). It quickly gained an important status as the technique involving down-regulation of desired genes, and earned the prestigious Nobel Prize in Physiology or Medicine for its discoverers in 2006.The earlier RNAi research primarily focussed on the functional genomics studies that aided in deciphering the functions of several novel genes (reviewed in McGinnis 2010). Later works led the world scientific community to witness large miracles orchestrated by the small RNAs in the field of plant improvement that included improved yield, nutrition and resilience to biotic and abiotic stresses in some of the major crops such as rice, cotton, tomato, maize and wheat (reviewed in Rajam 2020). For instance, over-expression of *miR156* suppressed the expression of *SPL* genes in transgenic rice plants, which showed altered plant architecture and highly improved yield (Jiao et al. 2010). Studies for the nutrient quality improvement of crops comprised of their biofortification, such as increasing the essential amino acid content in rice (Yang et al. 2016) and corn (Planta and Messing 2017), antioxidant levels in canola (Wei et al. 2009), and mustard (Yu et al. 2008), and enhancing the stability of edible oils (Li et al. 2016; Zaplin et al. 2013). Some other important commercial applications of RNAi involved the development of male sterile lines (Fujii and Toriyama 2008), enhanced fruit shelf life (Zhang et al. 2018; Yang et al. 2017), secondary metabolite production (Allen et al. 2008) and modification of flower colour and scent (Nakatsuka et al. 2010) among others. Advanced techniques such as deep sequencing analysis have helped in deciphering the novel miRNAs in several plants and the gained information is being deposited in the public repository known as the miRbase (Kozomara et al. 2019). Apart from this, in-depth bioinformatics analyses have helped to identify the target seed sequence for providing homology-dependent gene silencing in the transgenic plants with highly reduced off-target effects. Designer RNAi crop plants that are developed with an aim for enhanced precision and reduced risks ensure global food security for the teeming millions. Thus, RNAi has rightly been called as the breakthrough technology that has immense potential in revolutionizing crop improvement strategies.

19.7 Indian National Status

The powerful approach of targeted gene silencing by RNAi provided the directional impetus to many Indian labs to focus on its numerous applications for plant improvement. Host-induced silencing of vital genes in several pathogens including fungal (Pareek and Rajam 2017; Tetorya and Rajam 2018; Singh et al. 2020) and insect pests (Yogindran and Rajam 2016; Israni and Rajam 2017) along with the development of male sterile lines using the RNAi technology (Sinha and Rajam 2013) has been reported from the Department of Genetics, University of Delhi South Campus (UDSC), New Delhi, India. Expression profiling and characterization of

abiotic stress-related microRNAs in rice (Sharma et al. 2015; Sanan-Mishra et al. 2009) was reported by the Plant Molecular Biology Group, International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi, India. Research labs at Indian Agricultural Research Institute, New Delhi, India reported RNAi-mediated silencing of the target gene(s) that provided resistance against the Tomato leaf curl virus (Praveen et al. 2007; Ramesh et al. 2007). RNAi suppression of rice MATE family transporter gene, *OsMATE2*, resulted in reduced accumulation of toxic arsenic content (36.9–47.8%) in transgenic rice (Das et al. 2018). Apart from crop protection strategies involving RNAi, several other groups are working on enhancing the nutritional aspect of crops. For instance, seed-specific silencing of inositol polyphosphate 6−/3−/5-kinase gene by siRNA (Punjabi et al. 2018) and *GmMIPS1* (Kumar et al. 2019) resulted in the production of low phytic acid soybean, resulting in increased mineral bioavailability. Similar studies were also reported in wheat (Aggarwal et al. 2018) as well as in rice (Ali et al. 2013a, b). High-throughput sequencing has helped to reveal several novel miRNAs in wheat (Pandey et al. 2014; Ragupathy et al. 2016), rice (Sasi et al. 2019), chickpea (Kohli et al. 2014) as well as in potato (Lakhotia et al. 2014). Several studies encompassing the potential role of RNAi for crop improvement as well as improvisation of strategies for its applications are underway in key Indian labs. This includes investigation of nanoparticles as agents for dsRNA delivery, designing of efficient RNAi constructs with the aid of bioinformatics analysis, production of marker-free transgenic plants and development of reliable validation methods. This will provide a long-lasting momentum for the advancement of the RNAi technology for crop improvement in the Indian perspective.

19.8 Conclusions and Future Prospects

In the modern era, food security poses an important impact on the economy of a nation. With the ever-increasing population along with the depleting resources, it is a herculean task to feed an entire nation. Breeding strategies for crop improvement which increases the yield and nutritional value of the crop have been a part of the agricultural practices for a long time from now. With the increasing demand of food supply, the breeding strategies need to be clubbed with newer technologies such as RNAi and gene editing tools like CRISPR-CAS9, etc. for crop improvement programmes. RNAi is a technique which uses the small non-coding RNAs as a powerful gene silencing tool for incorporation of desirable traits, which are heritable. It has proved effective in understanding the functions of genes and also providing different insights to the molecular breeders with an aim to produce improved crop varieties. The sRNAs can be silenced, over-expressed or manipulated by introduction of artificially synthesized miRNAs targeting the gene of interest. Every technology comes with its own pros and cons. Manipulation of desired genes using the sRNAs might at times lead to undesirable pleiotrophic changes in the plant growth and development. The off-target effects should be avoided to reduce unwanted effects on the plants and also on the ecosystem.

RNAi has been successful in the development of crops having improved productivity (biomass and yield), enhanced nutritional value, resistance against biotic (bacteria, fungi, virus, insect pests and nematodes) and abiotic factors (drought, salinity, etc.). The application of RNAi technology in crop improvement has been possible with the help of the knowledge gathered by the research on the role of small non-coding RNAs, especially siRNAs and miRNAs. This has given valuable information regarding the different pathways and mechanisms of gene regulation. Additionally, the on-going sequencing of various crop species has given valuable insights about different genes and their functions, which can be altered for crop improvement.

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miRNA-Mediated Regulation of Biotic and Abiotic Stress Responses in Plants

20

Swati Verma and Ananda K. Sarkar

Abstract

MicroRNAs (miRNAs) are small, 20–24 nucleotide long, non-coding RNAs involved in post-transcriptional regulation of gene expression through mRNA cleavage or translational repression of targets. miRNAs are known to play some fundamental roles in regulating plant growth and development. Various phytopathogens and changing global environments significantly affect plant growth worldwide. In the past decade, plant miRNAs have emerged as important regulators of biotic and abiotic stress responses in plants. Application of high-throughput sequencing has identified conserved and non-conserved miRNA-target pairs differentially expressed during specific or combined stresses. Though still in its infancy, functional characterization of miRNAs has revealed their crucial roles in plant stress management. Different miRNA-target modules are known to work both independently and in group for generating a stress-related response. Some miRNAs show same expression patterns in different species, under same or different stresses, while some exhibit species-specific or even tissue-specific responses. Expression of various miRNAs is also known to be regulated by the nature of stress. Different studies indicate huge potential of engineering miRNA-mediated gene regulation for developing improved biotic or abiotic stress-tolerant crop varieties, without compromising plant growth and productivity. In this chapter, we have discussed the current knowledge on the diverse roles of miRNAs in regulating biotic and abiotic stress responses in plants and their potential utilization for crop improvement.

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Abbreviations

ABA	Abscisic acid
ATAF	*Arabidopsis* transcription *activation factor*
APS	ATP sulphurylases
AFP	Anti-freezing protein
AGO	Argonaute
AmiR	Artificial microRNA
AMP1	Altered meristem program 1
AP 2	Apetala 2
ARF	Auxin response factor
CRISPR	Clustered regularly interspaced short palindromic repeats
CSD	Chaperones for superoxide dismutase
CSP	Cold shock proteins
DCL1	Dicer-like1
EREBP	Ethylene-responsive element binding protein
ETI	Effector triggered immunity
EXPO5	Exportin 5
FSD1	Iron SOD 1
GPA	Green peach aphid
HEN1	Hua enhancer 1
HLB	Huanglongbing
HR	Hypersensitive response
HSF	Heat shock transcription factors
HSP	Heat shock proteins
HST	Hasty
HYL1	Hyponastic leaves 1
JA	Jasmonic acid
KTN1	Katanin 1
LAO	L-ascorbate oxidase
LEA	Late embryogenesis abundant
LOX	Lysyl oxidase
LRR	Leucine-rich repeat
MiRNAs	MicroRNAs
MITE	Miniature inverted-repeat transposable elements
MYB	Myeloblastosis
MYMIV	Mungbean yellow mosaic India virus
NAC	NAM/ATAF/CUC
NAM	No apical meristem

NBS	Nucleotide binding site
NF-YA	Nuclear transcription factor-Y subunit alpha
PAMP	Pathogen associated molecular pattern
PCD	Programmed cell death
phasiRNAs	Phased secondary siRNAs
PPR	Pentatricopeptide repeat
Pre-miR	Precursor-microRNA
PTI	PAMP triggered immunity
RISC	RNA induced silencing complex
ROS	Reactive oxygen species
RSV	Rice stripe virus
SA	Salicylic acid
SBP	Squamosa promoter binding protein-like
SBPH	Small brown planthopper
SE	Serrate
SMV	Soybean mosaic virus
SOD	Superoxide dismutase
SRBSDV	Southern rice black-streaked dwarf virus
SULTR	Sulphate transporter
STTM	Short tandem target mimic
TCP	Teosinte branched1/cincinnata/proliferating cell factor
TF	Transcription factors
TIR	Toll interleukin 1 receptor
TuMV	Turnip mosaic virus
VAT	Virus aphid transmission
VCS	Varicose
YMD	Yellow mosaic disease

20.1 Introduction

Extreme weather conditions due to increased human activity and phytopathogens pose constant stress to plant growth. In plants, these biotic/abiotic stress factors significantly reduce crop productivity. Many stress-responsive genes, hormones and other regulatory molecules are known to play fundamental roles in regulating stress responses in plants (Pandey et al. 2017). Already known to be indispensable for their roles in plant growth and development, small non-coding RNAs have emerged as important regulators of plant stress responses in the past decade (Chen 2009; Ghildiyal and Zamore 2009; Shriram et al. 2016; Chauhan et al. 2017).

miRNAs are small, 20–24 nucleotide (nt) length, non-coding RNAs known to regulate post-transcriptional silencing of genes by transcript cleavage or translational repression (Axtell 2013). Since the first report of *lin-4* in *Caenorhabditis elegans* (Lim et al. 2003), many years of further research has led to the discovery of large

number of miRNAs in plants. miRNAs are known to play fundamental roles in regulating growth, organogenesis, organ patterning, nutrient homeostasis and hormone signalling in plants (Li and Zhang 2015). The role of several miRNAs is being investigated in regulating biotic and abiotic stress pathways (Jatan and Lata 2019).

20.2 Abiotic and Biotic Stresses: Impact on Plants and the Plant Stress Response

Abiotic factors such as heat, cold, salinity and drought affect plant growth and cause huge losses to crop yield worldwide. On perceiving a stress signal, plants channelize their molecular machinery to generate a specific response (Shriram et al. 2016). Abiotic stresses lead to decreased rate of photosynthesis, closure of stomata, reduced leaf growth, increased root length and high reactive oxygen species (ROS) scavenging in plants. Biotic stresses posed by various pathogens like bacteria, fungi, viruses, insects, nematodes, arachnids and weeds cause stomatal closure, production of ROS and decreased photosynthesis (Melloto et al. 2006; Bilgin et al. 2010). Rapid production of ROS is often followed by hypersensitive response (HR) during pathogen infection. Production of toxic compounds like phytoalexins and localized cell death check pathogen spread (Wojtaszek 1997). After perceiving external stress signal, a cascade of events activates the internal signalling components. The signal is ultimately sent to nucleus for generating a specific response (Mittler et al. 2004; Apel and Hirt 2004; Miller et al. 2007). Often regarded as the first line of response, the external stimuli are sensed by the calcium sensors (Edel and Kudla 2016). Mitogen-activated protein kinases (MAPKs) are activated in a phosphorylation cascade, which further activates the downstream molecular machinery to generate a specific response (Ichimura et al. 2006; Xu and Zhang 2015).

Various phytohormones and transcription factors (TFs) play key roles in generating a particular defence response against environmental stresses (Shigenaga and Argueso 2016; Nguyen et al. 2016). During pathogen attack, salicylic acid (SA) and JA pathways and ethylene signalling are induced (Yoshida et al. 2015; Banerjee and Roychoudhury 2017). ABA acts antagonistically with SA (de Torres et al. 2009; Jiang et al. 2010; Ding et al. 2016) and often increases plant susceptibility to pathogens (Xiong and Yang 2003; Yasuda et al. 2008; Lievens et al. 2017; Peskan-Berghöfer et al. 2015). Various transcription factors (TFs) are induced in ABA-dependent and ABA-independent manner (Yoshida et al. 2015; Banerjee and Roychoudhury 2017). These TFs induce or repress expression of various genes to make plants develop some adaptive physiological changes (Kim et al. 2010; Silva et al. 2018; Huang et al. 2018; Wang et al. 2018). Together, these hormonal crosstalks along with various stress-responsive TFs channelize the plant machinery towards mitigating the effects of abiotic stresses or defence against plant pathogens.

20.3 Origin and Evolution of miRNAs

miRNA origins have been traced back to primitive organisms. They are encoded by unicellular green alga *Chlamydomonas reinhardtii*, suggesting their appearance prior to the evolution of land plants. In land plants, many miRNA and target pairs are found to be conserved (Bartel and Bartel 2003; Molnar et al. 2007; Jones-Rhoades 2012; Zhang et al. 2013). miRNAs can be broadly divided into two groups based on their conservation and diversification during the evolution of plant kingdom. First group is of highly expressed and evolutionarily conserved ancient miRNA families, and second ones are the young miRNA families expressed at comparatively low levels. Also, the young miRNAs are often expressed in lesser species under specific conditions, thus are evolutionarily non- or less-conserved (Qin et al. 2014).

MIR genes might emerge from expressed genes by aberrant replication, transposition or recombination events. Also, *MIR*s could be lost during evolution. Therefore, there could be frequent birth and death of *MIR*s in plant species (Fahlgren et al. 2007). Three events have been proposed for the appearance and evolution of *MIR* genes in plants. First, miRNAs might originate by the inverted duplication events of their target genes (Allen et al. 2004; Maher et al. 2006); second, miRNAs could originate by spontaneous evolution from fold-back sequences which are present in the genomes (de Felippes et al. 2008) and third, miniature inverted-repeat transposable elements (MITEs) could fold back and make imperfect stem-loops to ultimately form a precursor sequence for new miRNA (Piriyapongsa and Jordan 2008).

20.4 miRNA Biogenesis in Plants

Biogenesis of miRNA majorly comprises the following steps: (a) transcription of *MIR* gene; (b) processing of transcript by various proteins and (c) loading onto RNA induced silencing complex (RISC). *MIR* genes are mostly located in intergenic regions, but could also be present in sense or antisense orientation within the introns. Some miRNAs are polycistronic. RNA polymerase II (Pol II) transcribes long primary transcripts, termed pri-miRNAs from *MIR* genes. Like canonical Pol II transcripts, these are 5′-end capped and 3′-end polyadenylated (Bartel 2004; Xie et al. 2005; Jones-Rhoades et al. 2006; Rogers and Chen 2013). pri-miRNAs are basically hairpin structures comprising a terminal loop, upper stem, lower stem and flanking region. The stem-loop regions are variable in length. DICER-LIKE1 (DCL1), in assistance with other accessory proteins like SERRATE (SE) and HYPONASTIC LEAVES 1 (HYL1) catalyse the production of most of the miRNAs in the model plant *Arabidopsis thaliana* (Fang and Spector 2007; Dong et al. 2008). Other than DCL1, other DCLs like DCL3 or DCL4 may be involved in processing of pri-miRNAs in other plant species (Rajagopalan et al. 2006; Wu et al. 2010). HUA ENHANCER 1 (HEN1) methylates the 2′-OH position which protects the miRNAs from uridylation and subsequent degradation (Yu et al. 2005; Yang et al. 2006).

RISC majorly assembles in nucleus and is finally exported by EXPO1 to cytosol (Bologna et al. 2018). However, some recent data also includes the possibility of miRNA/miRNA* duplexes being exported out of nucleus and later assembled into RISC. The guide strand (miRNA) of miRNA/miRNA* duplex gets associated with ARGONAUTE (AGO), while the passenger strand (miRNA*) is degraded. By base complementarity, the miRNA guides RISC to the target transcript and carries out post-transcriptional gene silencing (Zhang et al. 2015).

20.5 Modes of Plant miRNA Action

miRNAs regulate the expression of target genes by transcript cleavage or translational repression (Chen 2005; Chen 2009; Voinnet 2009; Rogers and Chen 2013). miRNA-mediated RNA cleavage is done at a particular position on target mRNA (Llave et al. 2002). The P-element induced wimpy (PIWI) domain of AGO protein has endonuclease activity which accomplishes cleavage. Subsequently, the 5′ and 3′ fragments generated after cleavage are degraded by exonucleases (Mi et al. 2008; Montgomery et al. 2008; Takeda et al. 2008; Ji et al. 2011; Maunoury and Vaucheret 2011; Zhu et al. 2011). miRNA-mediated translational inhibition is accomplished by KATANIN 1 (KTN1), processing body (P body), VARICOSE (VCS), GW-repeat protein SUO (Brodersen et al. 2008; Yang et al. 2012) and ALTERED MERISTEM PROGRAM 1 (AMP1) (Li et al. 2013). In plants, miRNAs have a nearly perfect complementarity with their target mRNAs, therefore, transcript cleavage was considered to be a prevalent mode of miRNA action earlier (Chen 2005; Jones-Rhoades et al. 2006). But miRNA-mediated translational inhibition could also be seen in such pairings having high sequence complementarity. Therefore, although a high degree of miRNA-target sequence complementarity is likely to result in RNA cleavage, it might not necessarily be refractory to translational repression (Brodersen et al. 2008; Yang et al. 2012; Li et al. 2013).

Some miRNAs trigger generation of phased secondary siRNAs (phasiRNAs). phasiRNAs function in *trans* to suppress the level of their target transcripts (Fei et al. 2013). Some studies demonstrate that miRNAs regulate gene expression by directing epigenetic changes like DNA and histone methylation (Bao et al. 2004; Khraiwesh et al. 2010; Wu et al. 2010).

20.6 Role of miRNAs in Biotic Stress Responses in Plants

20.6.1 miRNAs in Viral Pathogenesis

Viral diseases of plants cause considerable losses to crop production. Factors like rapid evolution ability, international trade and climate changes are leading to frequent emergence of new viral diseases in plants. The role of miRNAs in regulating plant responses to virus attack is being widely studied across the world (Niu et al. 2006; Ding and Vionnet 2007; Qu et al. 2007; Bester et al. 2017; Kundu

et al. 2017; Bao et al. 2018). Upon Turnip mosaic virus (TuMV) infection, *Brassica rapa* showed upregulation of bra-miR158 and bra-miR1885. bra-miR1885 is known to target toll interleukin1 receptor-nucleotide binding site-leucine-rich repeat (TIR-NBS-LRR) disease-resistant gene transcripts, indicating about its role in viral pathogenesis (He et al. 2008). The homeostasis of AGO1 is known to be controlled by miR168 in plants. Virus-mediated induction of miR168 and inhibition of AGO1 protein accumulation was proposed to serve as a defensive strategy against viral infection by Varallyay et al. (2010). Rice stripe virus (RSV) affects rice growth and production in many countries. The insect vector, small brown planthopper (SBPH) transmits RSV into plants. RSV-infected rice showed increased abundance of seven putative novel miRNAs and decreased abundance of two putative novel miRNAs (Guo et al. 2012). Later, another study also showed decreased abundance of miR2118 family members upon RSV infection. miR2118 members are known to target certain NBS-LRR genes (Lian et al. 2016). Increased expression of miR160 and miR393 resulted in repression of auxin signalling genes to generate a defence response against soybean mosaic virus (SMV) (Yin et al. 2013).

miR164, miR396, miR530 and miR1846 were reported to target rice genes responsible for symptoms of southern rice black-streaked dwarf virus (SRBSDV) infection. miR164 mediates the regulation of no apical meristem (NAM)/*Arabidopsis* transcription *activation factor* (ATAF)/cup shaped cotyledon (CUC) (NAC1), NAM and SA-induced protein. On SRBSDV infection, this module regulates appearance of various symptoms like excessive tillering, aerial rootlets, branches on stem nodes and SA-mediated virus resistance (Xu et al. 2014). In another study, miRNA expression patterns were also studied in the roots and leaves of RBSDV-infected rice. miR408, miR827 and miR1428e showed decreased abundance, while miR156a-j, miR166abcdf and miR169hijklm showed increased abundance in both roots and leaves. Tissue-specific miRNA expression patterns revealed increased expression of miR164abf and miR398 in leaves as compared to roots (Sun et al. 2015b).

Yellow mosaic disease (YMD) caused by mungbean yellow mosaic India virus (MYMIV) is one of the most devastating and widely spread diseases affecting legume production. The expression of pathogen-responsive miRNAs was studied in MYMIV-infected *Vigna mungo*. The expression of miR396, known to target lysyl oxidase (LOX), was observed to be induced on MYMIV infection. LOX is known to be involved in JA synthesis in plants. Increased expression of miR396 was reported to suppress JA-mediated pathway and in turn activate SA-mediated pathway. An enhancement was also observed in the expression of miR159, while isoforms of miR319 and miR166 showed downregulation on MYMIV inoculation. The abundance of miR482 transcripts declined resulting in an increase in the levels of target NB-LRR genes. The study also revealed altered abundance of miRNAs involved in regulating the genes of auxin signalling pathway and photosynthetic machinery (Kundu et al. 2017). Later Patawa et al. (2018) identified 422 miRNAs in MYMIV-infected *Phaseolus vulgaris*. The expression of miR482 reduced in resistant genotype, the simultaneous upregulation of resistance gene encoding NB-LRR might be involved in improved resistance to MYMIV.

20.6.2 miRNAs in Fungal Pathogenesis

During fungus attack, there is a fight to acquire nutrition by the fungal pathogen and fight to generate a counter-defence response for survival by host plant. A complex array of molecular interactions occur during these plant–fungus interactions. miRNAs have been reported to be involved in regulating these plant–fungus interactions (Ramachandran et al. 2020). Decreased miR1444 levels on *P. syringae* infection in tomato, leading to higher levels of polyphenol oxidase enzymes showed increased resistance of tomato plants to *P. syringae* (Li and Steffens 2002). Many miRNAs showed significant decrease in expression in the fusiform rust fungus (*Cronartium quercuum*) infected galled stem in *Pinus taeda*. (Lu et al. 2007). Another study identified 24 miRNAs upon *Erysiphe graminis* f. sp. *tritici* (*Egt*) infection in wheat (*Triticum aestivum* L.). The expression was found to be different in susceptible lines and near-isogenic lines having a resistant R-gene. While a decrease in miR156 expression was seen in both the cultivars, expression of miR164 decreased only in resistant lines. miR393 known to target auxin response factors also showed downregulation in resistant lines (Xin et al. 2010). Kulcheski et al. (2011) performed the expression profiling of different miRNAs in soybean [*Glycine max* (L.) Merrill] upon infection with *Phakopsora pachyrhizi*. The necrotrophic oomycete, *Pythium aphanidermatum* causes the *Pythium* soft rot of turmeric. Eighteen conserved and 3 novel miRNAs were identified during the *Pythium* soft rot disease of turmeric. Namely, miR159, miR164, miR393 and miR482 were found to be involved in regulating plant defence against this fungal pathogen. miR164 abundance was found to decrease, which led to increments in NAC expression known to be involved immune responses of plants. Decrease in miR167 and miR393 expression led to the upregulation of auxin-responsive genes (Chand et al. 2016).

More than 65 miRNA families showed differential expression in resistant and sensitive cotton varieties upon infection with *Verticillium dahliae*. The expression of miR1917 and miR2118 was downregulated during fungal infection. The target genes for miR1917 are known to be involved in ethylene signalling and miR2118 modulates a putative NB-LRR protein suggesting their role in regulating plant defence responses during *V. dahlia* infection (Yin et al. 2012a). Sequencing of small RNAs in eggplant (*Solanum melongena* L.) identified 99 known miRNAs families and two novel putative miRNAs upon *V. dahliae* infection (Yang et al. 2013). Later, 37 novel and 443 conserved miRNAs were identified in an upland cotton variety infected with *V. dahlia,* moderate virulence and high virulence strains. Some predicted targets of the novel miRNAs were known to be involved in plant–pathogen interactions (He et al. 2014). *Sclerotinia sclerotiorum* is the causal agent of white mold disease of *Brassica napus*. Fifty-three novel and 227 known miRNAs were identified in *B. napus* upon infection with *S. sclerotiorum*. Various TFs known to be involved in plant development and defence responses were identified as targets for these miRNAs. miR1885a, miR6030 and miR168a_L + 1R_1 known to target TIR-NBS-LRR, At1g12290- like disease resistance proteins and small glutamine-rich tetratricopeptide repeat protein 1 (SGT1a)-phosphatase-like proteins,

respectively, were known to be involved in effector triggered immunity (ETI) responses in plants (Cao et al. 2016). Small RNA profiling of *Allium sativum* upon infection with *Fusarium oxysporum* L. revealed 45 conserved miRNAs and 6 novel miRNAs. Overexpression of miR164a, miR168a and miR393 showed decreased fungal growth and increased expression of genes involved in plant defence (Chand et al. 2017). Recently (Ramachandran et al. 2020), expression analysis of two wheat cultivars infected with *Puccinia striiformis* f. sp. *Tritici* revealed the expression of 163 novel miRNAs, 182 known miRNAs and 91 variants of miRNAs. Differential expression was observed to be cultivar-specific for TA078/miR399b.

20.6.3 miRNAs in Bacterial Pathogenesis

Advances in genomics techniques have improved our understanding of bacterial pathogen–host interactions in plants. Several studies have shown that miRNAs play crucial roles in regulating the plant defence responses against various bacterial pathogens (Jodder et al. 2017). Few reports are discussed in this section.

The *Agrobacterium tumefaciens* C58-induced tumours exhibited reduced accumulation of miR167 and miR393 suggesting their role in bacterial pathogenicity responses (Dunoyer et al. 2006). Bacterial elicitor, flg22, was shown to induce the expression of miR393 leading to suppression of auxin signalling, subsequently resulting in pathogen associated molecular pattern (PAMP)-triggered immunity (PTI) (Navarro et al. 2006).

High-throughput small RNA profiling revealed that a non-pathogenic *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 strain with a mutated type III secretion system, *hrcC,* induced the expression of miR393, miR167 and miR160. miR393 overexpression resulted in restricted growth of *Pst* DC3000 in *Arabidopsis*. miR160 and miR167 are known to regulate the expression of auxin response factors (ARFs) which regulate auxin signalling pathways. Auxin signalling is known to be modulated during pathogen defence responses indicating the role of these miRNAs in regulating *P. syringae* infections (Fahlgren et al. 2007 and Yang et al. 2013). On flg22 treatment, AGO1-bound small RNA profiling revealed the role of miR160a, miR398b and miR773 in callose deposition, suggesting their role in PTI responses (Li et al. 2010). Different miRNA families were found to be differentially expressed upon *Pst* DC3000 *hrcC* (non-pathogenic), *Pst* DC3000 EV (virulent) and *Pst* DC3000 *avrRpt2* (avirulent) infections. Most of the differentially expressed miRNA targets were known to be involved in hormone biosynthesis and signalling pathways, suggesting the roles of these miRNAs in defence signalling (Zhang et al. 2011a). In *Manihot esculenta,* a total of 56 conserved and 12 novel miRNAs families were found to be differentially expressed on infection with *Xanthomonas axonopodis* pv. *manihotis* (*Xam*). Some upregulated miRNAs were known to regulate defence responses by regulating auxin response factors (ARFs) (Perez-Quintero et al. 2012).

Huanglongbing (HLB) disease is caused by bacteria *Candidatus liberibacter* asiaticus. Rawat et al. (2015) identified HLB-associated miRNAs in citrus. The

HLB-responsive miRNAs comprised miR156, miR166, miR167, miR172 and miR393. In another study, *Bacillus cereus* pre-treated plants exhibited repression of miR825 and miR825* expression and upregulation of corresponding target TIR-NBS-LRR. The results also indicated that a particular bacterial infection might help imparting immunity against other bacterial species (Niu et al. 2016). In *Malus hupehensis*, the expression of miR168a was found to be upregulated upon *Botryosphaeria dothidea* infection (Yu et al. 2017).

20.6.4 miRNAs in Plant–Insect Interactions

Alongside attracting pollinators and other beneficial insects, plants also need to defend themselves against the insect herbivores, which cause crop damage via direct feeding or acting as viral agents. To minimize the tissue losses by sap-sucking or chewing insects, plants have evolved various defence mechanisms (Zhu-Salzman et al. 2005; War et al. 2012; Fürstenberg-Hägg et al. 2013; Poelman 2015; Aljbory and Chen 2018; Li et al. 2018). On the other hand, insects have evolved counter-defence mechanisms against such responses (Zhu-Salman et al. 2005; Alba et al. 2011). Currently, the knowledge on role of miRNAs in these interactions is in its infancy. Barah et al. (2013) revealed several miRNAs regulating the expression of transcription factors and target genes involved in SA, JA and ethylene signalling pathways during susceptible interaction between green peach aphid (GPA) and *Arabidopsis.* Kettles et al. (2013) also reported that resistance against GPA in *Arabidopsis thaliana* seems to involve the role of miRNA-regulated expression of phytoalexin camalexin. Flower production is adversely affected on *Chrysanthemum* (*Chrysanthemum morifolium* Ramat) infestation by *Macrosiphoniella sanbourni* (Gillette). Aphid infestation and mock puncture treatment in *Chrysanthemum* induced the expression of miR159a, predicted to target *GAMYB-like 2*. *GAMYB-like 2* is known to regulate gibberellic acid (GA)-signalling. Possibly, miR159a played a role in aphid attack-induced programmed cell death (PCD). The study also showed decreased expression of miR160a and miR393a during this interaction (Xia et al. 2015).

Virus aphid transmission (*Vat*) gene imparts resistance against *Aphis gossypii* in melon (*Cucumis melo*). A study by Sattar et al. (2012) showed that miR156, miR157, miR159 and miR162 were induced during the early stages of resistant interaction in Vat^{+}, whereas miR166 and miR2111 were induced, and miR156, miR159 and miR162 were suppressed during late stage of aphid infestation in Vat^{+}. While miR408 increased in abundance during early stage of infestation during susceptible interaction (Vat^{-}), miR56, miR166, miR168, miR169, miR171, miR172 and miR396 were induced during the late stage. Sattar et al. (2016) further identified 70 miRNA-target modules in *A. gossypii* resistant melon lines, which included 28 novel miRNA-target modules. Out of the 11 miRNAs found to be involved in regulating phytohormone pathways, six regulated auxin interactions. *A. gossypii* Glover causes economic losses, both by virus transmission and direct feeding on cotton and cucurbit crops. miRNA sequencing of apterous *A. gossypii* adults fed on

an allelochemicals containing artificial diet, identified a total of 292 miRNAs. The targets of miRNAs were predicted to be involved in the metabolism of plant allelochemicals (Ma et al. 2017). Using deep sequencing, Jeyaraj et al. (2017) identified 130 known and 512 novel miRNAs upon interaction between tea plants and *Ectropis oblique*. The miRNAs were predicted to be involved in stress signalling mechanisms.

20.6.5 miRNAs in Plant–Nematode Interactions

The interaction among plants and parasitic nematodes has made plants evolve remarkable adaptations during these host–parasite interactions. Small RNA sequencing has lead researchers identify many miRNAs involved in plant–nematode interactions (Medina et al. 2017; Kaur et al. 2017; Tian et al. 2017). Specifically, miR396b and some members of miR167 family exhibit downregulation in *Arabidopsis* roots infected with *Heterodera schachtii* at 4 and 7 dpi (Hewezi et al. 2008). High-throughput sequencing has revealed the expression of many miRNAs (miR156, miR159, miR172, miR319, miR393, miR396 and miR996) during *M. incognita* infection (Cabrera et al. 2016). Later, the expression of miR396 was also found to be repressed in *Arabidopsis* roots infested with *H. schachtii* during onset of syncytium formation. During later stages of infestation, miR396 showed upregulation (Hewezi et al. 2012).

The miRNAs involved in the plant root knot nematode infection are being studied in *Arabidopsis* (Cabrera et al. 2016 and Medina et al. 2017). *Arabidopsis miR159abc* mutant exhibited decreased susceptibility to *M. incognita*. However, the overexpression of miR159 in galls showed repression of its target MYB33, suggesting the role of miR159 in regulating *M. incognita* infection responses (Medina et al. 2017). Recently, Pan et al. (2019) reported differential expression of 16 miRNAs in *M. incognita* infected whole cotton roots. In another recent study, Noon et al. (2019) studied the role of miRNA396-growth regulating factor (GRF) module in soybean *H. glycines* infection in soybean. Their results suggested that miRNA396-regulated *GRF* homeostasis plays role in governing the productive soybean-cyst nematode infections. The involvement of different miRNAs in various biotic stress responses in plants is represented (Fig. 20.1).

20.7 Role of miRNAs in Abiotic Stress Responses in Plants

20.7.1 miRNAs in Heat Stress

Global warming is posing heat stress to vegetation, affecting plant growth worldwide (Long and Ort 2010; Ding et al. 2020b). Various hormones, ROS, heat-shock proteins (HSPs) and heat-shock transcription factors (HSFs) are induced upon heat stress in plants. In the quest for deciphering the molecular regulators of heat stress responses, miRNAs have also emerged as important molecules (Mittler et al. 2012;

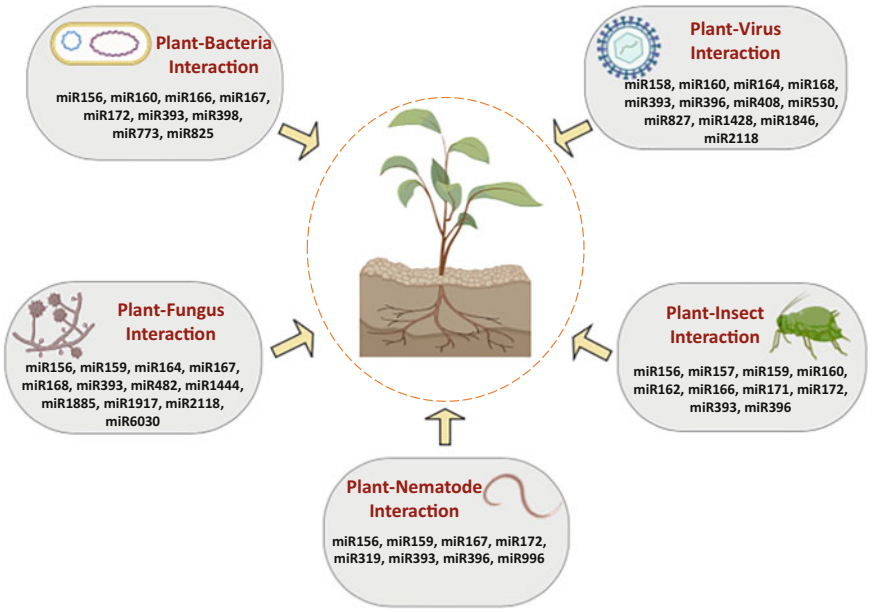

Fig. 20.1 Biotic stress-responsive miRNAs in plants (Image created on BioRender.com)

Ding et al. 2020b). miR5175 was identified to be heat stress-responsive in barley by Kruszka et al. (2014). Zhou et al. (2016) reported that under moderately elevated temperatures, *Hsp70* is targeted by spi-miR6300_gma, while *Hsp60-3A* is targeted by spi-miR166c-3p and spi-miR166g-3p_osa in tomato at acutely elevated temperatures. In another study, Liu et al. (2017) identified 102 heat-responsive miRNAs from the panicles of heat-tolerant and -sensitive rice varieties. Guan et al. (2013) reported that induced miR398 expression resulted in reduction of its target copper chaperones for superoxide dismutase (CSD1, CSD2 and CCS) transcripts. Moreover, miR398-resistant plants for CSD1, CSD2 and CCS showed more sensitivity to heat stress, establishing miR398 as an important regulator of heat stress responses (Guan et al. 2013). Various studies have identified miR827 to be heat stress responsive in different crops (Lin et al. 2010; Xin et al. 2010; Hivrale et al. 2016).

As discussed, prior to targeting a particular transcript, mature miRNAs require loading onto AGO and form RISC. Reduced expression levels of miR168 during heat stress and increased expression of AGO1 are indicative of high miRNA activity. The results suggested that comparatively high miRNA activity is required during elevated temperatures to counter heat stress (Vaucheret et al. 2006; Chen et al. 2012a; Liu et al. 2017; Zhou et al. 2016). Also, heat stress-tolerant tomato plants showed decreased expression of miR168 under heat stress resulting in increased availability of AGO1 (Zhou et al. 2016). Overexpression of miR400 shows increased sensitivity of *Arabidopsis* plants to elevated temperatures. Two pentatricopeptide repeat (PPR) genes show upregulation during heat stress which

are reported to be the targets of miR400. This suggests the role of miR400-PPR module in heat stress responses in plants (Yan et al. 2012; Li et al. 2014a; Park et al. 2014). Recently, Keller et al. (2020) identified several novel and already known miRNAs in the developing and heat-stressed pollens of tomato. Target analysis predicted many heat stress transcription factors and HSPs as their putative targets. The study thereby established miRNAs as important elements of thermotolerance during various developmental stages of plants.

20.7.2 miRNAs in Cold Temperature Responses

Plants have evolved certain physiological and molecular mechanisms to fight cold stress (Steponkus et al. 1998; Kaplan and Guy 2004, 2005; Kaplan et al. 2007). Some low molecular weight solutes, proline and soluble sugars serve as osmolytes to protect plants from the cold-induced damage. Expression and accumulation of late embryogenesis abundant (LEA) proteins, cold shock proteins (CSPs) and anti-freezing proteins (AFPs) prepare plants for freezing tolerance (Ruelland et al. 2009). The role of some miRNAs in regulating these processes is discussed below.

During an initial study, Sunkar and Zhu (2004) reported repression of miR319c and miR398a expression and increment in miR393 expression under cold stress in *Arabidopsis* seedlings. In another study, the microarray expression analysis of cold-treated *Arabidopsis* by Liu et al. (2008) revealed increased expression of miR165, miR168, miR169, miR171, miR172, miR319, miR393, miR396, miR397 and miR408. The response of some miRNAs could be species-specific, for e.g., the expression of miR169 was found to decline in rice, wheat, grapevine and poplar (Lv et al. 2010; Chen et al. 2012c; Tang et al. 2012; Sun et al. 2015a), but found to increase in almond, *Arabidopsis* and *Brachypodium* under cold stress (Zhou et al. 2008; Liu et al. 2008; Zhang et al. 2009; Karimi et al. 2016). In other reports, the expression of miR395 decreased at 2 h in poplar, but same miRNA showed a mildly elevated expression in grapevine at same time point. Therefore, along with species-specific, the miRNA expression patterns have also been known to be effected by duration of cold exposure (Chen et al. 2012c; Sun et al. 2015a). Barakat et al. (2012) identified 108 miRNAs from the young emerging leaves and chilled vegetative buds of peach. Some miRNAs revealed tissue-specific expression patterns. While only 10 miRNAs specifically expressed in buds, 25 miRNAs were unique to leaves. The highest expression was shown by miR167 and miR395 in buds. In another study, Karimi et al. (2016) also revealed tissue-specific miRNA expression patterns in cold-treated ovary and anthers of almond. miR159-5p, miR160f-3p and miR7723-3p revealed ovary- and cold-stress specific expression and miR393 revealed anther- and cold-stress specific expression. miR482d-3p showed increased abundance in anther and decreased abundance in ovary. Zeng et al. (2018) identified 269 conserved and 84 putative novel miRNAs from the leaves and roots of cold-tolerant and cold-sensitive winter turnip rape varieties. The identified targets of some of these miRNAs were found to be involved in various stress and metabolic pathways of plants. Recently, Abla et al. (2019) identified 168 conserved miRNAs and

14 putative non-conserved miRNAs under cold stress in *Astragalus membranaceus*. Among 27 cold-stress responsive miRNAs, four conserved miRNAs were induced, 17 conserved miRNAs were repressed and six non-conserved miRNAs were found to be induced under cold stress.

20.7.3 miRNAs in Drought and Salinity Stress

In the area of stressed agriculture, salinity and drought stress have gained more attention and emphasis. Plants exhibit certain similar responses under drought and salinity stress. It has been observed that the altered osmotic effects in the first phase of salinity stress are quite similar to drought stress. The signal transduction and hormonal regulation of both stresses often crosstalk. Increased ROS levels under salinity and drought are followed by changes in the redox homeostasis of cells (Uddin et al. 2016). The role of miRNAs in regulating these processes is being discussed.

Identification of 98 miRNAs belonging to 27 families was done in salt sensitive maize cultivar (Huangzao4) and salt tolerant genotype (NC286). The results showed reduced expression of miR156, miR164, miR166, miR167 and miR396, and increased expression of miR162, miR168, miR395 and miR474 in maize roots under salt stress conditions (Ding et al. 2009). Yin et al. (2012b) analysed the miRNA expression profiles of salt tolerant (SN-011) and salt sensitive (LM-6) cotton cultivars. The miRNAs exhibited genotype-specific expression patterns. The expressions of miR167, miR397 and miR399 were significantly repressed in LM-6, whereas miR156, miR169, miR535 and miR827 showed induction in this cultivar. Recently, Parmar et al. (2020) identified 75 conserved and 200 novel miRNAs from the roots and shoots of the seedlings of a salt tolerant rice variety (Pokkali) on NaCl treatment. Target analysis revealed that several miRNAs targeted transcription factors like ARF, NAC, HD-Zip III, nuclear transcription factor Y subunit alpha (NF-YA), apetala2/ethylene-responsive element binding protein (AP2/EREBP) domain protein, myeloblastosis (MYB), teosinte branched1/cincinnata/proliferating cell factor (TCP) and squamosa promoter binding protein-like (SBP), known to regulate salt and other abiotic stress responses in plants. A novel miRNA, osa-miR12477 regulated the expression of L-ascorbate oxidase (LAO), indicating its role in regulating salt tolerance.

During initial studies, Zhao et al. (2007) observed upregulation of miR169g and miR393 expression during drought stress in rice. Later, Trindade et al. (2010) reported induction of miR398a/b and miR408 expression in shoots and roots, and repression of miR169 expression in roots under drought conditions in *Medicago truncatula*. The upregulation of miR398a/b and miR408 correlated with downregulation of their respective targets revealing the roles of these miRNA-target modules in drought regulation responses. In another study, 13 switchgrass-specific and 4 conserved miRNAs were identified under drought stress in switchgrass (Xie et al. 2014). Later, it was reported that novel barley-specific

miRNAs (hvu-miRX33, hvu-miRX34 and hvu-miRX35) showed significant induction on drought treatment (Hackenberg et al. 2015).

While a particular miRNA may show induction on drought or salt treatment in one particular species, it may show repression in another. For e.g., *Arabidopsis* plants showed higher expression of miR156 under salinity stress (Liu et al. 2008), but repression of miR156 expression was observed in maize (Ding et al. 2009). Similarly, NaCl treatment resulted in higher expression of miR396 in maize (Ding et al. 2009) and *Arabidopsis* (Liu et al. 2008), but similar expression patterns were not observed in rice (Zhou et al. 2010). Also, drought treatment induced the expression of miR168 and miR396 in *Arabidopsis* (Liu et al. 2008) and tobacco (Frazier et al. 2011), but expression of same miRNAs was repressed in rice under drought conditions (Zhou et al. 2010). Expression of miR408 was repressed upon drought treatment in rice (Zhou et al. 2010), peach (Eldem et al. 2012) and cotton (Xie et al. 2015), but induced in *Arabidopsis* (Liu et al. 2008), *Medicago* (Trindade et al. 2010) and barley (Kantar et al. 2011).

20.7.4 miRNAs in Heavy Metal Stress

Contamination of soils with heavy metals has become a major concern worldwide. Heavy metals include iron (Fe), copper (Cu), manganese (Mn) and zinc (Zn) as essential metals and cadmium (Cd), aluminium (Al), mercury (Hg), arsenic (As) and lead (Pb) as non-essential metals. While essential metals are required for various physiological processes of plant growth and development, excess of them could be toxic. Excess of non-essential metals in soil poses potential risk to plant growth and health. In plants, several miRNAs have been identified to regulate these heavy metal stress-related responses (Ding et al. 2020a). Various metal-responsive elements have been identified on the promoters of different miRNAs and the abundance of these elements has been reported to be family-specific (Ding et al. 2011). Under heavy metal stress, decreased expression of miR166 and miR398, and increased expression of miR393, miR171, miR319 and miR529 were recorded in *Medicago truncatula* (Zhou et al. 2008). Under Hg stress, many conserved and non-conserved miRNAs showed differential expression in *M. truncatula* seedlings (Zhou et al. 2012). Xie et al. (2007) reported downregulation of miR156, miR171, miR393 and miR396 during Cd stress in *Brassica napus*. In a later study, upregulation of miR528 and downregulation of miR156, miR162, miR168, miR166, miR171 and miR390 were observed during Cd stress in rice (Ding et al. 2011).

Decreased abundance of miR159, miR160, miR319, miR390 and miR396 was observed during Al toxicity in *M. truncatula* (Chen et al. 2012b). In *Nicotiana tabacum*, the expression of miR159, miR162 and miR396 increased on treatment with Al_2O_3 nanoparticle which is a newly emerging pollutant. While, in *M. truncatula*, the expression of same miRNAs decreased on $AlCl_3$ treatment (Chen et al. 2012b; Burklew et al. 2012). miR1508, miR1515, miR1510/2110 and miR1532 are being reported Mn-responsive by Valdes-Lopez et al. (2010). Similarly, Liu and Zhang (2012) identified 67 new miRNAs in indica rice roots as

As-responsive. In another study by Srivastava et al. (2013), miR156, miR162, miR165, miR167 and miR390 were found to be arsenate-responsive in *B. juncea*.

20.7.5 miRNAs in Metal-Ion Starvation Responses

Plants often grow in soils that are poor in certain nutrients. This results in an imbalanced and insufficient uptake of nutrients by plants. Plants have developed various strategies to thrive in nutrient-deficient environments. miRNAs play crucial roles in maintaining metal-ion homeostasis in plants. This majorly occurs by modulation of other nutrient uptake and metabolism pathways when a particular nutrient is scarce (Liang et al. 2015). During Pi-deprivation, the expression of miR156, miR399, miR778, miR827 and miR2111 is increased, while decrease in the expression of miR169, miR395 and miR398 is being observed in *Arabidopsis* (Hsieh et al. 2009). miRNA 399 has been found to be governing Pi-homeostasis by regulating the expression of PHO2 encoding ubiquitin-conjugating E2 enzyme, UBC24. During Pi-starvation, the upregulation of miR399 leads to decreased expression of UBC24 which inhibits uptake and translocation of Pi under low Pi environments. In *Arabidopsis*, these miRNA-mediated responses help in regulating Pi-homeostasis (Fujii et al. 2005; Bari et al. 2006).

Nitrogen (N) availability also modulates miRNA expression in plants. Under sufficient N-availability, miR167 expression is suppressed; as a consequence, the overexpression of ARF8 initiates lateral root formation in *Arabidopsis* (Gifford et al. 2008). miR169, miR171, miR395, miR397, miR398, miR399, miR408, miR827 and miR857 showed reduced expression upon N-starvation, while miR160, miR780, miR826, miR842 and miR846 were induced under N-starvation in *Arabidopsis* (Liang et al. 2012). miR395 is known to target ATP sulphurylases (APS1, APS3 and APS4) and sulphate transporter 2;1 (SULTR2;1/AST68). Both the target gene families regulate sulphate metabolism in plants. miR395 shows increased abundance upon sulphate starvation, indicative of its role in regulating sulphate metabolism (Allen et al. 2005; Jones-Rhoades and Bartel 2004). Yamasaki et al. (2007) proposed that under Cu-limiting conditions, miR398 targets copper/zinc superoxide dismutase and regulates Cu-homeostasis in plants.

20.7.6 miRNAs in Oxidative Stress Response

Change in redox state due to the accumulation of reactive oxidation species (ROS) occurs in both abiotic and biotic stresses encountered by plants. The amount of ROS production and turnover determines the levels of ROS accumulation, which greatly decides whether ROS would function as a defensive or destructive molecule. Imbalance in ROS accumulation during stress can lead to various types of damage to cell macromolecules and put plants under further stress (Apel and Hirt 2004; Mittler et al. 2004 and Miller et al. 2007). Under oxidative stress, Sunkar et al. (2006) reported decrease in the expression of miR398 and increments in the

expression of superoxide dismutase (SOD) proteins in *Arabidopsis*. Li et al. (2011) revealed that miR528 was downregulated, while miR169, miR397, miR827 and miR1425 were upregulated in H_2O_2-treated rice seedlings. The involvement of miRNAs in various abiotic stress responses in different plants is listed in Table 20.1.

20.8 Modulation of miRNA-Target Module Expression for Biotic and Abiotic Stress Tolerance in Plants

It has been established that miRNAs are important players in regulating biotic and abiotic stress responses in plants. In the recent past, miRNA overexpression, mutant, target-mimic and resistant target lines have been deployed for functional characterization of miRNAs and generation of plants showing better biotic and abiotic stress tolerance. Transgenic *Arabidopsis* plants overexpressing miR398 exhibited decreased resistance to *P. syringae* infection (Li et al. 2010). Overexpression of miR7695 in rice confers resistance against *Magnaporthe oryzae* infection (Campo et al. 2013). Li et al. (2014b) demonstrated that overexpression of miR160a and miR398b in rice showed increments in H_2O_2 accumulation at the site of infection, led to upregulation of some plant defence genes and resulted in decreased growth of fungus *M. oryzae*. Overexpression of either miR400 or miR844 in *Arabidopsis* showed decreased resistance to pathogenic bacteria (*P. syringae*) or fungi (*Botrytis cinerea*) (Park et al. 2014; Lee and Yeom 2015). Recently, Zhang et al. (2019) demonstrated that transient expression of *Md-miRln20* in susceptible apple variety leaves led to reduced expression of *Md-TN1-GLS* and reduced incidence of glomerella leaf spot disease of apple. Various strategies are being developed to protect plants from herbivorous insect attack. Recently, in one such attempt, Bally et al. (2020) engineered pre-microRNA (pre-miR) of insect and expressed them in transgenic *Nicotiana benthamiana* plants to produce artificial microRNAs (amiRs) which could target insect genes. Abnormal development along with increased mortality rates and delayed growth rates were observed for *H. armigera* fed on the leaves of these transgenic plants.

The miR169 overexpressing tomato plants exhibited less water loss due to decreased stomatal opening and reduced transpiration rates, conferring drought tolerance in tomato (Zhang et al. 2011b). Overexpression of miR159 in rice resulted in increased sensitivity of plants to heat stress, hence reduced expression of miR159 might contribute to increased tolerance of rice to heat stress (Wang et al. 2012). Enhanced stress tolerance under both Pi starvation and salt treatments was observed in tobacco plants overexpressing miR408 (Bai et al. 2018). Overexpression of miR166-resistant target forms in rice exhibited higher resistance to drought, similarly the short tandem target mimic of miR166 (STTM166) also showed significantly higher drought tolerance (Zhang et al. 2018). The miR164b-resistant mutant for *OsNAC2* showed significant increase in drought tolerance (Jiang et al. 2019). Ghr-miR414c is known to target iron SOD (*GhFSD1*) in *G. hirsutum* plants. While ectopic expression of *GhFSD1* exhibited increased salinity tolerance in *Arabidopsis*, plants with constitutive ghr-miR414c expression exhibited

Table 20.1 List of abiotic stress-responsive miRNAs in plants

Heat		
miR400	*Arabidopsis*	Yan et al. (2012); Li et al. (2014a); Park et al. (2014)
miR398	*Arabidopsis*	Guan et al. (2013)
miR5175	Barley	Kruszka et al. (2014)
miR827	*Triticum, Panicum*	Lin et al. (2010); Xin et al. (2010); Hivrale et al. (2016)
miR168, miR166c-3p, miR166g-3p, miR6300	Tomato	Zhou et al. (2016)
Cold		
miR319c, miR398a, miR393	*Arabidopsis*	Sunkar and Zhu (2004)
miR169	Rice, wheat, grapevine, poplar	Lv et al. (2010); Chen et al. (2012c); Tang et al. (2012); Sun et al. (2015a)
miR167 and miR395	Peach	Barakat et al. (2012)
miR159-5p, miR7723-3p, miR160f-3p, miR393	Almond	Karimi et al. (2016)
Drought and salinity		
miR169g, miR393	Rice	Zhao et al. (2007)
miR156	*Arabidopsis*	Liu et al. (2008)
miR156, miR164, miR166, miR167 and miR396 miR162, miR168, miR395, miR474	Maize	Ding et al. (2009)
miR398a/b, miR408, miR169	*Medicago*	Trindade et al. (2010)
miR396	Rice	Zhou et al. (2010)
miR168 and miR396	Tobacco	Frazier et al. (2011)
miR408	Peach	Eldem et al. (2012)
miR156, miR169, miR535, miR827	Cotton	Yin et al. (2012b)
miRX33, miRX34, miRX35	Barley	Hackenberg et al. (2015)
miR408	Cotton	Xie et al. (2015)
miR166	Rice	Zhang et al. (2018)
miR160 and miR165/166	*Arabidopsis*	Yang et al. (2019)
Osa-miR12477	Rice	Parmar et al. (2020)
Heavy metal		
miR393, miR171, miR156, and miR396	*Brassica*	Xie et al. (2007)
miR162, miR166, miR171, miR390, miR168, miR156, miR528	Rice	Ding et al. (2011)
miR159, miR160, miR319, miR396, and miR390	*Medicago*	Chen et al. (2012b)
miR159, miR162 and miR396	*Nicotiana*	Burklew et al. (2012)
miR156, miR162, miR165, miR167, and miR390	*Brassica*	Srivastava et al. (2013)
Metal-ion starvation		
miRNA 399	*Arabidopsis*	Fujii et al. (2005); Bari et al. (2006)

(continued)

Table 20.1 (continued)

Heat		
miR400	*Arabidopsis*	Yan et al. (2012); Li et al. (2014a); Park et al. (2014)
miR398	*Arabidopsis*	Yamasaki et al. (2007)
miR167	*Arabidopsis*	Gifford et al. (2008)
miR156, miR399, miR778, miR827 and miR2111, miR169, miR395 and miR398	*Arabidopsis*	Hsieh et al. (2009)
miR169, miR171, miR395, miR397, miR398, miR399, miR408, miR827, miR857	*Arabidopsis*	Liang et al. (2012)
Oxidative stress		
miR398	*Arabidopsis*	Sunkar et al. (2006)
miR169, miR397, miR827, miR1425, miR528	Rice	Li et al. (2011)

comparatively less salinity tolerance. Double mutant of miR160 and miR165/166 showed compromised drought tolerance as compared to their individual STTM counterparts (Yang et al. 2019).

20.9 Current Global Status of miRNA-Based Research on Plant Stress Responses

Increased human intervention due to industrialization, international trade and tourism, and unplanned urbanization has led to changes in global climatic conditions. These changes have affected agricultural productivity worldwide. Increase in international trade has led to the introduction plant diseases caused by various pests to new geographical locations. This has also led to occupation of agricultural land by invasive alien weeds and other alien plant species posing threat to native biodiversity of that area (Hulme 2009; Ding et al. 2020b). The current worldwide scenario of plant miRNA-based research is still mainly focused on analysing the miRNA expression under certain specific/combined stresses. Such studies are mainly being accomplished using high-throughput miRNA sequencing and bioinformatics tools for elucidating miRNA expression, and target prediction (Wani et al. 2020). Though vast number of conserved and novel miRNAs have been identified in different plant species, the functional characterization of miRNAs using transgenic lines (mutant, overexpression, STTM) has only been done for few candidate miRNAs. Worldwide, researchers have generated and used these transgenic lines to study miRNA-responsiveness to bacterial and fungal infection, heat, drought, metal-ion, salinity stresses in various plants (Li et al. 2010; Zhang et al. 2011b; Wang et al. 2012; Park et al. 2014; Lee and Yeom 2015; Bai et al. 2018).

Recently, Tripathi et al. (2019) elucidated preferential expression patterns of miRNAs in three natural populations of *A. thaliana* growing among wide altitudinal gradients of Indian Himalayas. More of such natural plant population-based studies

could be important in further understanding of miRNAs-mediated modulation of plant physiology and architecture which cold suit growth in a particular geographical location. Modulation of such candidate miRNA expression could be helpful in development of native plant cultivars which could adapt to stresses without compromising other valuable quality and quantity traits specific to them. In conclusion, though the advances in genomic techniques have made available vast amount of miRNA expression data in different crops worldwide, the ultimate success will depend on utilization of this information for improvement of crops for stress tolerance.

20.10 Conclusions and Future Perspectives

Different studies have shed light on the conserved and novel role of miRNAs in regulating the plant biotic/abiotic stress responses. The perception of external stress stimulus by plants is followed by crosstalks among various hormonal and molecular regulatory pathways. These crosstalks ultimately channelize the plant machinery for miRNA-mediated regulation of gene expression to generate a counter stress response. High-throughput sequencing has identified large number of conserved and novel miRNAs showing differential expression during specific or combined stresses. The predicted miRNA-target genes have been found to play diverse roles in disease resistance, hormonal regulation and metal-ion homeostasis. Modulation of miRNA expression by generating miRNA overexpression, mutant, target-mimic and resistant target lines has begun to be further deployed for functional characterization and development of stress-tolerant plant varieties. This has led to a paradigm shift in our understanding of post-transcriptional regulation of stress management in plants. The already available knowledge invites further functional characterization of miRNA-target modules for in depth understanding of their roles in stress management. Today, the advancements made in bio-engineering techniques have made gene characterization processes more effective and quick. Application of genome editing tools like clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 for engineering miRNA-mediated gene regulation could pave way for development of stress-tolerant crop varieties.

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The Role of Nanofertilizers in Smart Agriculture: An Effective Approach to Increase Nutrient Use Efficiency

21

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Abstract

Outburst of world population in the recent times has forced the agricultural sector to increase crop productivity to satisfy the huger of billions of people especially in under-developed and developing countries. The use of large quantities of chemical fertilizers increased crop production, but on the other hand disturbed the soil mineral balance and decreased soil fertility. In recent years, nanotechnology has extended its relevance in all the sectors including agriculture. Nanofertilizers are the novel, low-cost, eco-friendly fertilizers having the potential role in agriculture. The role of these smart fertilizers in plant and soil systems has been well documented and can act efficiently for enhancement of agricultural productivity. Nanoparticles have high surface area, sorption capacity and controlled-release kinetics to targeted sites making them "smart delivery system". Nanofertilizers are the important tools in agriculture to improve crop growth, yield and quality parameters with increased nutrient use efficiency, reduced wastage of fertilizers and cost of cultivation. Nanofertilizers increase crop growth up to optimum concentrations and further increase in concentration may inhibit the crop growth due to the toxicity of nutrient. The rapid progress of nanotechnology in other key industries may over time be transferred to agricultural applications as well and

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facilitate their development. In this chapter, we have summarized the recent advancements in the nanofertilizers research, viz. synthesis and its applications.

Keywords

Nanotechnology · Nanofertilizers · Nutrient use efficiency · Smart fertilizers

21.1 Introduction

Nanofertilizers are modified version of traditional fertilizers being prepared by encapsulating plant nutrients into nanomaterials employing thin coating of nanomaterials on plant nutrients and delivering in the form of nano-sized emulsions. Nanotechnology offers an unmatched opportunity in developing unique and concentrated source of plant nutrition which is high in absorption rate and more utilization efficacy as smart delivery system (Pirzadah et al. 2020). The term "nano" adapted from the Greek word meaning "dwarf" means 10^9 or one billionth part of a metre, thus nanotechnology is directed towards understanding and creating improved materials, devices and systems that exploits nanoscale properties. Nanoparticles have high surface area to volume ratio, nanometre regime and unique properties, which make them highly applicable in various fields such as electronics, energy, remediation, automobile, space technology and life sciences. It has great potential in biological and medical applications such as gene and drug delivery, biosensing, diagnostic and tissue engineering. Nanotechnology provides new interdisciplinary venture into agriculture and food sciences by converging science and engineering. It promises significant contribution to agricultural research, which can lead to new avenues for solving numerous agricultural problems (Mikkelson 2018). Nanoparticles are having potential applications in agriculture system, viz. detection of pollutants, plant diseases, pests and pathogens; controlled delivery of pesticide, fertilizers, nutrients, and genetic material; and can also act as nanoarchitects in formation and binding of soil (Iqbal 2019). Nanoparticles directly or indirectly affect plant growth and development. Nanoparticles can have varied compositions from being composed of metal oxide, ceramics, silicates, magnetic materials, quantum dots, lipid, polymers and dendrimers to emulsions (Solanki et al. 2015).

Composition of nanoparticles plays a significant role in their application right starting from agrochemical carrier using polymer-coated particles to soil remediation and sensor technology using metal nanoparticles(Aslani et al. 2014). Outburst of world population in the past decade has forced for higher agriculture productivity to satisfy the needs of billions of people especially in developing countries. Widespread existence of nutrient deficiency in soils causes both great economic losses for farmers and significant decreases in nutritional quality and overall quantity of grain for human beings and livestock. In addition, the utilization of most of the macronutrient is very low due to their inversion to insoluble form in soil. In general, only half of the total micronutrient present in chemical fertilizer is used by plants. The remaining minerals may leach down and become fixed in soil or contribute to air

pollution. So taking long run into account, chemical fertilizers may not a suitable option as they affect soil health as well as human health simultaneously to an extent which is irreparable. Considering the above-mentioned points, there is an urgent need to develop smart materials that can systematically release chemicals to specific targeted sites in plants which can be beneficial in controlling nutrition deficiency in agriculture. "Smart delivery system" means combination of specifically targeted, highly controlled, remotely regulated and multifunctional characteristic to avoid biological barriers for successful targeting(Nair et al. 2010). However, being an infant technology, the ethical and safety issues surrounding the use of nanoparticles in plant productivity are limitless and must be carefully evaluated before adapting the use of the so-called nanofertilizers.

21.2 Conventional Fertilizers Versus Nanofertilizers

Conventional fertilizers are generally applied by directly onto crops (through spraying) or indirectly through soil (through broadcasting and soil application). However, one of the major factors that decides the mode of application is the final concentration of the fertilizers reaching to the plant. In practical scenario, very less concentration (much below to minimum desired concentration) reaches to the targeted site due to leaching of chemicals, drift, runoff, evaporation, hydrolysis by soil moisture and photolytic and microbial degradation. It has been recorded that 40–70% of N, 80–90% of P and 50–90% of K present in applied fertilizers are leached out in the environment instead of reaching to plant. These problems have initiated repeated use of fertilizer and pesticide in a single crop cycle, which affects the nutrient balance present in the soil. World demand is projected to reach 192.8 Mt. by 2016–2017. Tilman et al. (2002) reported that excess use of fertilizers and pesticide increases pathogen and pest resistance, reduces soil micro-flora, diminishes nitrogen fixation, contributes to bioaccumulation of pesticides and destroys habitat for birds. Hence, it is very important to understand and reduce the use of chemical fertilization in providing crop nutrition and to reduce the associated environmental risks. Accordingly, it can be favourable that other methods of fertilization may also be tested and used to provide necessary nutrients for plant growth and yield production, while keeping the soil structure in good shape and the environment clean(Miransari 2011).

Nanotechnology has provided the feasibility of exploring nanoscale or nano-structured materials as fertilizer carriers or controlled-release vectors for building of the so-called smart fertilizers as new facilities to enhance the nutrient use efficiency and reduce the cost of environmental pollution (Solanki et al. 2015). For example, encapsulation inside nanomaterials coated with a thin protective polymer film or in the form of particles or emulsions of nanoscale dimensions. Surface coatings of nanomaterials on fertilizer particles hold the material more strongly due to higher surface tension than the conventional surfaces and thus help in controlled release. Materials such as sand and gravel are employed in commercial applications. In this case, nutrient solution is flushed from one end and old solution is removed from the

other end. The disadvantages with this method are frequent pathogen attack and high moisture rates, which may cause over wilting of soil-based plants.

21.3 Smart Fertilizer: Nanofertilizer

Fertilizer delivery to plants is like drug delivery system for human being and animals. Fertilizers are material that provide nutrients to plants and also improve soil fertility. They are the most productive way of promoting crop production and to upgrade the quality of food. Fertilizers help to increase plant growth by providing boosts of the much-needed macronutrients N, P and K to the plant as well as other nutrients. But plants do not get the chance to use more than half of the nutrients in fertilizers because the compounds escape through leaching. For example, greenhouse gas emissions and hypoxia are just two of the negative environmental effects of the too much application of chemical fertilizers for major crops. Hypoxia is the scientific word for a "dead zone" area in a body of water, nearly deprived of oxygen as the outcome of agricultural water runoff carrying nitrates and phosphorus from fertilizer, e.g., the 6–7000 square mile dead zone in the Gulf of Mexico. The usage of nanotechnology could lead to the solution of such problems. The use of a variety of plant materials for the biosynthesis of nanoparticles is considered a green technology because it does not involve any harmful chemicals. For the tech-savvy farmers or gardeners, nanofertilizer could soon solve the problem of nutrient depletion and environment. Molecular modified materials, which are synthesized with the help of nanotechnology, used to amend the soil for a better yield and enhance crop quality and quantity, are called nanofertilizers. Nanofertilizers are substances that release nutrients as plants need them. Nanoscience has found utilization in controlling the release of nitrogen, characterization of soil minerals soil development, nature of soil and nutrient ion transport in soil-plant system. Nanotechnology has accelerated new ways to boost nutrient efficiency and minimize costs of environmental safety. Nanofertilizers can be further classified in three classes as shown in Fig. 21.1. Nanofertilizers reduce nitrogen loss as leaching, emissions and long-term incorporation by soil microorganisms by slow and controlled release of fertilizers

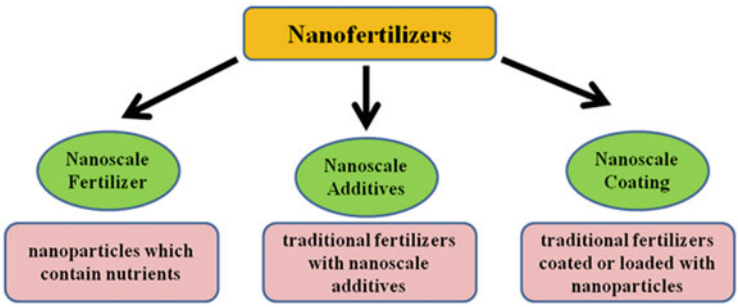

Fig. 21.1 Classification of nanofertilizers

hence, soil become more porous by decreasing toxic effects related with fertilizers over use. Using nanofertilizer to control delivery of nutrients can be a powerful tool towards attaining sustainable agriculture and environment. Nanofertilizers with quick absorption and optimized release of nutrients to the plant are going to replace conventional fertilizers. Nanoparticles have made little progress in improving soil quality and disturbed land. Little progress has been made respecting the use of nanoparticles to improve soil quality and to reclaim barren lands. Nano-fertilizers among one of the technologies whose followers claim to raise crop yields while reducing the environmental damage. Possibly no claim is more appealing than increasing the efficiency of fertilizers and reducing the negative environmental effects due to chemical fertilizers through nanotechnology. Agricultural plants need optimized managements as well as appropriate weather and soil condition for maximum usage of environment potentials and therefore, best performance. Production of inorganic nano-materials from biological systems such as plants and microorganisms have several advances over other methods because it is simple, cost-effective, relatively reproducible, and often results in more stable materials. The cellular extracts from these organisms can be used to synthesize nanoparticles with different size, properties and chemical compositions. Biosynthesis of metal nanoparticles can be squeezed from distinct parts of the plant, which is the most efficient method of synthesis at a very low cost (Pattanayak and Nayak 2012). Dr. JC Tarafdar of the Central Arid Zone Research Institute under the Indian Agriculture Research Institute (IARI), New Delhi used bottom up approach (Sol–gel method) to develop ZnO nano particle to check the effect of ZnO nanoparticles (spherical, 16–30 nm) on production of polysaccharides, phosphatases and phytase on two fungi *Aspergillus terreus* CZR1 (JF 681300) and *Aspergillus flavus* CZR2 (JF68130) and results suggested that ZnO nanoparticles were very effective in synthesis of fungal polysaccharides and phosphatases compared to bulk ZnO.

21.4 Methods of Application of Nanofertilizers

Nanofertilizers are applied either to soil and/ or leaves (Fig. 21.2). Soil is the important natural resource, which provides macro- and micronutrients to the plants. It supports plant health for growth and development. Increased productivity can be attaining through supplemental plant nutrition by the means of soil application (root feeding) and foliar application (foliar feeding). Soil application of nutrients is the most common approach, but it has many drawbacks with respect to availability of nutrients to the plants. The inorganic nutrients are trapped in the soil as insoluble forms, which are subjected to leaching by rain and irrigation water (Alshaal 2017). These all limitations can be overcome by foliar application. In addition to that, foliar feeding has proved to be the fastest way of providing nutrient deficiencies and increasing yield and quality of crop (Roemheld 1999), thereby minimizing environmental pollution and improving nutrient utilization by reducing the amount of fertilizers supplemented to the soil (Abou-El-nour 2002). Even though leaves permit gaseous exchange, but cuticle present in the leaves restricts the penetration of

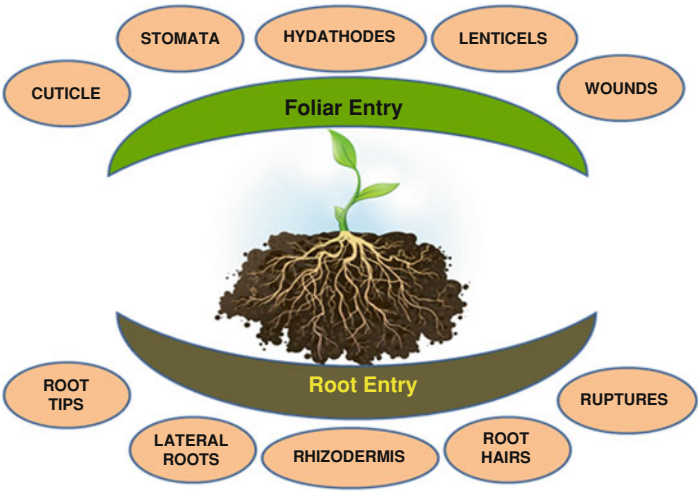

Fig. 21.2 Method of application and mode of entry of nanofertilizers into plant systems

substances (Perez-de-Luque 2017). The nano-coated substances increase the penetration via stomata with a size exclusion limit above 10 nm. The nanocarriers deliver the nutrients in the exact place and at the right time, which reduce the extra amount of active chemicals deposited in the plant system and increase the nutrient use efficiency. Hence, foliar application of nanofertilizer leads to higher nutrient use efficiency (NUE) and provides rapid response to the growth of crop plants.

21.5 Biosynthesis of Nanoparticles

21.5.1 Phosphate-Based Nanoparticles as Fertilizers

Phosphorus is an important nutrient and phosphate-based nanoparticles have capacity to be used as phosphorous nanofertilizer for agriculture use. These are applicable for heavy metal remediation by forming highly insoluble and stable phosphate compounds. Vivianite (Fe3 (PO4)2·8H2O) particles (~10 nm) and apatite (Ca5 (PO4)3Cl) particles (<200 nm) are phosphate-based nanoparticles. Vivianite nanoparticles synthesized with sodium carboxymethyl cellulose (CMC) as a stabilizer for in situ immobilization of lead in soils. Vivianite can effectively reduce the TCLP (toxicity characteristic leaching procedure) leach-ability and PBET (physiologically based extraction test) bio-accessibility of Pb2+ in calcareous, neutral and acidic soils. Compared to soluble phosphate used for in situ metal immobilization, use of the vivianite results in 50% decline in phosphate leaching into the environment (Liu and Zhao 2007).

21.5.2 Nitrogen-Based Nanoparticles as Fertilizers

Nitrogen is a key nutrient source for biomass production in agriculture and most important element in fertilizers. Conventional fertilizers are with particle size dimensions greater than 100 nm. These are missing to the soil via leaching, emission as ammonia and nitrogen oxides and soil microorganism-mediated incorporation into soil organic matter over time. Nowadays, the emerging nano-strategies point out that due to the high surface area to volume ratio, nanofertilizers are expected to be far more effective than even polymer-coated conventional slow-release fertilizers. A nano-strategy involving a slow-release fertilizer combination based on urea-modified hydroxyl-apatite (HA) nanoparticles encapsulated into the cavities exist in soft wood. HA (Ca_{10} $(PO_4)_6(OH)_2$) nanoparticles are rated as one of the outstanding candidates in agricultural applications, which can supply phosphorus nutrient.

21.5.3 Iron-Based Nanoparticles as Fertilizers

Iron is the metal used at the active site of many essential redox enzymes dealing with cellular respiration and oxidation reduction in plants and animals (Tarafdar et al. 2012). Iron is abundant in soil and its compounds make up to 5% of earth crust weight. In spite of large amount, due to it less accessibility, its shortage is common for plants. Most of present iron is insoluble. Only a small amount of iron is soluble ($Fe(OH)_2$, $Fe(OH)^{+2}$, Fe^{+3}, Fe^{+2}). Iron is a crucial element for growth of plants; lack of iron causes chlorosis and significantly reduction in photosynthesis activity and consequently biomass produced. Iron chelate nanofertilizers can be recognized as a rich and decisive source of bivalent iron for plants because of its high stability and slow release of iron in a broad pH range (3–11). Absence of ethylene compounds in its structure is a benefit of this nanofertilizer. Ethylene enhances growth progress and restricts chlorotic leaves. Second advantage of these nanofertilizers is increasing ratio of ferrous iron to ferric iron in chelate surface, which results in increasing synthesis of chlorophyll in plant.

21.5.4 Zinc-Based Nanoparticles as Fertilizers

Zinc deficiency has been identified as one of the main problems limiting agricultural productivity in alkaline calcareous soils. Thus, zinc is often included in macronutrient fertilizers to enhance crop production and quality. The addition of zinc fertilizers to soil, zinc transforms gradually from more active and available fractions into less available species such as precipitates (i.e., $ZnCO_3$) and adsorbs to oxide phases (e.g., Fe-, Al-oxides). Moreover, availability of zinc to plants in calcareous soils mainly depends on the diffusion of zinc from fertilizer granules to the plant root. Nanomaterials could be applied in designing more soluble and diffusible sources of zinc fertilizer for increased plant productivity. The smaller size, higher specific

surface area and reactivity of nano-particulate ZnO compared to bulk ZnO may affect zinc solubility, diffusion and hence availability to plants.

21.5.5 Titanium-Based nanoparticles as Fertilizers

The nano TiO_2 (anatase) improved light absorbance and promoted the activity of rubisco activase thus accelerated plant growth. Anatase improved plant growth by enhanced nitrogen metabolism and accelerating conversion of inorganic nitrogen into organic nitrogen, thereby fresh weights and dry weights increase. Effects of anatase on the content of light harvesting complex II on thylakoid membranes showed an increase in LHC II content (Liu and Zhao 2007). These promote energy transfer and oxygen evolution in photo-system II. It has also been found that anatase promoted antioxidant stress by decreasing the accumulation of superoxide radicals, hydrogen peroxide, malonyldialdehyde content and enhanced the activities of superoxide dismutase, catalase, ascorbate peroxidase, guaiacol peroxidase. The ability of anatase to improve the light harvesting complex content of plants is highly comparable with the use of TiO2-quantum dot (QD) assembly for the conversion of solar energy. Uptake and distribution of QD by the plant cells can be exploited for increased solar energy trapping that might improve the photosynthetic efficiency of plants.

21.5.6 Aluminium-Based Nanoparticles as Fertilizers

Nano-aluminium has been used in various industries and hence there is great chance for the interaction of such nanomaterials with higher plant that constitutes a major portion of the ecosystem. Pure alumina nanoparticles (13 nm) without any modifications reduced root elongation in various plants like *Zea mays, Cucumis sativus, Glycine max, Daucus carota* and *Brassica oleracea,* thus potentially retarding the growth of plants. However, it was surprisingly observed that when nanoparticles were loaded with phenanthrene (polycyclic aromatic hydrocarbons), their toxicity significantly decreased showing no adverse effects on roots of plants. This showed the relevance of proper surface modifications, which reduce the phytotoxicity of nanoparticles. The impact of aluminium oxide and aluminium oxide with carboxylate ligand coating particles (100 nm) on plants shows no adverse effect on the growth. Aluminium concentration in rye grass was increased 2.5-fold above control tests, whereas no uptake of aluminium was observed in kidney beans which inferred the difference in uptake and distribution efficiency of even the same kind of nanoparticles by different plants.

21.5.7 Copper-Based Nanoparticles as Fertilizers

The seed germination in the presence of Cu NPs showed an increase in shoot to root ratio compared to control plants. Different flora and fauna gives versatility in their response to nanoparticles. It is necessary to evaluate the secure effective concentration of each group of nanoparticles before their application that lower the risks of eco-toxicity to a very large extend.

21.5.8 Silver-Based Nanoparticles as Fertilizers

Silver nanoparticles have antimicrobial properties to control various phytopathogens. Effect on the seed germination and root growth of plants in hydroponic solution enhanced with Ag NPs showed no negative effects. On the other side, a decrease in plant biomass and transpiration was observed on prolonging their growth in presence of Ag NPs (Stampoulis et al. 2009). The cytotoxic and genotoxic impacts of Ag NPs were analysed with root tips of onion. It was observed that Ag NPs impaired the stages of cell division and caused cell disintegration. These results show the requirement for a more cytotoxic and genotoxic evaluations by considering the properties of nanoparticles, their uptake, translocation and distribution in different plant tissues (Malik and Kumara 2014).

21.6 Advantages

Enhancement in fertility and the instinct of self-preservation greatly enhance the productivity, quality and reliability of a crop. This leads to three major areas of experiments:

1. *Yields*: Nanofertilizers increase yields by an average of 20% and for some crops even more. These numbers indicate overall growth in plants collectively. For example, in some experiments, sunflower grain yields increased by 50% and in cucumber trials yield increased up to 25%.
2. *Nutritional value*: Results showed an increasing effect of about 10% in both protein and sugar content of treated plant for most types of crops plant.
3. *Health*: Overall health of the plant is enhanced, making it more resistant to severe weather and extreme environmental conditions. Immunological response is allowing the plant to fight with disease and prevent infections.

21.7 Potential Risks

Although nanotechnology has incredible potential to revolutionize many aspects of human life, the benefits may come with some price. One of the major questions faced by the world before accepting nanotechnology is whether the unknown risks of

nanoparticles involving their environmental and health impact prevail over their potential benefits. The risks associated with the application of nanoparticles are yet to be evaluated before fully implementing this technology. This consideration has resulted in consideration of "nanotoxicology", which is responsible for understanding of toxicological potential and promoting safe use of nanoparticles. A systematic and thorough quantitative analysis regarding the potential health impacts, environmental clearance and safe disposal of nanoparticles can lead to improvements in designing further applications of nanotechnology (Meng et al. 2009). Although no direct human disease has been linked to nanoparticles so far, early experimental studies indicate that nanoparticles could initiate adverse biological responses that can lead to toxicological outcomes. A nanoparticle being an ultrafine particulate matter enters into the human/animal body through respiratory or through skin. There is also a common assumption that small size of nanoparticles can easily enter into tissues, cells and organelles and interact with functional biomolecular entities (i.e. DNA, ribosomes) due to the actual physical size of an engineered nanostructure and its similarity to many biological molecules. A corollary is that the entry of the nanoparticles into vital biological systems could cause damage, which could subsequently cause harm to human health. However, one of the most disgustful scenarios is the lack of concrete technical data on toxicological aspect of nanoparticles giving opportunity to both nanotechnology proponents and opponents to make contradictory, unscientific and sweeping conclusions about the safety of nanoparticles. This atmosphere of uncertainty is precisely the feature of nanotechnology that causes cynics the greatest concern. So due to this, arises need for proper characterization and understanding appropriate exposure protocols and methods for assessing nanoparticles outcome with respect to environment, as well as in living organisms. Once these issues are addressed, optimal experimental conditions could be established in order to identify if a particular nanoparticle poses a threat to human health. Thorough research between materials scientists, environmentalists and life scientists can be utilized to overcome these shortcomings in identifying hazards of nanotechnology. Sadly, the risk assessments of nanotechnology are partly subjective and likely to be highly politicized. Disastrously, no single scenario for describing risks and controls can be universally applied to conclude the outcome due to the heterogeneous and developmental nature of nanotechnology. Absence of standardized methods and SOPs makes it difficult to compare the safety/toxicity from different research organizations. Moreover, maintaining specificity regarding experiments design, alternative assessments are required for consideration of ethical, social and political values relating to policies involving in nanotechnology. Before studying data related to toxicology, it is essential to calculate and determine the expected concentrations of nanoparticles that may be present in the ecosystem. The use of nanotechnology in agriculture is significantly important as it directly affects humans. Hence, the concentration of nanoparticles present in nano-fertilizers should be considered with respect to the critical exposure and toxicity to other biological systems. As discussed in most of the studies regarding the use of nanoparticles for promoting growth of plants with a focus on using lower concentrations of

nanoparticles, it can be argued that it will pose insignificant health and environmental damage.

Many countries are investing significantly in its applications to food production as they have identified the potential of nanotechnology in the food and agriculture sectors. However, owing to our limited knowledge of the human health effects of these applications, these countries recognized the need for early consideration of the food safety implications of nanotechnology. As suggested by the scientific committee of the European Food Security Authority (EFSA), "the risk assessment paradigm is applicable for nanoparticles". It is most likely that different types of nanoparticles vary with respect to their toxicity. The available data on consequent toxicity are limited. The risk assessment of nanoparticles has to be performed on an individual basis. Various parameters may be included in deciding the risk associated with the use of any particular nanoparticle in food and feed. These include physico-chemical characterization of nanoparticles, its stability in the food and feed, exposure scenario of the nanoparticles from food and feed, and toxicokinetics (absorption, distribution, metabolism/biotransformation, and excretion/elimination) within the human and animal systems. The Nanotechnology Regulatory Science Research Plan of the US Food and Drug Administration (FDA) lays out a frame-work and implementation plan to provide coordinated leadership on regulatory science activities and issues related to FDA-regulated products that either contain nanoparticles or otherwise involve the application of nanotechnology to address key scientific gaps in knowledge, methods or tools needed to make regulatory assessments of these products.

21.8 Current Status of Research on Nanofertilizer Across the Globe

Researchers across the globe are focused on the future role of nanofertilizer for solving agricultural issues related to productivity and nutrition of crops. Most of the studies showed the positive impact of application of nanofertilizers such as improved nutrient absorption capacity, increased crop growth, seed yield and other yield components of agricultural crops. Naseem et al. (2020) synthesized mesoporous nanocomposite of zinc aluminosilicate ($ZnAl_2Si_{10}O_{24}$) using co-precipitation method. They loaded the nanocomposite with urea to study the delivery of both zinc and urea to *Oryza sativa L.* They found significantly higher yield in case of urea loaded $ZnAl_2Si_{10}O_{24}$ nanocomposite as compared to conventional fertilizers. The potential of nano copper (Cu) compounds has been checked for improvement of the physiological performance and agronomical parameters of *Medicago sativa* by Ruiz et al. (2020). They found plants treated with bulk/nano Cu showed better agronomical responses and improved the physiology of alfalfa. Further, the Fe and Zn content in roots and Fe content in leaves were increased as compared to controls. Ramirez-Rodriguez et al. (2020) prepared K- and N-doped nanoparticles by green synthesis. These nano U-NPK nanoparticles were tested to study the reduction of supply of nitrogen to durum wheat. They found that the amount of nitrogen supplied to the plants reduced by 40% with respect to a conventional treatment. Jahangirian

et al. (2020) studied the effect of smart fertilizers on crop yield and soil productivity. They prepared Zeolite/Fe2O3–NCs using quick green precipitation method. These nanocomposites can act as an iron smart nanofertilizer as they slowly released iron ions and they are non-toxic in nature. Sharma et al. (2020) reported the effect of chitosan nanofertilizer comprising of copper (Cu) and salicylic acid (SA) on maize source activity. They applied this nanofertilizer by seed treatment and through foliar route on maize plants. They found that the source activity in developing maize plants was significantly up-regulated. Experiments were conducted by Ghasemi et al. (2020) to study the effect of nanofertilizers on the yield components and antioxidant properties of Dragon's head. The highest grain yield per plant, essential oil percentage and mucilage percentage were observed in nanofertilizers incorporated treatments. Abd El-Azeim et al. (2020) conducted field experiments to study the efficacy of nanofertilizers as compared to conventional chemical fertilizers. They found that the application of nanofertilizers in lower rate is comparable to full dose of conventional chemical fertilizers. Further application of these nanofertilizers significantly improved the potato productivity and quality.

Yusefi-Tanha et al. (2020) studied the impact of ZnONPs on seed yield of soil-grown soybean (*Glycine max* cv. Kowsar). They found significant influence of ZnONPs on seed yield, lipid peroxidation and various antioxidant biomarkers in soybean. Priyanka and Venkatachalam (2020) investigated the effect of CuO nanoparticle (CuONPs) on plants growth, physiological, biochemical and nutrients changes in shallot grown in soil amended with CuONPs. They found increase in shoot and root growth, biomass, photosynthetic pigments and antioxidative enzymes in CuONPs exposed plants as compared to the control. Muhemed and Mijwel (2020) studied the effect of nanofertilizer on Baher and Jamela hybrids of cucumber. They observed the increase in the percentage of dry matter, early yield weight and total yield weight of cucumber as compared to mineral fertilizers. Mazumder et al. (2020) synthesized and characterized zinc oxide nanoparticles (ZnONPs) and studied their beneficial effect in the growth and development of *Brassica juncea*. They concluded that nanoparticles can be used as an alternative of chemical fertilizers as they show positive impact on the growth of *Brassica juncea.* Few reports on worldwide researches on effect of nanofertilizer on plant growth and yield attributes of different crops are summarized in Table 21.1.

21.9 Conclusion

Widespread existence of nutrient deficiency in agricultural soils has resulted in significant decrease in crop productivity and great economic losses in agriculture. Conventional fertilizers increase productivity of crop, but their large-scale usage is not sustainable for long run as they are not fully accessible to plants. In addition, the utilization of most of the macronutrient is very low due to their inversion to insoluble form in soil. Delivery of macro- and micronutrients to the plants is an important aspect of application of nanotechnology in agriculture. Nanoscale or nano-structured materials as fertilizer carrier or controlled-release vectors for building of the

Table 21.1 Worldwide studies on effect of nanofertilizers on growth and yield of plant

	Different Nanomaterials	Size (nm)	Concentration (ppm)	Method of preparation	Treatment	Test Plant	Applications	References
1.	ZnO	36.8	20	Microwave assisted hydrothermal	Foliar spray	Squash	Improved plant growth and yield	Ahmed et al. (2019)
	Fe_2O_3	51.6		Green microwave assisted hydrothermal			Organic acids, protein, lipids, and energy were recorded at higher level	
	Mn_3O_4	56		Microwave assisted hydrothermal			Enhances plant growth and yield	
2.	ZnO	12–24	1000 and 2000 mg/l	Sonicator	Foliar spray	Habanero pepper plants (Capsicum chinense Jacq.)	At 1000 mg/l plant growth and development was promoted and at 2000 mg/l fruit quality, soluble solids and titratable acidity were increased.	Lopez et al. (2019)
3.	$ZnSO_4$ + nano-sized manganese hollow core shell	117.7–155.3	1.3–3.8	Layer by layer adsorbtion of opposite charge polyelectrolyte	–	Rice	Zn efficiency improved, while reducing the loss of nutrients and minimizing environmental pollution.	Yuvraj and Subramanian (2015)
4.	Nano zinc	18.5	–	Hydrothermal technique	Foliar spray	Pearl millet *cv*.HHB	Green eco-friendly approach to enhance crop production	Tarafdar et al. (2014)
5.	TiO_2	43	100 mg/l	Gel coating	Seed treatment	Eggplant, pepper, tomato	Promotes crop performance and nutrient uptake, accelerates the germination	Younes et al. (2020)

(continued)

Table 21.1 (continued)

	Different Nanomaterials	Size (nm)	Concentration (ppm)	Method of preparation	Treatment	Test Plant	Applications	References
6.	CuO	20–26	300 550 800	Co-precipitation	Seed treatment	Maize	Shoot and root elongation, increased chlorophyll content	Toqeer et al. (2020)
7.	CuO	100–1000	200 mg/kg	Mixed with soils	Soil treatment	Lettuce	Improved photosynthesis and biomass production	Wang et al. (2019)
8.	CuO	10–100	100 and 1000μg/mL	Spraying	Fruit spray	*Prunus domestica* fruits	Suppressed grey mould symptoms caused by *B. cinerea* and soil borne diseases	Malandrakis et al. (2019)
9.	AgO	6–36	5 10	Nanopriming solution	Seed treatment	Rice	Acceleration of seed germination and seed growth, enhanced starch metabolism and dehydrogenase activity	Mahakham et al. (2017)
10.	Ag		50 mg/l 75 mg/l	Mixed with pot soils	Soil treatment	Wheat	Accelerated growth and tolerance to heat stress	Iqbal (2019)
11.	Ag	9×10^{-4} M	50 mg/l 75 mg/l	Spraying	Foliar application	Cowpea Brassica	Enhanced growth and biomass by stimulating root nodulation and soil bacterial diversity	Pallavi et al. (2016)
12.	Al_2O_3	20	400 mg/l	Spraying	Foliar application	Tomato	Successfully controlled *Fusarium* root rot in tomato	Shenashen et al. (2017)
13.	Urea-hydroxyapatite (6:1)	15–20	–	Scale-up process		Rice	Urea released over longer period of time (up to one week) and slow release of nitrogen and other important nutrients	Kottegoda et al. (2017)

							compared to that of pure urea	
14.	Urea-hydroxyapatite	40–60	–	Sol–gel technique	Soil application	IR-36 rice	Enhanced crop growth and productivity without environmental damage and act as a potential alternative of conventional N and P fertilizers	Pradhan et al. (2020)
15.	Chitosan-K	–	–	–	–	Maize	Sustained nutrient release synchronize with crop demands reduced fertilizer requirement and increased productivity	Kubavat et al. (2019)
16.	Chitosan-NPK	10		CS-PMAA	Foliar spray	Wheat	Improved wheat grain quality	Abdel-Aziz et al. (2018)

so-called smart fertilizers can enhance the nutrient use efficiency and reduce the cost of environmental pollution. A nanofertilizer accurately releases their active ingredients in respond to environmental trigger changes and biological demands. Both in vitro and in vivo methods can be used for nanofertilizer delivery to the plants. Still, the uptake and translocation of nanoparticles in plants are not known, which result in the rise of various ethical and safety issues surrounding the use of nanofertilizers in crop plant productivity. Quantitative analyses are required to understand potential health impacts, environmental clearance and safe disposal of nanomaterials, which will lead to improvements in designing applications of nanofertilizers.

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Shifting Paradigm Towards the Crops: From Model Plants to Crops and Employing the Genome Engineering to Target Traits

22

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Abstract

Genome editing has become one of the most promising genetic engineering functional tools for crop traits improvement. As crops are the major staple food consumed globally and hence crop improvement in terms of yield and nutritional quality is requisite to feed a large population. Much work has been done for crop improvement using traditional breeding approaches yet because of the high labour and high time consumption, much faster methods are required. Also, the polyploidy of crops and the complex genome is still a bottleneck for improved trait development. Besides this, a difficult genetic transformation and low transformation efficiency are holding back the production of better crops. However, genome engineering has been proven to be an efficient and much faster biotechnological approach to overcome these challenges. The first-generation tools including zinc finger nuclease (ZFN) and transcription activator-like effector nuclease (TALEN) have been employed to a precise genome manipulation. But, because of complicated protein engineering and being expensive techniques, much simpler tools are required. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) technology is the recent and revolutionary technique that can create a precise and targeted genetic manipulation in the genome. The recent advances in CRISPR have accelerated genetic engineering even in polyploid crop systems. In this chapter, we provide an insight into the development of Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9 (CRISPR/Cas9) system as a genome engineering tool and various types of native and modified CRISPR effector proteins and their different application.

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The major focus of the chapter is the use of CRISPR technology and its advancement for the plant trait modifications.

Keywords

Genome-editing · Crops · CRISPR/Cas9 · Hexaploid wheat · Biofortification

22.1 Introduction

CRISPR/Cas9 system is an adaptive immune system in bacteria against phage infections (Marraffini and Sontheimer 2010; Deveau et al. 2010; Koonin and Makarova 2013; Makarova et al. 2013; Barrangou and Marraffini 2014). The bacteria capture small fragments of DNA from the attacking virus and generate an array of short palindromic repeated sequences containing amid viral DNA. This array is known as CRISPR array and the viral DNA fragments are called protospacers. The protospacers are short DNA sequences generally of 20 base pairs. This array makes the bacteria to keep a memory of invading viruses or the closely related ones. Furthermore, the bacteria transcribe RNA fragments against the same viral DNA from the CRISPR array that guide Cas9 protein to cleave the target viral DNA and makes it dysfunctional. The array was discovered in 1987 for the first time in *Escherichia coli* during the study of genes involved in phosphate metabolism (Ishino et al. 1987), but its function was not known until 2007 (Barrangou et al. 2007). In *E. coli,* certain unusual DNA sequences were found by Ishino et al. (1987), but due to the lack of genomic information, it was difficult to predict the function of the array. Due to the high similarity and conservation of the CRISPR array, it was also observed in archaebacteria and other eubacteria. The first important clue of the function of CRISPR array was found in the mid-2000s when the protospacers were studied and they were found to be similar to viral DNA. The study was conducted by three groups individually (Mojica et al. 2005; Pourcel et al. 2005). Further studies also confirmed similar observations (Bolotin et al. 2005). Subsequent studies revealed that the CRISPR system is involved in a similar mechanism in bacteria as RNAi in eukaryotes (Makarova et al. 2006). Finally, in 2007, it was experimentally proved that the CRISPR system is an acquired immune system of bacteria against bacteriophages (Barrangou et al. 2007). The study was conducted in *Streptococcus thermophilus*, a short fragment of bacteriophage DNA was inserted as a spacer DNA inside the CRISPR array of *Streptococcus thermophilus* that made the bacteria resistant to that particular bacteriophage. Furthermore, this phenomenon was confirmed when a fragment of plasmid was inserted into the spacer DNA in *E. coli* and it did not let that plasmid to transform into the same bacteria (Marraffini and Sontheimer 2008). This paved new ways to use this system as a functional tool for gene silencing and led to discover an in vitro system to edit the genome. In 2012, it was demonstrated that the CRISPR/Cas system can cleave DNA in vitro (Jinek et al. 2012; Gasiunas et al. 2012). This led to the engineering of this system for further use as an RNA-guided DNA editing tool.

22.2 Types of CRISPR System

The CRISPR system is classified into two major classes—Class 1 and Class 2. This classification is based on the structure, the number of subunits and functional domains that provide the nuclease activity to different effector proteins. The Class 1 is a multi-subunit system that involves multiple Cas operons to perform its function. However, Class 2 is a single, multi-domain effector protein system (Makarova et al. 2015). Furthermore, based on the type of effector protein involved, each of the class is divided into three types. Class 1 and 2 are further classified as Type I, Type III, Type IV and Type II, Type V, Type VI, respectively. These subtypes have different effector proteins including Cas3, Cas9, Cas10, Csf1, Cpf1 and Cas13 for Type I, II, III, IV, V and VI, respectively (Table 22.1). In type I CRISPR system, Cas3 is the trans-acting helicase-nuclease that is recruited by RNA-guided multi-subunit complex known as Cascade (CRISPR-associated complex for antiviral defence). Although the Type I system is the most abundant CRISPR type in archaea and bacteria, however, this system is not used extensively as a tool for genome editing. Type II CRISPR system, however, is the extensively understood and majorly used genetic engineering tool. The effector protein of this

Table 22.1 Multiple types of CRISPR system

Class	Types	Subtypes	Cas Protein	PAM	Function
1 (multi sub-unit complexes of different Cas proteins)	I	1-A 1-B 1-C 1-D 1-E 1-F 1-U	Cas3		Cleavage: Double-stranded DNA
	III	III-A III-B, III-C III-D	Cas10	No PAM required	Cleavage: Single-strand break both in DNA and RNA
	IV	–			
2 (single multi-domain effector protein)	II	II-A, II-B, II-C	Cas9	3-NGG (SpCas9), 3-NNGRRT(SaCas9), 3-NNNNGATT (NmCas9)	Cleavage: Blunt-ended dsDNA break
	V	V-A (Cpf1), V-B (C2c1), V-C (C2c3)	Cas12	5-TTTN (FnCas12a)	Cleavage: 5 nt 5′ overhang dsDNA break
	VI	VI-A VI-B VI-C (C2c2)	Cas13	3-H (LshCas13a), 5-D and 3-NAN or NNA (BzCas13b), none (RfCas13d)	Cleavage: ssRNA

system is Cas9 that has nuclease activity for the double-stranded DNA break (DSBs) due to the presence of two domains HNH (His-Asn-His) and RuvC. The domains are responsible for cleavage at sense and anti-sense strands, respectively. Type II is the first CRISPR/Cas9 system used as a functional tool for RNA dependent genome engineering followed by Type V.

22.3 CRISPR/Cas9 Mechanism

Type II is the most widely used genome editing system. The native CRISPR/Cas9 defence mechanism of bacteria is comprised of the following components: (1) CRISPR array: is the part of bacterial genomic DNA that amid the viral DNA known as protospacer or spacer DNA flanked by repeated palindromic sequences. In general, the size of the protospacer is 20-23 nucleotides. (2) crRNA: is the processed spacer RNA that guides the Cas9-tracrRNA-crRNA complex to the target site and hybridize with the target site. (3) tracrRNA: is the trans-activating crRNA and contributes to the maturation of crRNA by directing it to cleave individual crRNA and besides this, it is also responsible for mobility of the complex to the target site. (4) RNase III: RNase III functions as a host that facilitates the maturation of crRNA (5) Cas9 protein: is the major effector protein of the CRISPR/Cas9 system that creates a DSB at the targeted site. In the native defence mechanism of bacteria through CRISPR/Cas9, there are three major steps involved in the cleavage of the invading foreign DNA. The very first step is to produce a mature crRNA also known as the guide RNA (gRNA). The second step of the mechanism is to produce a ribonucleoprotein complex of crRNA-Cas9-tracrRNA. The final step is the mobility of this RNP complex to create a DSB at the target site. In the first step, each protospacer DNA gets transcribed into individual crRNA. The protospacer is fully or partially complementary to the targeted foreign DNA. The complementarity between gRNA and target is the major basis of the CRISPR/Cas9 mediated immune system in bacteria. The gRNA will guide the Cas9 nuclease to its target cleavage site. The CRISPR array is transcribed into pre-crRNA. Furthermore, pre-crRNA is processed into an individual crRNA by a specialized trans-activating RNA which shows homology to the palindromic CRISPR repeats. The tracrRNA mediates the recruitment of Cas9 and RNAseIII enzymes. This enforces the separation of individual crRNA generating an individual ribonucleoprotein complex of crRNA-Cas9-tracrRNA. This mobile RNP complex binds to the target site which is complementary to the gRNA/crRNA. Apart from the complementarity protospacer adjacent motif (PAM) is required for the cleavage. Among the multiple Cas9 discovered from different bacterial strains, *Streptococcus pyogenes* CRISPR/cas9 (SpyCas9) system is the most understood mechanism. The PAM for spyCas9 is 3'-NGG. Once the complex is guided to its target, Cas9 creates a DSB at the target site. To sustain the host system it is important to repair this DSB. Now, if the break is repaired through non-homologous end joining (NHEJ), there is a high chance of insertion or deletion mutation known as INDEL mutations that would make the gene dysfunctional (Symington and Gautier 2011). Alternatively, if the repair mechanism is facilitated

by another template DNA that possesses homologous sequence flanking to the target site than it will be repaired through homologous direct repair (HDR) that enables DNA insertion at the target gene and hence enabling the gene insertion or replacement (Symington and Gautier 2011).

22.4 Genome Editing: From Model to Crop Plants

Since the discovery of CRISPR/Cas9 system as a genome editing tool, it has been used to edit different mammalian cell lines (Cong et al. 2013; Mali et al. 2013). Soon after these studies, in 2013, CRISPR/Cas9 system was used to edit plant genome and successful plant genome editing led to extensive use of this system as an efficient genome editing tool in plants. Initially, most of the studies were conducted on the model plant systems (Li et al. 2013; Nekrasov et al. 2013), however, a few studies were conducted on crop systems also (Shan et al. 2013). After these initial studies, multiple studies were conducted showing CRISPR/Cas9 based genome editing in plants (Mao et al. 2013; Upadhyay et al. 2013; Feng et al. 2013; Miao et al. 2013; Jiang et al. 2013). Genome editing in wheat has various hurdles to cross and hence the initial studies in wheat genome editing were lacking a stable plant transformation. Before wheat, CRISPR/Cas9 was used in rice for a stable transformation and the plants were generated from the transformed protoplast (Shan et al. 2013). But wheat being a recalcitrant crop, it is difficult to regenerate plant for the protoplast. Nonetheless, in 2014, stable transformation with genome editing was shown in wheat and powdery mildew resistant wheat plant was generated by editing *TaMLO* gene (Wang et al. 2014). Although the researchers produced a homozygous line with editing in all the A, B and D genomes (*aabbdd*), however, the transformation efficiency was very less. Furthermore, the same research group produced stable genome-edited and transgene-free lines using transiently expressed CRISPR/Cas9 DNA or RNA (Zhang et al. 2016). These studies led to the further advancement of genome editing in wheat.

22.5 Working with CRISPR in Crops

Working with crop systems could be challenging because of the complex genome and difficult genetic transformations. Moreover, polyploid crops like wheat might show much more off-targets that would be difficult to map. However, with the advancement in CRISPR technology, new optimized methods and various online tools, it has become possible for researchers to overcome these challenges. In wheat, various homoeologs contribute differently to gene expression. Hence, two kinds of genome editing could be employed in wheat that includes either to target specific homoeologs or the conserved region among all the homoeologs of a gene. Using the online tools that are linked to the genomic database of crops has made it possible to avoid off-targets and identify an efficient and unique target site. Some of the available online tools include CRISPR plants (Xie et al. 2014), CHOP CHOP

(Labun et al. 2019), BREAKING CAS (Oliveros et al. 2016), etc. These selected target sites could be validated both in in vitro using RNP complex and in vivo using protoplast transient transformation. The most efficient target site is considered and targeted in the host plant genome. Furthermore, genetic transformation remains the final barrier. Tissue culture-based genetic transformation has been extensively used for genetic transformation either with *Agrobacterium* or with particle bombardment. With a selection free tissue culture, it is now possible to generate non-transgenic plants with desired genome editing. However, a stable transformation has been a major obstacle in functional genomics studies in wheat. The transformation methods for wheat are very time-consuming. The most favoured method for wheat transformation is callus mediated and for this immature embryo serves as an explant. Producing callus from an immature embryo needs extra effort to grow plants in a controlled environment to avoid any kind of contamination to the plants. Also, there is a very short time frame to collect immature wheat seeds. Moreover, the collected immature seeds cannot be stored for a longer period and hence making it a very hectic and laborious task. There is a continuous need for immature embryos for regular experiments of transformation. However, using mature seed embryos potentially could reduce this dependency and also increase the frequency of the experimental setups. Additionally, morphogenic regulators including Baby boom and Wuschel proteins have been shown to increase the transformation efficiency in monocots (Lowe et al. 2016). Using these new advancements in wheat could reduce the cost, time and it could increase the efficiency of transformation in wheat. Besides the tissue culture based methods, a new breeding based method has been used for the genetic transformation known as the pollen magnetofection system (Zhang et al. 2019b). These improved transformation approaches have increased the transformation efficiency and with the addition of CRISPR technology, it is now possible to get homozygous edited lines at the T1 generation, even in polyploid crop plants. A flowchart showing the strategies to work with CRISPR/Cas9 system in crops is shown in Fig. 22.1.

22.6 Delivering Genome Editing Constructs to Cells

There are three modes of the CRISPR technique—DNA based, RNA based and ribonucleoprotein (RNP) based. The CRISPR technique was pioneered with CRISPR/Cas9 system from *Streptococcus pyogenes* using DNA based approach. Initially, a binary vector system was used to target a gene of interest. In this system, two different vectors were used to express Cas9 and single-guide RNA (sgRNA) and co-transformed inside the host plant cells. This system was found to be an efficient method to create a precisely targeted editing at the target site. In later advances in this area, researchers designed a single vector system, in which both the sgRNA cassette and Cas9 was cloned in a single vector and transformed into the host plant cells. Furthermore, multiplexing of target sites for a single gene was used for high confidence genome editing. Although this is the most widely used and successful method yet due to the limitation that filler DNA gets integrated at random places in

Fig. 22.1 Working with CRISPR/Cas9 system in crop plants: the flowchart summarizes the approaches to be used for genome editing in crop systems. When selecting the gene and region to be targeted, it is better to choose a functional domain of the protein, however, the conserved domains should be avoided to reduce off-targets. The target site close to 5′ end of the gene facilitates an early mutation and hence avoids the formation of any functional truncated protein. The selection of gRNA based on predicted 'on-target' and off-target frequency could be done using a blast analysis or available online tools including Breaking Cas (linked to Ensembl), CRISPR-P V.2.0 (linked), Chop Chop (linked), etc. Prefer a unique target site. Choose a target site such that the closest homolog must have 2-3 mismatches at 3′ end in the seed sequence (the last 12 nucleotides of the target site are known as seed sequence). Synthesize and clone desired gRNAs, there are three modes of CRISPR/Cas9 system as a genome editing tool including DNA based, RNA based and RNP (ribinucleoprotein) based. For a DNA based approach, commercialized vectors are available for plants with various promoters and selectable markers. Various vector modules are also available for multiplexing (multiple target sites for a single gene in a single construct). Validate genome editing in an in vitro validation setup: Amplify the amplicon harbouring target site and incubate it with in vitro transcribed and translated construct to check the functionality of construct as well as the efficiency of the target site for mutation. In vivo validation: deliver the construct in respective protoplasts or callus and check the mutation efficiency. Finalized gRNA is delivered into the plant system

the genome and are difficult to trace or identify. Also, the integration of foreign DNA makes the plant transgenic. To overcome these problems, RNA based method was used. In this method, Cas9 and sgRNA were in vitro transcribed and delivered into the host cells. This method was able to create a mutation in the target site without the integration of any foreign DNA into the host cells. But with this method, the transformation frequency was very low due to the less stability of RNA. Hence, ribonucleoprotein (RNP) based approach was developed in which sgRNA was

in vitro transcribed and the Cas9 was expressed in *E. coli* and further purified and a complex of sgRNA and purified Cas9 was prepared and delivered into the plant callus. The use of RNP is highly efficient in creating non-transgenic plants along with the edited genome.

22.7 Role of Genome Editing to Improve Agricultural Traits

The importance and impact of agriculture in nourishing human civilizations have been tremendously great. Crop systems are valuable necessities because they provide fuel and food. But with the ever-increasing human population which is expected to cross 9 billion by 2050, fluctuating climatic conditions, depleting water resources and reduced agricultural land, more robust and innovative breeding technologies are required to develop plant systems with higher yield and better quality (Clarke and Zhang 2013). Modern agriculture which focuses on making the desired genetic changes in plant species via crossing donor and elite varieties for some beneficial trait such as disease resistance is followed by successive rounds of backcrossing of the hybrid with donor parent, a technique known as cross-breeding. This approach is limited to plants that offer a substantial genetic variability for a particular trait and cannot be employed for genetic traits for which variability is low such as those for micronutrients such as Fe and Zn (Connorton and Balk 2019). Another method known as mutation breeding is based on the treatment of plant with physical and chemical mutagens to introduce necessary genetic changes is truly based on random mutation events (Ma et al. 2016). Transgene breeding is another aspect of modern agriculture which is based on the insertion of foreign gene fragments carrying the required trait in the plant's genome. These techniques are, however, time-consuming and labour intensive taking several years to complete and requiring extensive screening of multiple genotypes and phenotypes. Cross-breeding and mutation breeding are moreover non-targeted approaches causing random insertions/mutations. Transgene breeding although has the benefit to be employed in case of plants that offer reproductive barriers, but the acceptability of genetically modified organisms (GMOs) in terms of health, environment, economic and public regulations is again a lengthy and costly process (Smyth and Phillips 2014). Hence a more quick, precise and targeted approach with lower risk and cost is needed to fill the demand and supply gap of population. Genome editing is a sustainable approach which introduces fewer genetic changes which are at par with those occurring in natural populations. Genome editing tools comprise of ZFNs, TALENs and CRISPR/Cas. The components of this toolkit are a type of sequence-specific nucleases (SSNs) which introduce double-stranded break (DSB) that are repaired by plant's endogenous repair system either by NHEJ causing indels or HDR that results in gene replacements and insertions (Symington and Gautier 2011). ZFNs were used for the first time in 2005 in the tobacco plant, as evidence for homologous recombination-mediated genome engineering (Wright et al. 2005). To date, ZFNs have been used to modify *Arabidopsis*, *Nicotiana*, maize, *Petunia*, soybean, rice, rapeseed and apple. TALENs were first employed for crop improvement in rice,

Table 22.2 Comparison of ZFN, TALEN and CRISPR/Cas as a genome editing tool

Property	ZFNs	TALEN	CRISPR/Cas
Existence	Archea and eukaryotes	Prokaryotes	Archaea and prokaryotes
Discovery as genome editing tool	1996	2011	2013
Example	Cys2-His2	TALEN	CRISPR/Cas9, CRISPR/Cas12, CRISPR/Cas13.
Size	30 AA	33 to 35 AA module	–
Target recognition	Single ZF domain can recognize 3 bases. Longer sequence can be recognized by linking multiple ZF domains.	Single TALE motif target a single nucleotide	20 nucleotide target site + PAM
Functionality	Heterodimer (zinc finger domain and fokI endonuclease domain)	Heterodimer fusion protein (bacterial TALE and Fok1)	Complex of Cas protein and noncoding RNA
Target interaction	Protein-DNA	Protein-DNA	RNA-DNA/RNA
Cleavage	Double-strand break	Double-strand break	Single/double-strand break
Cost	High cost	Moderate	Cheaper
	More tedious and time-consuming as require high knowledge of protein engineering to design the array of ZF domains	Moderate as compare to ZFNs and TALEN.	Simple
Cloning/can be used as	Linkages between ZF motifs required	Do not require linkages. TALE motif can clone separately	SgRNA = direct RNA molecule or in DNA expression vector. Cas9 = as DNA expression vector or direct Cas9 protein OR Preassembled SgRNA-protein complex

wherein bacterial blight susceptibility gene *OsSWEET14* was disrupted resulting in blight-resistant rice. Subsequently, TALENs have been applied successfully in *Arabidopsis*, *Nicotiana*, *Brachypodium*, barley, potato, tomato, sugarcane, flax, rapeseed, soybean, rice, maize, wheat (Ran et al. 2017; Martínez-Fortún et al. 2017). However, ZFNs and TALENs are restricted in application in terms of construction complexity and variable efficiency rate (Table 22.2).

In 2013, a more versatile genome editing technique—CRISPR/Cas came into the picture when three independent groups successfully established gene knockouts via targeted mutagenesis. In one group led by Li et al. (2013), Arabidopsis and *Nicotiana benthamiana* protoplasts were transfected by co-expressing plant codon optimized SpCas9 and gRNA against *Phytoene desaturase* (*PDS*) gene of respective plants. Another group performed transformation of rice protoplasts with codon optimized Cas9 and two sgRNAs targeting different strands of *PDS* gene (Shan et al. 2013). Nekrasov et al. (2013) targeted *N. benthamiana* leaf tissue for *PDS* by using *Agrobacterium tumifaciens* as a vector driving GFP-Cas9 and sgRNA cassette. Since then, CRISPR/Cas has been employed multidimensionally for yield improvement, enhancing nutritional quality and resistance to abiotic and biotic stresses (Table 22.3).

22.8 CRISPR in Yield Improvement

Crop yield is a factor determined by grain weight, grain number, grain size, etc. Many of the improvements about yield have targeted genes having a direct or indirect impact on yield performance (Table 22.3). For example, when *LAZY 1* gene was knocked out in rice, a phenotype with a greater number of tillers was generated (Miao et al. 2013). Also when other negative regulators of yield were targeted, viz., GRAIN NUMBER (Gn1a), DENSE AND ERECT PANICLE (DEP1), Grain Size (GS3), it resulted in rice plants with enhanced grain number, larger grain size and dense erect panicles (Li et al. 2016b). Another report has described knocking out of an essential gene—*Grain weight 2* (*GW2*) from all the three homeologs of wheat (*Triticum aestivum* L.) resulting in a phenotype with an increase in thousand-grain weight (TGW), flour protein content and gluten strength (Zhang et al. 2018b).

22.9 CRISPR in Biofortification

CRISPR/Cas9 has been employed for nutritional quality improvement in a variety of plants. For example, targeted mutagenesis of starch branching enzyme—SBEI and SBEIIb in rice resulted in de-branching of amylopectin (Sun et al. 2017). This modulated and fine-tuned the structural properties of starch and created rice with high amylose content and resistant starch which confers potential health benefits in terms of easy digestibility and controlled blood sugar levels, especially in case of diabetic individuals. In another study, *GBSS* (granule bound starch synthase gene) was knocked out in maize and potato which resulted in the absence of amylose expression resulting in a product with improved levels of amylopectin thus conferring easy digestibility (Andersson et al. 2017). CRISPR system has also been used to decrease the level of undesirable polyunsaturated fatty acids (PUFAs) and improve oleic acid content in oilseed plant *Camelina sativa* resulting in oil with healthier and oxidatively stable properties (Jiang et al. 2017).

Table 22.3 Trait development in Crops system using CRISPR/Cas system

Crop	Genome editing tool	Gene	Resulting Trait	References
Wheat	TALENs	*TaMLO*	Resistance to powdery mildew	Wang et al. (2014)
	CRISPR/Cas9	*TaGW2*	Increase in grain weight and protein content	Zhang et al. (2018b)
	CRISPR/Cas9	*EDR1*	Resistance to powdery mildew	Wang et al. (2014)
	CRISPR/Cas9	*TaCKX2-D1*	Yield increase	Holubová et al. (2018)
	CRISPR/Cas9	*Alpha-gliadin*	Low gluten content	Sánchez-León et al. (2018)
	CRISPR/Cas9	*TaNFXL1*	Resistance to Fusarium graminearum	Brauer et al. (2020)
	CRISPR/Cas9	*TaALS*	Herbicide resistance	Zhang et al. (2019a)
	CRISPR/Cas9 (CBE)	*TaACCase*	Herbicide resistance	Zhang et al. (2019a)
Rice	ZFNs	*OsQQR*	Trait stacking	Liu et al. (2020c)
	TALENs	*OsSWEET14*	Resistance to bacterial blight	Li et al. (2012)
	CRISPR/Cas9	*eIF4G*	Resistance to pathogen	Macovei et al. (2018)
	CRISPR/Cas9	*OsSWEET13*	Resistance to bacterial blight	Xu et al. (2019)
	CRISPR/Cas9	*SBEIIb*	Amylose content increased	Sun et al. (2017)
	CRISPR/Cas9	*Gn1a,GS3, DEP1*	Increase in grain number, grain size and dense erect panicles	Li et al. (2016b)
	CRISPR/Cas9	*OsERF922*	Rice blast resistance increased	Wang et al. (2016)
	CRISPR/Cas9	*AAP3*	Increase in yield	Lu et al. (2018)
	CRISPR/Cas9	*ALS*	Resistance to herbicide	Kuang et al. (2020)
	CRISPR/Cas9	*EPSPS*	Resistance to herbicide	Li et al. (2016a)
	CRISPR/Cas9	*OsGS3 OsGW2 OsGN1a*	Increase yield	Zhou et al. (2019)
	CRISPR/Cas9	*OsPIN5b OsGS3*	Increase yield	Zeng et al. (2020b)
	CRISPR/Cas9	*OsLOGL5*	Increase yield	Wang et al. (2020)

(continued)

Table 22.3 (continued)

Crop	Genome editing tool	Gene	Resulting Trait	References
	CRISPR/Cas9	*OsGW5*	Increase yield	Liu et al. (2017b)
	CRISPR/Cas9	*5'UTR & Promoter of OsGBSSI*	Low amylose content	Zeng et al. (2020a)
	CRISPR/Cas9	*Wx allele*	Increased β content	Dong et al. (2020)
	CRISPR/Cas9	*OsFAD2-1*	Monounsaturated omega-9-fatty acid	Abe et al. (2018)
	CRISPR/Cas9	*OsPLDα1*	Decreased phytic acid content	Khan et al. (2019)
	CRISPR/Cas9	*OsSF3B1*	Resistance to herbicide	Butt et al. (2019)
	ABE	*OsTubA2*	Resistance to herbicide	Liu et al. (2020b)
	CBE	*Promoter of OsGBSSI*	Low amylose content	Huang et al. (2020)
	Abe, CBE	*OsALS1*	Herbicide-resistant	Liu et al. (2020c)
	Abe, CBE	*OsACCase*	Herbicide-resistant	Li et al. (2020), Liu et al. (2020c)
Maize	ZFNs	*ZmIPK1*	Resistance to herbicide and reduction in phytate level	Shukla et al. (2009)
	ZFNs	*ZmTLP*	Trait stacking	Ainley et al. (2013)
	TALENs	*ZmGL2*	Decrease in epicuticular wax in leaves	Char et al. (2015)
	TALENs	*ZmMTL*	Induction of haploid plants	Kelliher et al. (2017)
	CRISPR/Cas9	*Wx1*	Increase in amylopectin content	Bull et al. (2018)
	CRISPR/Cas9	*TMS5*	Thermosensitive male sterile	Waltz (2016)
	CRISPR/Cas9	*ALS*	Resistance to herbicide	Svitashev et al. (2015)
	CRISPR/Cas9	*ARGOS8*	Tolerance to drought	Shi et al. (2017)
Soybean	TALENs	*FAD2-1A,1B and 3A*	Increase in oleic acid and decrease in linoleic acid level	Haun et al. (2014)
	CRISPR/Cas9	*GmFAD2*	Oleic acid proportion increases	Do et al. (2019)
	CRISPR/Cas9	*ALS*	Resistance to herbicide	Li et al. (2015)
Cassava	CRISPR/Cas9	*EPSPS*	Resistance to herbicide	Hummel et al. (2018)

(continued)

Table 22.3 (continued)

Crop	Genome editing tool	Gene	Resulting Trait	References
	CRISPR/Cas9	*MePTST1 MeGBSS1*	Decreased amylose content	Bull et al. (2018)
		eIF4E	Potyviridae resistance	Gomez et al. (2019)
Potato	CRISPR/Cas9	*Wx1*	Increase in amylopectin content	Andersson et al. (2017)
	CRISPR/Cas9	*ALS*	Resistance to herbicide	Veillet et al. (2019)
	CRISPR/Cas9	*StGBSSI*	Decreased amylose content	Andersson et al. (2018)
	CRISPR/Cas9	*stSBE1, StSBE2*	Increase amylose content	Zhan et al. (2019), Andersson et al. (2018)
	CRISPR/Cas13	*PVY*	PVY virus-resistant potato	Zhan et al. (2019)
Tomato	TALENs	*ANT1*	Anthocyanin rich tomatoes	Čermák et al. (2015)
	CRISPR/Cas9	*SlMLO1*	Resistance to powdery mildew	Nekrasov et al. (2017)
	CRISPR/Cas9	*SlJAZ2*	Resistance to bacterial speck	Ortigosa et al. (2019)
	CRISPR/Cas9	*SlCLV3 promoter*	High yield	Rodríguez-Leal et al. (2017)
	CRISPR/Cas9	*SlENO*	High yield	Yuste-Lisbona et al. (2020)
	CRISPR/Cas9	*SlSGR1, SlBlc1*	Lycopene enriched tomato	Li et al. (2018c)
	CRISPR/Cas9	*SlCAT9 SlGABA-TP1, SlGABA-TP3*	γ aminobutyric enriched tomato	Li et al. (2018b)
	CRISPR/Cas9	*SlALC*	Increased shelf life	Yu et al. (2017)
	CRISPR/Cas9	*SlCCD8*	Broomrape resistance	Bari et al. (2019)
	AID	*OsALS1*	Herbicide resistance	Veillet et al. (2019)
Flax	CRISPR/Cas9	*EPSPS*	Resistance to herbicide	Sauer et al. (2016)
Cucumber	CRISPR/Cas9	*eIF4E*	Resistance to virus	Chandrasekaran et al. (2016)
Orange	CRISPR/Cas9	*CsLOB1*	Resistance to citrus canker	Peng et al. (2017)
Lettuce	CRISPR/Cas9	*LsGGP2*	High ascorbate lettuce	Zhang et al. (2018a)
Mushroom	CRISPR/Cas9	*PPO*	Anti-browning phenotype	Zhang et al. (2018a)

22.10 Resistance to Abiotic and Biotic Stresses

CRISPR/Cas9 has been employed to create plants resistant towards biotic stresses such as bacterial, viral and fungal pathogens. For example, knockout of *TaEDR1* (Enhanced Disease resistance 1) from all the wheat homoeologs resulted in powdery mildew resistant wheat (Zhang et al. 2017). Similarly, Mildew Locus O (*SlMLO1*) knockout resulted in powdery mildew resistant tomatoes. Also, rice with blast resistance was developed by knocking out Ethylene Response Factor—*OsERF922*. When eukaryotic translation initiation factor 4E was subjected to disruption in cucumber, broad virus resistance was conferred. Moreover, resistance towards abiotic stresses such as herbicides has been achieved in rice by disrupting *C287* gene. The resulting rice plants were resistant towards the herbicide—Imazamox (IMZ). In cassava, glyphosate tolerance was achieved by mutating an essential gene of shikimate pathway, i.e. 5-enolpyruvylshikimate-3-phosphate synthase EPSPS.

22.11 Improvement in Breeding Technologies Via CRISPR/Cas

Improvement of crops via traditional breeding technologies is a time-consuming and laborious process. To introduce a desired trait into a crop, it requires many generations of crossing for the trait to be stably introduced into a cultivar. Haploid induction is a method to create homozygous lines within a lesser number of generations. It involves the development of a haploid plant which could be possibly achieved by microspore or megaspore culture in vitro or irradiation of pollen to deactivate the sperm cells so that after fusion with the egg cell it develops into haploid embryo. The haploid plant can be made diploid spontaneously or artificially (by using chemical agents such as colchicine). Haploid induction (HI) was first achieved in maize protoplasts via CRISPR/Cas to generate loss of function/knockout mutants by targeting the gene—*NOT LIKE DAD (NLD) /MATRILINEAL (MTL) / ZmPHOSPHOLIPASE A1 (ZmPLA1)* (Liu et al. 2017a). This gene is pollen-specific and plays a crucial role in pollen development. Subsequently HI via CRISPR Cas was also extrapolated to rice and wheat for *MTL* orthologs (Yao et al. 2018; Liu et al. 2020a).

Hybrid vigour is another aspect of plant breeding that focuses on preventing inbreeding depression and generates more robust crops with beneficial traits combined from distant varieties into one. For this, crops are to be made male sterile to prevent self-pollination. Introducing this aspect via breeding is a lengthy process and difficult for the crops with inbreeding habits like wheat. Therefore, the genes responsible for male fertility have been targeted by CRISPR/Cas. Knockout of *male sterile 1 (Ms1)* and *Ms45* which encode for glycosylphosphatidylinositol-anchored lipid transfer protein and strictosidine synthase-like enzyme in wheat resulted in abolishing male fertility (Singh et al. 2018; Okada et al. 2019).

22.12 CRISPR: Further Developments and Applications

Traditional genome editing via SSNs offers the problem of multiple base-pair insertions or deletions in the target sequence. This arises due to the endogenous repair of DNA as a result of DSBs created by SSNs. However, the trait variations that occur in elite plant populations are having base changes that differ by a single nucleotide. Therefore, advanced genome editing solutions that create point mutations are required. This has been achieved by fusing Cas9 nickase or dead Cas9 with enzymes having base conversion capability such as—Cytidine deaminases and Adenine deaminases causing C.G to T.A substitutions and A.T to G.C substitutions. This is referred to as base editing technology and is composed of cytosine base editors (CBEs) and adenine base editors (ABEs). The cytosine base editing scheme is composed of Cas9 nickase carrying a mutation—D10A in its RuvC nuclease domain. This impaired enzyme is fused to Cytosine deaminase and Uracil DNA glycosylase inhibitor (UGI). The complex is guided by sgRNA which binds to the target DNA and the base pairing between RNA and DNA displaces a segment of ssDNA creating a loop-like structure. Cytosine deaminase modifies cytosine into uracil on the non-target strand and uracil DNA glycosylase inhibitor prevents the correction of this mistake by inhibiting the cellular uracil DNA glycosylases. nCas9 then creates a nick in the target strand followed by the activation of DNA mismatch repair pathway causing repair of non-edited strand by using edited strand as template resulting in C.G to T.A base transition. The base editing systems were developed in human cell lines and their version of cytosine deaminases has been extrapolated to plant systems as well. *Petromyzon marinus* cytidine deaminase 1 (CDA1)-based CBE was applied successfully in rice and tomato by introducing multiple point mutations in ALS (Acetolactate synthase) for herbicide resistance in the former and on-target mutations in *DELLA* and ethylene receptor (*ETR1)* genes in the latter (Shimatani et al. 2017). Human activation-induced cytidine deaminase (AID)-based CBEs have also been applied for efficient base editing in rice leaf sheath protoplasts causing frequent C > T conversions within the editing window (Ren et al. 2018). A truncated version of human APOBEC3B (hAPOBEC3B) has also been applied successfully for precision editing in rice plants with fewer off-target effects (Jin et al. 2020). CBEs components are also be used in conjunction with apurinic or apyrimidinic (AP) lyases which excise the uridine and form a DSB, recruiting the base excision repair machinery and result in precise deletion between the Cas9 nick and excised uridine. This tool comprises of Cas9, UDG and AP lyase and is known as 'APOBEC–Cas9 fusion-induced deletion systems' (AFIDs).

Base editing via adenine base editors consists of adenosine deaminase fused to nCas9 (D10A) modifying adenine to inosine, the latter being recognized by the repair system as guanosine resulting in A.T to C.G transition. There are no adenosine deaminases known to modify adenosine in DNA. The first adenosine deaminase evolved from *E. coli* using codon optimized BE2 which was composed of APOBEC1 cytidine deaminase, Cas9 nickase and UGI and was able to rescue the antibiotic sensitive phenotype by reversing the point mutations in antibiotic

resistance genes (Gaudelli et al. 2017). The APOBEC was shown to exhibit homology to *E. coli TadA* which is t-RNA specific adenosine deaminase. ecTadA and nCas9 fusions have been successfully used to create precise editing in rice and wheat by targeting *OsACC1* (rice acetyl coenzyme A carboxylase) for herbicide resistance and *TaDEP1* and *TaGW2* genes in wheat for grain yield improvement (Li et al. 2018a). In *Arabidopsis,* ABEs have enabled generating single nucleotide changes in Flowering Locus T (FT) protein causing late-flowering phenotype (Kang et al. 2018). The improved versions of ABEs (ABE8) have recently been developed to offer better compatibility and efficient base editing with corresponding Cas9 nickases by techniques such as phage assisted evolution and directed evolution (Gaudelli et al. 2020; Richter et al. 2020).

CBEs and ABEs have also been combined to create a more robust gene-editing platform by performing dual base editing roles simultaneously. The components consist of APOBEC3A (cytidine deaminase), ecTadA–ecTadA* (an adenosine deaminase,) nCas9 (D10A) and a UGI. This complex is known as saturated targeted endogenous mutagenesis editor (STEME). A versatile technique—Prime editing technology can cause transversions in the target DNA segment which the base editors—CBEs and ABEs are unable to do. The tools of prime editor comprise of nCas9 (H840A), reverse transcriptase and prime editing guide RNA (pegRNA). pegRNA comprises of reverse transcriptase template and primer binding site. The primer binding site pairs with nicked strand and the RT template harbouring the changes is copied to create the desired change. Plant prime editor systems have been tested in rice and wheat protoplasts using codon optimized PPEs resulting in multiple insertions, deletions and all possible 12 base substitutions with significant efficiency (Lin et al. 2020).

CRISPR Cas constructs have also been introduced in plants as IVTs (in vitro transcripts) and RNPs complexes. Because earlier CRISPR constructs were delivered as DNA cassettes, they posed the problems of random integration and unwanted genetic changes such as on- and off-target cleavage as a result of constitutive expression of Cas9 (Kim et al. 2014). This is overcome by delivering RNPs and IVTs because they express transiently and avoid such off-target effects. This is referred to as DNA free genome editing and has been achieved by protoplast transfection and particle bombardment. The former technique has been successfully applied in tobacco, grape, lettuce, rice, etc. (Woo et al. 2015; Malnoy et al. 2016). However, some plants could not be propagated by the protoplast regeneration method and alternatively, recalcitrant plants like wheat have been subjected to particle bombardment with RNP complexes (Zhang et al. 2016; Liang et al. 2017). In maize, particle bombardment has also been used to create knockout mutants (Svitashev et al. 2016). Also, DNA free editing outcompetes the problem of transgene insertion enabling acceptance of crop systems from a commercial standpoint.

Conventional TypeII CRISPR/Cas recognizes target sequences that are upstream of protospacer adjacent motif (PAM) 5'-NGG-3′ thus restricting the number of target sites for which it could be employed. A new type V CRISPR/Cpf1 system surpasses this problem by recognizing PAMs that are T rich and unlike type II system which

results in blunt ends, it generates cohesive ends with 4-5 nucleotides overhangs. Cpf1 has many variants such as FnCpf1, LbCpf1, AsCpf1 (from *Francisella novicida*, *Lachnospiraceae bacterium* and *Acidaminococcus* sp. BV3L6). These have been successfully applied for improvements in rice, tobacco and soybean (Endo et al. 2016; Kim and Choi 2020). Base editing along with DNA free editing technology has been exploited together in wheat in which the construct carried rat cytidine deaminase—APOBEC1 fused to Cas9 nickase and uracil DNA glycosylase inhibitor (UPI).

22.13 Virus Mediated Genome Editing

Much recently, viruses have been proved to be efficient machinery for gene editing as they have a natural tendency to travel and infect inside the plant thus bypassing the need for tissue culture and regeneration steps. Several plants including both dicots and monocots have been subjected to viral-based editing. In the very first report, Cas9 overexpressing *Nicotiana benthamiana* plants were infected with Tobacco rattle virus-carrying sgRNA for *PDS* (*Phytoene desaturase*) gene with efficiency reaching 80%. TRV has two positive sense single-stranded RNAs—RNA1 and RNA 2, the latter of which can be used to carry external gene fragments (Ali et al. 2015). Other viruses like PEBV (pea early browning virus) and tobacco mosaic virus have also been employed to target dicots like *Arabidopsis* and *N. benthamiana* (Ali et al. 2018). In another study, barley stripe mosaic virus harbouring sgRNA against wheat and maize genes *TaGASR7* and *ZmTMS5* genes which regulate grain length, weight and pollen fertility was deployed with knockout efficiency of 77% and 91%, respectively (Hu et al. 2019). Viruses have proven to be promising candidates in terms of their broad host range and small genome size enabling sgRNA cloning easily. Additionally, their RNA genome does not offer the problem of integrating into the host genome. The only way gene editing by viruses poses hindrance is the requirement of Cas 9 overexpressing lines as the genome of RNA viruses has limited cargo capacity. Also, viruses cannot be propagated to the subsequent generations of plants because of their incapability to enter the reproductive tissues. These problems were worked upon by the scientists and in one study they were able to clone Cas 9 and sgRNA cassette adjacent to each other in barley yellow striate mosaic virus (Gao et al. 2019). To allow for carryover of viral editing in subsequent generations, researchers have performed multiplexing in TRV by fusing sgRNA and FT gene of *Arabidopsis thaliana* (Flowering locus T), which is transcribed in leaf and then moves to shoot apical meristem to induce flowering. It serves as a mobile genetic element for the viral doorway into the next generation. This approach led to the recovery of mutants with efficiency reaching up to 100% (Ellison et al. 2020).

22.14 Indian National Status

Since it is a new technique for genome editing in plants, not many Indian labs have reported their research work in this direction. First and foremost, utility of CRISPR-Cas9 mediated editing was shown in wheat suspension cells and tobacco (Upadhyay et al. 2013) at National Agri-Food Biotechnology Institute, Mohali, Punjab. Subsequently, phenotypic marker gene phytoene desaturase (PDS) was shown to be edited in Banana plants, thereby expanding its utility in fruit crops (Kaur and Alok 2018). Similarly, CRISPR-Cas9-mediated editing of the *CYP82E4-Nicotine N-Demethylase* (nnd) gene was also demonstrated in tobacco protoplasts (Shrestha et al. 2019). The selection of the Cas9 is also important to address the off-target effects of the editing system. The widely used Cas9 derived from *Streptococcus pyogenes* and its variants have been used successfully, for genome editing yet the concerns pertaining to their putative off-targeting at multiple loci across the genome remains unaddressed. At Institute of Genomics & Integrative Biology (IGIB, New Delhi), a *Francisella novicida* derived Cas9 was identified that showed a high specificity of binding to its intended targets and negligible binding to off-target loci (Acharya et al. 2019). This work is an important leap to demonstrate the applicability of Cas9 for stringent corrections of genetic disorders. Recently high level of Pro-vitamin A was achieved by performing genome editing in Banana. *LCYε* gene was edited using CRISPR/cas9 system that resulted in early termination of the LCYε protein and ultimately increasing six fold higher β-carotene content in cv. Rasthali (Kaur and Alok 2020). In the area of plant biology, novel means, including efficient design of system/constructs, delivery methods, validation methods and a stable editing in subsequent generations is still at the nascent age in the Indian perspective. This area needs to be nurtured and much impetus is required in this direction.

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QTLs and Gene Tagging in Crop Plants

23

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Abstract

QTL mapping by biparental crossing for accurate and precise positioning of QTLs in terms of nucleotide sequence is being followed in several crops. However, it is best possible in the crops whose genome sequencing has been established. Identification and validation of candidate genes through association mapping is not easy to be determined due to population structure of the panel of genotypes that are to be fully characterized for several aspects to achieve unbiased results. Complex traits controlled by large number of genes with additive effects do not show simple Mendelian inheritance and cannot be tagged easily. Two basic approaches, namely linkage mapping and association mapping, the first also known as positional cloning based on linkage analysis of nearly isogenic lines through a functional marker gene also called as candidate gene or a physical DNA marker based on molecular marking techniques like RFLPs, SSR, and later SNPs. The other is association mapping, which is based on linkage disequilibrium based fundamentally on the principle of genetic recombination or linkage in shared inheritance for a collection of individuals. Association mapping studies are well in progress especially in the well-established breeding centers of the crop in the world. If a number of polymorphic markers are mapped in the QTL region from sequenced genomes, then markers closest to QTL can be used to identify variation in nucleotides or mutants responsible for QTL in terms of quantitative trait nucleotide polymorphism (QTN). Recently many plant speceis are being sequenced for whole genome, the prospectus to identify genes for quantitative traits are brighter. However, association mapping approach is a little bit tedious

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and could be exploited with groups of people associated with specific crops where well characterized genotype panels are available cropwise. Both these approaches viz. RILs and association mappings are being used intentionally in major cereal crops such as maize and wheat.

Keywords

QTLs · Gene mapping · Crop plants · Linkage map · Positional cloning · Association mapping

23.1 Introduction

Yield potentials of crop plants are basically based on two main aspects of crop raising activity by targeting inherent capacity of biomass of any economic trait of interest and production in the most suitable environment under best nutritional conditions. The former is thus an activity to improve the seed for yielding abilities per se though at practical level each character is controlled by some specific factors in plant system called gene. Though plant acts as a holistic system where the entire genome system coordinates to produce higher or lower yield. Therefore, tagging or identifying genes for yield production is the crucial issue. Of the two main types of plant traits: one is simple oligogenic controlled Mendelian trait loci (MTL), the other is quantitative trait loci (QTL) where a large number of genes adjacent or wide spread over genome with small cumulative additive effects are present. However, the name quantitative trait loci (QTL) was first coined by Gelderman (1975) who defined QTL as chromosomal region responsible for specific complex traits like yielding ability or so and can be isolated and so be tagged with DNA based molecular markers. The QTL thus is a hallmark of tagging most suitable groups of genes for quantitative traits by using modern DNA technology. So tagging is possible to genomic regions. Therefore, it is possible to transfer to tagged region to produce better yielding lines, one of the very simplest ways as done for oligonucleo genes. In oligo gene approach, selection is made on phenotypic plant trait directly, which is comparatively easy for the improvement in this QTL selection approach. Here groupings of individuals are possible by genetic or DNA markers and the economic traits of interest are measured on group of marker genotypes to find out the specific chromosomal region of specific trait yielding ability.

23.2 Genesis of QTLs in Tagging Genes of Economic Interest

One of the advances of twentieth century is the clear-cut understanding of how individual gene controls simple traits called MTLs. Now it turns that we may succeed in full understanding of genes and alleles that control traits which can be identified, tagged, dissected, and improved if possible and then to transfer to a target

genotype for crop improvement (Flint-Garcia and Thornsberry 2003). The hosts of created DNA marker generation techniques like RFLPs, AFLPs, RAPDs, SSRs and ISSRs repeat polymorphisms as discussed by several authors and by Thakur (2000) and the most recent of the markers, simple nucleotide polymorphism (SNPs) have been described which can be used to mark MTLs that are qualitative trait loci. These can, however, be used to find linkages between these DNA markers and the traits of interest controlled by quantitative genes/QTLs. Tagging for MTLs is, however, simple and got just done by looking for the phenotypic value of this marker carrying genotype. The marking for QTLs, however, is much more complicated.

23.3 Tagging Genes as Complex Traits in QTLs

QTLs for oligo genes follow the Mendelian inheritance and a simple general scheme based on linkage studies and applicable to all crops having breeding system has been described by Young et al. (1992) is being prescribed here for general comprehension of QTLs with important traits of agronomic importance. The identificatio of QTLs is the determination of the association of genomic region on chromosoeme with the DNA markers as visulalised in the form of polymorphic bands on electrophoresis. In a basic technique of linkage detection, two inbred parental lines which differ in an agronomic trait value from each other substantially are genotyped for one or several DNA markers. If the DNA marker polymorphism is obtained between the two parents for such markers, say a very high yielding inbred genotype has a DNA marker M1 and a very low yielding one M2, the controlled artificial sexual hybridization between two such parents having marker genotypes M1M1 and M2M2 is allowed to be made. And then in the simplest case such as back cross (F2 and recombinant inbred lines, RILs), the population can be grouped into individuals that are homozygous for physical marker alleles of recurrent parent having M1M1 and those of individuals which are heterozygous for the marker alleles from recurrent and the donor parents say having M1M2 which could be done by genotyping for the physical marker alleles in the back cross generation/ individuals by molecular technique.

For determining the association of markers with quantitative traits, on the basis of phenotypic values the whole populations is divided into two subpopulations and these two subpopulations can be compared by analysis of variance or regression. If the difference between these two subpopulations is significant (that is if group of M1M1 individuals shows higher yield than the group of M1M2), the marker that is M1 here may be linked to complex trait/QTL for the yield and so virtually identified. Such analysis is done over other physical marker loci genome-wide to find linkage between all those other markers and the agronomic traits that is yield. Such QTL analysis can be made for all agronomically complex traits of plants associated with yield or any other characteristics of importance. More rigorous methods to detect such markers is the method of log of likelihood (LOD) score propounded by Lander and Botstein (1989) which has been adapted to allow interval mappings of QTLs.

This basic method was for simple situations but has been generalized for a wide variety of settings (Lander and Schork 2006). Genome-wide QTL analysis by interval mapping was first applied to fruit characteristics in tomato by Paterson et al. (1988). Different mathematical models to detect linkages of markers with QTLs have been developed by Kersey and Hyne (1994) and Hyne and Kearsey (1995).

23.4 Genetic Linkage Map and Gene Tagging

Character expressions were first postulated to be regulated by John Mendel (1866) and it was later when Sutton and Boveri (1902) argued that these factors were present on thread like genetic material, which organize themselves into a definite number in a diploid cell during its somatic or reproductive nuclear divisions and this number is 2 N where N is the total number of threads in a haploid cell. These threads were dubbed as chromosomes on which the factors or genes are present and this type of inheritance through chromosomes is called chromosomal theory of inheritance. Thus all genes are present in a single set of chromosome number called haploid (N) number in all the species. All the genes present on one chromosome are joined and hence inherited together and the group is called one linkage groups. Hence, there are total N linkage group in any species where genes are located in each chromosome group wise. Finding the location of a single specific gene in relation to other genes on a single chromosome adjacent or nearby is called gene mapping or chromosome mapping. It is possible to locate any gene or piece of DNA of an organism to one of the total number of chromosomes. Virtually it is possible to locate or map any gene with known or unknown function to any of the linkage group. Therefore, linkage map construction is one of the basic precepts for identifying the location of any known functional gene per se linked to a genetic marker or physical DNA marker or with any gene of unknown function. The quantitative genes/loci responsible for complex traits are similarly located on any of the chromosomes linked to physical DNA marker. So virtually, given resource and time any complex trait controlled by a stretch of DNA sequences as multiple loci is possible to be located in relation to other known or unknown genes and any number of marked QTLs identified. This linkage map is the primary repository of oligogenes and polygenes linked with genetic marker and physical DNA marker which is called QTL marker. Thus integrated genetic linkage map is the most crucial resource from where any piece of DNA for MTL or QTL could be referred for its future detailed analysis and from here structural and functional analysis of tagging genes become possible and is done by interval mapping already described.

23.5 The Concept of Basic Linkage Map Construction of Any Organism

The sets of genes present on a chromosome are expected in a specific linear order, some genes present adjacent to very near to far off on the same chromosome. The order of these linked genes is, however, detected by finding the distance between them. The relative distance between genes is measured in terms of recombination frequency among different genes, which is facilitated by exchange of genetic material between non-sister homologous chromosomes during meiosis called crossing over. Closer genes having possibility of less crossing over will show lesser amount of recombination frequency than far off genes. In far off genes, more distance between genes will help crossing over during meiosis with much higher frequencies. One percent recombination frequency between two genes is considered a relative distance of one centiMorgan (cM), a unit of relative distance between genes. Therefore, recombination frequency up to 50% cM will be the maximum relative distance measured between any genes present on the same linkage group. After 50% recombination frequency, genes may look as if they are present on different chromosomes or unlinked though the genes are present in the same linkage group. Therefore, the distance in cM will provide an order of genes on chromosomes serially from one location to another.

For further measuring the distance on the same linkage group, the recombination frequency is to be determined by taking a gene within another range of 50 cM from some different location on the same linkage group. Hence, all genes or markers qualitative or physical DNA markers are possible to be assigned to a particular linkage group. Actually preliminary gene mapping on chromosome started as early as 1913 by Sturtevant. Finding the locations of trait expressing genes on chromosomes was then named as gene mapping or locating genes or it is just finding of positions of genes on particular site in long chromosomes. The external phenotype corresponding to that gene location is called genetic marker or tag and it is from here, the concept of gene tagging was initiated. Such genetic markers are typically single gene mutation at that location defining two alternative forms of a single gene called alleles with readily distinguishable phenotypes. The exact relative map positioning of two genes for two different phenotypes was started by Morgan (1940) where he mapped the relative distance between the positions of eye color and wing shape in Drosophila by the method of conventional crossing by measuring the recombination percentage between two genes in segregating populations that is specifically F2 or backcross progeny. This phenomenon is explorable for crop breeding and varietal improvement by breeders in any sexually reproducing species. However, these are simple traits controlled by oligo genes and often not traits that are particularly valuable for crop breeding purposes. Mapping or tagging complex agronomic traits directly is much more complicated. In general, such phenotypic marker or genetic markers are limited in number and is a basic bottleneck in tagging so many genes in any species. Another types of markers that are identified by using DNA based techniques by molecular biologists are the physical DNA markers. The pertinent benefit of these markers is that these can be observed directly without looking for

phenotypic expression avoiding complications associated with phenotypic evaluations such as environmental requirements, incomplete dominance, epistasis or pleiotropy. Like the genetic markers, polymorphism must be there and this polymorphism is to be detected by molecular technique rather than phenotypic expression. The polymorphism can be detected by isolating DNA from the progeny and inheritance of physical markers studied. In most of the cases, these are codominant and easily distinguishable among different individuals.

One of the significant advantage of these DNA based markers is that these are because of nucleotide variability present in DNA chains and are considered as created markers and hence, actually can be created by the scientists as by varying methodology using different reaction ingredients like that of restriction enzymes for cutting DNA and by using different sets of primers in amplifying DNA sequences to create different gametes of markers. Here virtually these markers are or could be unlimited in number vis a vis limited number in phenotypic or genetic markers, thus providing a dynamic marker exploration technique. With these markers, integrated linkage map with genetic marker construction is possible and nowadays, linkage map construction is done by program like the one JOINMAP 4.1 program (van Ooijen 2006). Several DNA markers, viz. RFLP, RAPD, AFLP, SSR, ISSR, SCAR, STS, PCR-RFLP, CAP, SAP and SNP, etc. have been developed and discussed by several workers (Thakur 2000). Of these, SSR or microsatellite markers and ISSR (inter simple sequence repeats) have been envisaged to be widely used by most of the laboratories without much technical sophistication and could be used in marker assisted selection (MAS) for crop improvement (Wang et al. 1999) and has become facility in many plant species. The crucial to the dissection of complex traits related with yielding potentials will be the construction of dense integrated linkage maps with large number of physical as well as genetic markers in the times to come which can be further enriched with more and more DNA markers and then to find association between genetic and physical markers and quantitative traits of agronomic importance which in reality is the gene tagging approach.

Quantitative traits have been the focus of crop improvement from the beginning of the concept of factors controlling plant traits from the time of Mendelian concepts or even pre-Mendelian era. Thoday (1961) demonstrated that simply inherited gene marker can be used as tag to locate quantitative trait controlling regions. However, impetus to use markers to identify quantitative traits and that was dubbed as QTLs was there when physical DNA markers were developed. These markers helped to breed traits controlled by polygenes by treating marker as if the trait is controlled by a single Mendelian trait (Tanksley 1983; Paterson et al. 1988).

23.6 Tagging and Dissection of Quantitative Trait Loci

This is also called the genetics of the complex traits. Complex traits do not show classical Mendelian recessive and dominance inheritance as shown by a single locus. The simple correspondence between genotypes and phenotypes breaks down in quantitative traits due to effects of environment or interaction of multiple genes

with other genes where different phenotypes can result in the same genotypes and trait is said to be complex trait. This has been found to be influenced by multiple genetic factors and more prevalently by a large number of additive genes each with small additive effect, the linked or unlinked, the inheritance of which is not possible to detect by simple genetic analysis. However, it is often impossible to find a tag or a marker that shows perfect co-segregation with a complex trait and it is mostly because of incomplete penetration and phenocopy, genetic or locus heterogeneity specifically encountered in animal system than in plants and polygenic inheritance, where in some traits simultaneous presence of mutations in multigene is required preferably called polygenic traits or quantitative traits expressed as combined action of individual quantitative loci. Discrete trait may represent a threshold effect and produced whenever an underlying quantitative variable is influenced by multiple genes, exceed a critical threshold or a pure synthetic effect requiring the simultaneous and joint action of each of the several mutations (Lander and Schork 2006).

The study of complex traits is much more complicated in animal system like humans than in crop plants, unlike animals where desired selective mating is possible. We shall be confining our focus on plant systems rather than animal. However, Lander and Schork (2006) have discussed four available methods for genetic dissection of complex traits in animals, viz. linkage analysis, allele sharing, association studies, and genetic analysis of large crosses in model organisms such as mouse and rats. But in plant system, of these, linkage analysis by controlled breeding has been explored to the highest extent in tagging and dissecting quantitative trait loci in crop plant species and recently, association studies have also been initiated in some major crops especially in maize and Arabidopsis for fine QTL mapping and possibility of using linkage disequilibrium in crop plants reviewed by Flint-Flint-Garcia and Thornsberry (2003).

One of the foremost aim of molecular mapping is to produce sufficiently fine scale map to pin point the location of genes that play a role in determining important agronomic traits per se or associated with yielding ability linked closely to any physical DNA marker possible to be created by DNA manipulation technique that are QTLs. The primary QTL mapping is coarse and locates the gene within a chromosomal region known as QTL supporting interval which is of approximately 10–30 cM (Lee et al. 2002) in general and in maize, the resolution of these maps was limited to 5–10 cM about 10–20 million base pairs within hundreds of genes within that each QTL (Buckler and Thornsberry 2002). Map based strategies for positional cloning of genes that control the QTL have been reviewed by Yano (2001).

To identify the actual gene involved in the quantitative trait, two methods are available: (1) positional cloning and (2) association mapping.

23.6.1 Positional Cloning

In positional cloning, steps are taken to map the QTLs to a much finer resolution where mapping of genes in terms of DNA nucleotide sequences is achieved. This can be done by crossing near isogenic lines (NILs) in which parents are different with

only one gene. This way we can do more precise fine mapping and genetic markers can be refered as candidate genes. Pflieger et al. (2001) have reviewed the candidate gene approach in plant genetics. Now the concept of studying candidate proteins of a candidate gene has been developed (de Vinne et al. 1999). When several polymorphic genetic or physical molecular markers are mapped in the region, fine mapping of DNA nucleotide sequence of a gene is at present possible. That means genes for QTLs are tagged at the nucleotide level sequences of that gene only for those plants whose genomes have been sequenced or can be thought of being in the process of sequencing. The molecular marker closest to the QTLs is used to link the genetic map to physical map. So it will be possible to determine gene responsible from the candidate genes in the location by identifying the mutation responsible for the QTL effect that is in quantitative trait nucleotide polymorphism (QTN). The validation of candidate genes is physiological analysis of CG expression activity and by genetic transformations. However, it may be necessary to test each predicted coding sequence region functionally by overexpressing or downregulating this gene.

However, so far, relatively small number of genes responsible for QTLs have been identified and still several of these encode regulatory proteins, for example, genes involved in flowering time in Arabidopsis and heading time in rice and QTLs mapping to elucidate the genes involved in tomato fruit ripening (Salvi and Tuberosa 2005). The basic methodology has been already described earlier. QTL linkage mapping is abundant in nearly all crop species; we shall, however, be focusing on some of the studies from different crops just for demonstration of some important aspects of gene tagging by QTL approach.

As already referred, high density linkage map is crucial for the identification of QTLs, positional cloning and integrated genetic maps and physical marker map assembly. Linkage mapping enables identification of association between traits and markers for both simple Mendelian traits and QTLs (de Ron et al. 2015). Some of the most recent studies with relation to linkage map using the much suitable PCR based markers using RILs linkage analysis are by Kujur (2015); Gonzalez et al. (2016); Sheetal et al. (2017); Gept et al. (2015); Song et al. (2015), and Bohra et al. (2011). Recently QTL mapping for yield and yield related traits and micronutrients has been done by Liu et al. (2019) in rice, essential grain quality in wheat by Goel et al. (2019) and dought related parameters in horse gram by Chahota et al. (2020). As far as the historical aspects of mapping or tagging are concerned, Thoday (1961) demonstrated that a simple inherited gene marker can be used as tags to locate quantitative traits. In legumes quantitative traits have been studied since Mendel.

The accounts of the techniques for identification of QTLs by gene markers became more efficient with the availability of molecular markers or rather physical DNA markers and found the new name as the QTLs (Gelderman 1975). The identification of QTLs by physical markers allowed the analysis and selection of complex traits as a set of single traits has been reviewed by Tanksley (1983). With the advent of molecular markers initiated by RFLPs, the restriction cutting of DNA also called hybridization based markers was first applied to develop linkage maps. These methods were subsequently replaced with amplification of DNA by PCR based markers, both non-specific RAPD and specific primer based AFLP and locus

specific SSR and SNP markers. The DNA sequence technology has made major advances over the last decade making many of the previous marker based systems redundant and genome sequences are now available for several of the crop plants giving trait improvement opportunity through MAS, identification of QTLs, gene tagging, and discovery. That later shifted to more suitable PCR-DNA amplification techniques like RAPD, AFLP, and SSR, thus the study of quantitative traits became facility in many plant species (Wang et al. 1999). The applications of molecular markers to plant breeding using modern statistical methods (Malosetti et al. 2013) have allowed breeders to accurately estimate the position and effects of genomic regions associated with variations in quantitative traits (Persequinni et al. 2016). In wheat, detection and mapping of QTLs by linkage analysis for yield and yield related components have been reported by Garcia et al. (2019) and Tricker et al. (2018) reviewed QTLs detections in wheat under water limited environments, some of the recent such linkage studies have been reported by Wang et al. (2012) in rice, Goel et al. (2019) and Liu et al. (2019) in wheat also citing some more references in these crops.

23.6.2 Association Mapping

Of the two most commonly used approaches at present for dissecting complex traits that is linkage analysis and association mapping, the latter is based on linkage disequilibrium, offers an alternative method for mapping QTLs (Marikagas 1996; Mackey and Currie 2001; Yu and Buckler 2006). Association mapping has several advantages over linkage mapping in traditional biparental populations (Flint-Garcia and Thornsberry 2003). It has been considered a more efficient approach to complement map based cloning and in this approach application of association tests is made to naturally occurring population (Risch 2000). The advantages of association tests include their speed as number of mapping population needs to be created and it gives high resolution, which depends upon the structure of disequilibrium. The complex breeding history of many crops and limited gene few in most wild plants created complex stratification within germplasm that complicates association studies (Sheubel et al. 2000). Linkage analysis explores the shared inheritance of functional polymorphism and adjacent markers within families or pedigree known success story as in humans. However, linkage analysis in plants has been typically conducted with experimental populations that are derived from biparental cross. Association mapping though based on the same fundamental principle of genetic recombination as linkage analysis, it examines the recombination or linkage in shared inheritance for a collection of individuals (populations) often with unobserved ancestry. As the unobserved ancestry can extend thousands of generations, the shared inheritance will only persist for adjacent loci after those many generations of recombination. Essentially, association mapping exploits historical and evolutionary recombination at population level (Ramington et al. 2001; Buckler et al. 2009). This approach of association mapping was first started in human (Corder et al. 1994; Karem et al. 1989). A linkage association mapping utilizes ancestral recombination events in

natural populations to make marker-phenotype associations and has been reviewed by Buckler and Thornsberry (2002) and Rafalski (2002). For QTLs analysis associataion mapping has been adapted to the statistical association between allelic variants at candidate loci within an existing set of genotypes (Inbred lines, breeding lines, cultivars or germplams accesions).

The use of SNPs, which are single base mutations but can also include insertions/deletions is a novel prospect to be used as marker to identify QTLs. ECO-TILLING (target induced local lesions in genomes) is a mutation detection tool offering the study at fundamental level of gene function and provide a powerful tool for identification of SNPs within target locus. This will allow the association between haplotype variation at a candidate gene and the quantitative phenotype to be detected. Haplotype is a particular combination of alleles in a defined region of some chromosome in haploid conditions and helps to study the association of DNA marker with associated alleles in the population. The SNPs in candidate genes with putative functions were found clustered near QTL in maize that contribute to phenotypic difference with teosinte, thus, association mapping is powerful tool to validate co-localization between candidate gene and QTL (Yu and Buckler 2006).

Linkage analysis and association mapping are, however, complementary to each other in terms of providing prior knowledge, cross validation, and statistical power (Wilson et al. 2004). Essentially, association mapping exploits historical and evolutionary recombination at population level (Ramington et al. 2001). In association mapping we explore deeper population genealogy rather family pedegree and require less time to draw conclusion than linkage analysis (Flint-Garcia and Thornsberry 2003; Yu and Buckler 2006). High resolution provided by association mapping is dependent upon the structure of linkage disequilibrium across the genome; LD, which refers to non-random association of alleles between genetic loci. This simply means that certain alleles for different loci within a population are found with specific phenotypes more frequency than expected, that is, these show linkages with marker (Flint-Garcia and Thornsberry 2003).

The term linkage and LD are often confused, though both these are related, these are distinctly different. Linkage refers to correlated inheritance of different loci through the physical connection on a chromosome, whereas linkage disequilibrium refers to the correlation between alleles in a population. Tight linkages result in high LD, but LD may be due to correlation between alleles present on genetic and non-genetic factors including recombination drift, selection, mating pattern, and admixtures (i.e. population of subgroups with different allele frequencies) offered the structure of linkage disequilibrium and precisely discussed by Flint-Garcia et al. (2005); Yu and Buckler (2006). The association studies, therefore, are best carried out in independent populations with large sample size.

The detailed methods of association mapping have been given by Whit and Buckler (2003) and additional information can be found at http://www.maize.genetics.net. The basic steps required are germplasm choice, estimation of population structure, trait evaluation, identification of candidate polymorphism, and statistical analysis (Flint-Garcia et al. 2005). To tag and dissect QTLs it is required first to estimate the structure of germplasm population by taking the all the factors like

heritablity, genotype x environmental interactios etc. Multidisciplinary approach is needed to choose candidate genes (CGs). The main aim of molecular genetics is to tag and isolate genes governing important traits. Of the three main approaches leading to cloning of genes of interest, that is positional cloning, insertional mutagenesis by transposon, and the positional candidate gene, the CG approach is alternative strategy of first two (Pfieger et al. 2001). The idea is to propose previously sequenced genes of known function that could correspond to major loci, i.e. MTLs and QTLs. The CGs may be structural genes or gene involved in regulation. The working hypothesis assumes that a molecular polymorphism within CG is related to phenotypic variation. The theoretical and practical applications of CG approach in plant genetics and breeding have been detailed by Pfieger et al. (2001). Choice, screening, validation, and application of CG approach for the simplified characterization of QTLs with linkage maps harboring high density of genes of non-function are available (Mitchel-Old and Pederson 1998).

The CG refers either to a cloned gene presumed to affect a given trait (functional CG) or the genes suggested by their close proximity on linkage map to loci controlling the trait (positional CG). The CG can be identified by looking for map co-segregation between CG and variation in the trait of interest. Candidate genes are genetic markers, while DNA markers are physical markers, both can be used to identify MTLs and QTLs. The CG may also be structural genes or gene involved in regulation of metabolic pathways. The working hypothesis assumes that a molecular polymorphism within the CG is related to a phenotypic trait variation and hence CG is a genetic marker of agronomic interest (Yu and Buckler 2006).

Studies involving mutagenesis, biochemical profiling, list of positional candidate, or candidate genes that fall within previously defined QTL intervals. A preliminary sequence for each candidate gene is needed to design overlapping primer pairs to amplify both upstream and in coding regions of the gene. Primers designed from the conserved regions may amplify paralogous regions of the genome; whereas in less conserved region due to extensive polymorphism in germplasm the priming may fail. Nucleotide sequence is determined by capillary electrophoresis. Sequence quality is assessed and sequences are contigued/joined. Alignment of contigs across the germplasm is created and polymorphism (Indels and SNPs) is identified.

Linkage disequilibrium should be examined to determine which polymorphism forms haplotypes within the candidate gene and to define the resolution of the association. This association is to be verified by evaluating candidate polymorphism in an entirely different population structure or by biochemical studies when the polymorphism causes a change in promoter activity or coding sequence and analysis of nearly isogenic lines.

23.7 Exemplary Association Studies in Crop Plants

Many plant association studies in plants have been compiled and reported in maize, sea beet ssp. maritime, Arabidopsis in different traits (Flint-Garcia et al. 2005). In the first candidate-gene association mapping study in plants dwarf 8 (D8), DNA

sequence polymorphisms within candidate gene D8 locus in maize were associated with flowering time QTL by Thornberry et al. (2001). It became the first empirical association study in any organism for which background molecular markers were used to control population structure (Pritchard 2001). Later studies of the same 102 inbred lines of Thornberry et al. (2001) associated the candidate gene sugary 1 (su1) with QTL for sweetness taste (Whit and Buckler 2003). Wilson et al. (2004) reported association of candidate genes brittle endosperm (bt2), shrunken 1(sh1) and shrunken 1(sh2) with kennel composition and QTLs for amylase extender (ae1) and sh2 were associated with starch pasting properties. In this study, principal component analysis was used to cluster phenotypic traits with three major groupings before association analysis, which served to reduce multiple testing and also facilitated the interpretation of results for many correlated traits. Szalma et al. (2005) have shown that candidate gene ae1 and whp1 were associated with maysin synthesis after controlling or a previously determined epistatic p locus illustrating the importance of incorporating known candidate genes in ensuring analysis. The candidate gene Y1 associated with maize endosperm color yellow was also substantiated by linkage analysis (Wong et al. 2004). Progress continues to be made deciphering the number of QTL under complex traits in maize, 50 QTLs for oil concentration and 62 consensus QTLs for flowering time. Gene discovery has been said to be initiated by analyzing existing data for pedigree maize inbred lines or hybrids.

Some of these examples illustrate that association mapping is especially useful for dissecting candidate genes involving MTLs (eg, Y1 for endosperm color and su1 for sweetness taste). This is because of their simple genetics and accurate phenotypic measurements and strong imposed selection. For more complex traits, candidate genes with relatively large effects on traits with relatively high heritability (e.g. D8) for lowering time will associate first. So far associations have however been successfully established for traits with only moderate heritability such as starch concentration in maize. Garcia et al. (2019) have reported the results of genome wide association study using wheat panel consisting of landraces, synthetic hexapods, and other accessions for contributing alleles that would increase yield in water limited environments such as those in Southern Australia by using 90 thousand SNPs. They have cited some other references like that of Singh et al. (2016) and Tyagi et al. (2014) who have also reported similar QTL studies in rice varieties and common wheat from India. Bennet et al. (2012) reported genetic dissection of grain yield and physical grain quality in bread wheat under salt limiting environments, whereas Baseggio et al. (2018) have reported results on genome wide association and genomic prediction models of tocochromanols in fresh sweet corn kernels.

23.8 National and International Status

The genes that control complex traits in plants have long been too difficult to identify or characterize precisely. Before the advent of QTL mapping, analyzing the genes that control complex traits was an overwhelming task (Camargo et al. 2018). Identification of quantitative traits is becoming easier through modern scientific

techniques, which has led to a new wave of optimism to accomplish this task. The most common strategies of QTL mapping use a large number of RILs, which are established for at least several generations of inbreeding (typically up to F6 or F7). Molecular markers based genetic maps are available for several economically important plants like Arabidopsis, maize, rice, wheat, sorghum, cowpea, tobacco, barley, tomato, potato, sunflower, pea, bean, rye, millet, cotton, soybean, turnip rape, cauliflower, alfalfa, carrot, sugar beet, coffee, grape, and sugarcane. Despite lack of precise information about the molecular nature of the QTL, introgression of QTLs into elite germplasm lines and marker assisted selection for QTLs in breeding could be undertaken in some crop plants such as maize, tomato, and rice with reasonable success. There are still some important cautions regarding QTL analysis. Only the QTLs of largest effect and those bordering to a marker locus will show statistically reliable association. Particularly significant is fine mapping or high resolution mapping of the QTL, if the QTL information is to be commendably applied in field (Dhingani et al. 2015). However, both the approaches RILs and association mappings are being used purposefully in major cereal crops that are maize and wheat (Goel et al. 2019; Liu et al. 2019 and Baseggio et al. 2018).

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Nanotechnology and Robotics: The Twin Drivers of Agriculture in Future

24

Amjad M. Husaini and Asma Khurshid

Abstract

Agriculture-based livelihood systems, which are already vulnerable to climate change, face formidable future challenges. Herein we explore the role of nanotechnology in crop production, protection, and sustainable agriculture. Additionally, robotics and drone-based spraying technology that can enable farmers to meet farm management issues and create high-value farms are also discussed. We must integrate the best of these modern technologies with crop biotechnology for creating a better food production model.

Keywords

Climate change · Food · Robotics · COVID-19 · Biotechnology · Nanotechnology

Abbreviations

COVID-19	Coronavirus disease 2019
U.N.	United Nations
FAO	Food and Agricultural Organization
OECD	The Organization for Economic Co-operation and Development

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24.1 Introduction

Food is a fundamental human right, and to grow food, we need healthy soil. The supply of nutrients in the form of fertilizers is necessary for improving soil fertility and crop productivity. However, the precise and judicious use of fertilizers is one of the most vital prerequisites for sustainability (Husaini and Tuteja 2013; Huang et al. 2017). Approximately 40% of the world's agricultural land has degraded due to intensive farming practices, leading to the loss of soil fertility (Kale and Gawade 2016). The nutrient use efficiency of conventional fertilizers is between 30 and 40% (Dijk and Meijerink 2014). For nitrogen the loss is 30–35%, for phosphorus it is 18–20%, and for potassium 35–40% (Subramanian et al. 2015). Actually, a meager amount reaches the targeted site due to high leaching losses, drift, runoff, hydrolysis, evaporation, photolytic and microbial degradation (Sabir et al. 2014).

Farming is currently facing formidable challenges in feeding a growing population in a sustainable way (Firbank et al. 2018). The situation has become complicated and worse in view of resource depletion, climate change, and challenges due to pandemics like COVID-19. There is an immediate need to explore modern ways to develop a robust agricultural system that would survive the challenges of climate change, resource shrinkage, environmental sustainability, and the constraints of labour shortages. Concurrently, some major high-tech, radical, and potentially game-changing technologies in biotechnology, robotics, nanotechnology, artificial intelligence, etc., can help address these challenges. These new technologies can lead to the next agriculture revolution, which is sometimes called 'Agriculture-4.0' (Rose and Chilvers 2018). International organizations like FAO, World Bank, and OECD look forward to harnessing these technologies' potential and their role in food and nutritional security (De Clercq et al. 2018; NFU 2019; Jouanjean 2019; Trendov et al. 2019; World Bank 2019). Herein, we discuss the future of agricultural production in view of modern technologies for ensuring food security: (1) use of nanotechnology in agriculture production and product development; (2) use of robotics and artificial intelligence-based technologies to increase precision, and profitability, while reducing health-hazards to humans, and problems due to labour shortages (Fig. 24.1). These options are discussed as follows:

24.2 Nanotechnology in Agriculture

In 1974, Professor Norio Taniguichi, Tokyo University of Science, coined the term 'nanotechnology' (Khan et al. 2017). Nanotechnology has emerged as a technology with the potential to revolutionize different aspects of human life. It can enable target-specific delivery of 'active molecules or nutrients' in a controlled manner. The use of nanotechnology can: (a) decrease market consumption of phyto-protective chemicals; (b) reduce nutrient losses from chemical fertilizers; (c) enhance yields; (d) improve nutrient release; (e) deliver precisely at the target; (f) reduce phytotoxicity, etc. There is growing evidence about the profound effect of nanoparticle application on crop growth (Table 24.1).

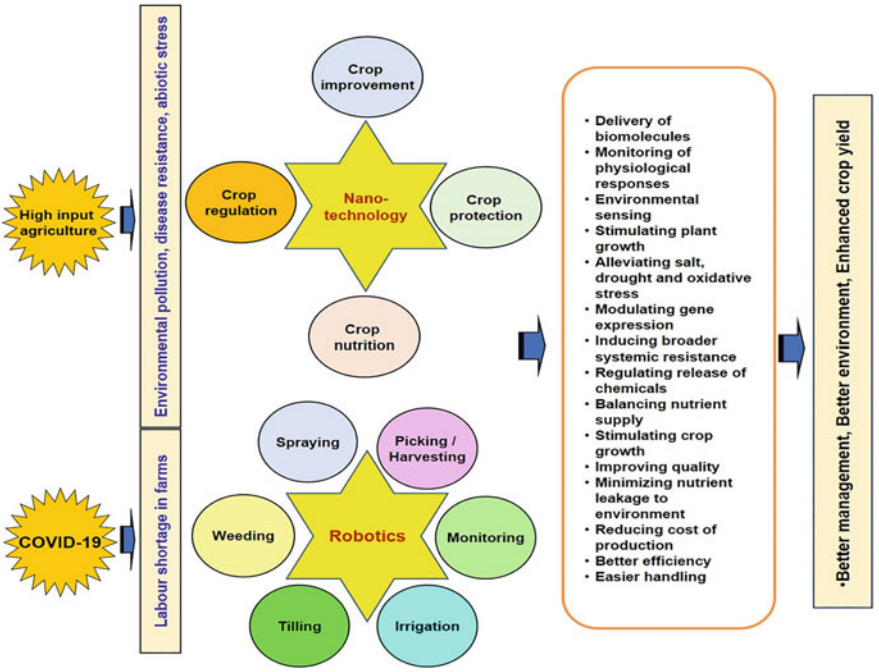

Fig. 24.1 Robotics and nanotechnology are the drivers of high precision agriculture

Nanoparticles act as 'bullets' loaded with agrochemicals for their controlled delivery into specific tissues. Further, nanoparticles can protect agrochemicals from the damage of external agents. This is illustrated with an example of Mesoporous Silica Nanoparticles (MSNs). Mesoporous silica nanoparticles have a honeycomb-like porous structure with hundreds of empty channels. These are able to encapsulate or absorb a large amount of agrochemicals. MSNs can load a pesticide (like avermectin) into its core which protects it from photo-degradation, and allows its sustained release (Li et al. 2007). MSNs get absorbed into the cuticular lipids of insects and protect plants against these pests. These damage their protective wax, causing dehydration and subsequently result in their death (Barik et al. 2008).

24.2.1 Nanofertilizers for Nutrient Management

Nanofertilizers contain fertilizers within nanostructured formulations that can be released in accordance with plant demands. Nano elements are a vital means to improve plant nutrition. Nano elements can be applied in two ways: (1) incorporate into a carrier-matrix like chitosan, clays, alginate, etc.; or (2) used to formulate nanoparticles per se. Micronutrients that are applied in nanoformulation have better solubility and quicker absorption. Nanoformulations enhance the nutritional quality

Table 24.1 Effect of nanoparticles of minerals applied via foliar route and soil route on plant growth, development, physiology, and disease tolerance

Active element	Effect on plant growth and physiology	Mode of application	Reference(s)
Ca	Enhances Ca uptake and controls insect pests of *Ziziphus mauritiana*	Foliar application	Hua et al. (2015)
	$CaCO_3$-nanoparticles enhance calcium uptake and growth in *Arachis hypogaea* seedlings	Soil application	Liu et al. (2005)
Mg	Increased growth, yield, mineral uptake, and enzyme activity in *Triticum aestivum* and *Vigna unguiculata*	Foliar application	Delfani et al. (2014), Rathore and Tarafdar (2015)
	Induction of tolerance against *Ralstonia solanacearum* through activation of salicylic acid and jasmonate signalling pathways in *Lycopersicum esculentum*	Soil application	Imada et al. (2016)
Zn	Increased fibre, carbohydrate, and fat in Spinacia oleracea and enhanced growth and yield in Arachis hypogaea, Pennisetum americanum, Vigna radiata, and Cicer arietinum	Foliar application	Burman et al. (2013), Kisan et al. (2015), Prasad et al. (2012), Tarafdar et al. (2014)
	Enhanced nutrient mobilizing enzyme content, yield, lycopene, and size in *Lycopersicum esculentum*	Foliar application	Raliya et al. (2015a)
	Increased Zn content in grains of *Triticum aestivum*	Foliar application	Deshpande et al. (2017)
	Enhanced growth and antioxidant status in Zea mays, Cicer arietinum, Cucumis sativus, Arachis hypogaea, *Lycopersicum* esculentum and Gossypium hirsutum	Soil application	Faizan et al. (2018), Venkatachalam et al. (2017), Pandey et al. (2010), Adhikari et al. (2015), Taheri et al. (2015), Zhao et al. (2014)
	Alleviated the toxicity caused by CuO nanoparticles in Phaseolus vulgaris	Soil application	Dimpka et al. (2015)
Fe	Increases carbohydrate and essential oils in Ocimum basilicum	Foliar application	Elfeky et al. (2013)
	Enhanced photosynthetic and transpiration rates in Glycine max and growth in Cucurbita maxima and Vigna unguiculata	Foliar application	Alidoust and Isoda (2013)
	Increased chlorophyll, protein, glycoprotein, and growth in Zea	Soil application	Ghafariyan et al. (2013), Pariona et al. (2017), Suresh et al. (2016)

(continued)

Table 24.1 (continued)

Active element	Effect on plant growth and physiology	Mode of application	Reference(s)
	mays, Glycine max, and Arachis hypogaea		
	Enhanced germination, growth of seedlings, antioxidant activity in Citrullus lanatus	Soil application	Li et al. (2013)
	Increased growth and malondialdehyde content in Oryza sativa and growth in Arachis hypogaea	Soil application	Gui et al. (2015), Rui et al. (2016)
Si	Increased chlorophyll, protein, phenol, and antioxidants in Zea mays	Foliar application	Suriyaprabha et al. (2014)
	Enhanced plant growth and achene number in capitulum in Carthamus tinctorius	Foliar application	Jan Mohammadi et al. (2016)
	Alleviated cadmium toxicity in Oryza sativa	Foliar application	Wang et al. (2015a, b)
	Enhanced germination, photosynthetic activity, and drought tolerance in Zea mays	Soil application	Karunakaran et al. (2013), Suriyaprabha et al. (2012), Rangaraj et al. (2014)
	Alleviated salinity stress and increased germination in Lycopersicum esculentum	Soil application	Haghighi et al. (2012)
Ti	Enhanced growth, protein, and chlorophyll content in Spinacia oleracea	Foliar application	Yang et al. (2007)
	Enhanced growth, chlorophyll content, and leaf protein in Vigna radiata	Foliar application	Raliya et al. (2015b)
	Enhanced chlorophyll, potassium, and phosphorus content in Cucumis sativus	Soil application	Servin et al. (2013)
	Increased shoot and root length, biomass, cysteine, methionine, and phosphorus in Lactuca sativa	Soil application	Zahra et al. (2015)
	Enhanced proline, malondialdehyde, and ROS scavenging enzymes in Brassica juncea	Soil application	Rao and Shekhawat (2016)
	Enhanced chlorophyll and photosynthetic efficiency in Lycopersicum esculentum	Soil application	Tiwari et al. (2017)
Ag	Enhanced growth of Brassica juncea and Vigna unguiculata	Foliar application	Pallavi Mehta et al. (2016)
	Enhanced growth of hydroponically grown Bacopa monnieri	Soil application	Krishna (2016)

(continued)

Table 24.1 (continued)

Active element	Effect on plant growth and physiology	Mode of application	Reference(s)
Au	Enhanced growth, redox status, and yield in Brassica juncea	Foliar application	Arora et al. (2012)
	Enhanced growth, yield, and ROS scavenging activity in Arabidopsis thaliana	Soil application	Kumar et al. (2013)
Carbon	Alleviated oxidative stress in Beta vulgaris grown under drought conditions	Foliar application	Borišev et al. (2016)
	Enhanced seed germination, root and shoot biomass in Lycopersicum esculentum	Soil application	Khodakovskaya et al. (2009)
	Enhanced yield, antidiabetic compounds, and lycopene content in Momordica charantia	Soil application	Kole et al. (2013)
	Increased root length in Medicago sativa	Soil application	Miralles et al. (2012)

of crops and reduce their environmental effect (Morales-Dıaz et al. 2017). Under hydroponic conditions, charged zein nanoparticles are an effective system for applying agrochemicals in sugarcane (Prasad et al. 2018). The nanoparticles have to cross cuticles to enter inside either through the hydrophilic or hydrophobic pathway. The hydrophilic nanoparticles penetrate through aqueous polar pores (2 nm) or stomata (>10 nm), while hydrophobic nanoparticles enter through cuticle wax by diffusion (Eichert et al. 2008).

Nanofertilizers prevent the premature volatilization of NH_3 from urea (Derosa et al. 2010). Urease inhibitors are loaded into MSNs for controlling the release of N from urea via hydrolysis (Hossain et al. 2008). Apatite nanoparticles are used as phosphorus (P) fertilizer for sustained low release of phosphorus. It reduces water eutrophication and environmental risks (Liu and Lal 2014).

Nanoformulations of fertilizers synchronize the release of fertilizer-elements with the crop's uptake demand. For instance, porous nanomaterials like zeolites, clay, or chitosan reduce the loss of nitrogen by regulating the demand-driven release and its subsequent uptake by plants (Panpatte et al. 2016; Millan et al. 2008;, Abdel-Aziz et al. 2016). Ammonium-charged zeolites increase the solubility of phosphate minerals and improve phosphorus uptake by plants (Dwivedi et al. 2016). Graphene oxide films prolong potassium nitrate release and minimize leaching losses (Shalaby et al. 2016). Sabir et al. (2014) demonstrated that nano calcite ($CaCO_3$—40%) application with nano SiO_2 (4%), MgO (1%), and Fe_2O_3 (1%) not only improves the uptake of Ca, Mg, and Fe but also increased the uptake of P, Zn, and Mn.

24.2.2 Nanoparticles for Plant Protection

Nanoformulation or encapsulation of pesticides can revolutionize the plant protection sector. Nano-encapsulation is the coating of pesticides with another material of different sizes at nano-range. The encapsulated materials form the 'internal phase' of the core material (pesticides) while capsulation nanomaterials form the 'external phase' (Nuruzzaman et al. 2016). Nanoformulations of pesticides boost crop yields by enhancing pesticide efficacy through regulation of pesticide transport (Petosa et al. 2017). Nanomaterials in pesticide formulation possess increased stiffness, permeability, thermal stability, solubility, crystallinity, and biodegradability which are important for sustainability of agriculture-environment system (Khan and Rizvi 2014; Haq and Ijaz 2019). It reduces the total amount of pesticides, a vital feature for sustainable pest management.

There are numerous examples about the successful use of nanoformulations in disease management. Some of these are discussed in the following passages. Strayer-Scherer et al. (2018) used nano-Cu nanoparticle composites with a Cu core–shell, multivalent Cu, and fixed quaternary ammonium copper to suppress bacterial spot caused by *Xanthomonas perforans*. *X. perforans* had acquired resistance against conventional copper bactericides. The nano-Cu composites were more effective than Cu-mancozeb. In an earlier report, Giannousi et al. (2013) engineered nano-CuO, Cu_2O, and Cu/Cu_2O composites against *Phytophthora infestans* in tomato. CuO nanoparticles were most effective in suppressing leaf lesions, followed by nano-Cu/Cu_2O composite. These nanoparticle-based products did not cause phytotoxicity and were better than commercial Cu-based fungicides. The antimicrobial activity of Zn nanoparticles has been reported too. Nano-Zn inhibits *Alternaria alternata*, *Botrytis cinerea*, *Fusarium oxysporum*, *Mucor plumbeus*, *Penicillium expansum, Rhizoctonia solani, Rhizopus stolonifer*, and *Sclerotinia sclerotiorum*. It has been reported that nano-MgO inhibits the germination of conidia of *A. alternata*, *F. oxysporum*, *R. stolonifer*, and *M. plumbeus* better than nano-ZnO (Wani and Shah 2012). Two nanoparticle formulations ZinkicideTM SG4 (a plate-like) and ZinkicideTM SG6 (particulate) have been found effective against citrus canker (*Xanthomonas citri* subsp. *Citri*), citrus scab (*Elsinoe fawcettii*), and melanose (*Diaporthe citri*) (Graham et al. 2016). ZinkicideTM SG6 decreased disease incidence more than the conventional cuprous oxide and cuprous oxide/zinc oxide bactericides.

A novel antimicrobial light-activated TiO_2/Zn nanoparticle composite has been developed by using photocatalyst technology and nanotechnology (Paret et al. 2013). It was found effective for controlling *Xanthomonas* sp. causing bacterial leaf spot on rose. Application of silver nanoparticles on cucumber and pumpkin leaves either 3–4 weeks before powdery mildew or after the infection was found highly effective (Lamsal et al. 2011a). The nanoparticles performed equally well as the commercial fungicide. Lamsal et al. (2011b) report that the application of nano-Ag (100 μg/mL) to peppers before anthracnose outbreaks is very effective in suppressing the disease. Park et al. (2006) combined nano-silver and nano-silicon with a water-soluble polymer and sprayed on cucumber leaves (0.3μg/mL) 3 days before exposing plants to *Podosphaera xanthii.* The pathogen was effectively

controlled. Gajbhiye et al. (2009) biosynthesized extracellular Ag nanoparticles from *A. alternata.* When combined with fluconazole (fungicide), the antifungal activity against *Phoma glomerata* increased but not against *Phoma herbarum* or *Fusarium semitectum.*

Elmer and White (2016) report that nano-Cu delivers a more active Cu load at significantly nominal rates and offers multiple benefits in plant disease management. Elmer et al. (2018) used foliar application of CuO nanoparticles to control *F. oxysporum* f. sp. *Niveum* in watermelon. Nanoparticles of the metalloid B were also effective against Fusarium wilt without having any adverse effect on yield. However, there was no disease-suppressive effect of nano-TiO, nano-FeO, nano-AlO, and nano-NiO on Fusarium wilt. Cu nanoparticles were the most effective at suppressing Fusarium wilt of tomato and Verticillium wilt of eggplant.

Nanoformulations combining polymeric nanocapsules and pyrethroid bifenthrin (nCAP4-BIF) possess better transport potential. Hence, nCAP4 can act as a promising delivery vehicle for pesticides like pyrethroid. It is due to the enhanced dispersion and wettability of nanoformulations. Some nanoformulations of pesticides widen the plant-based systemic acquired resistance (SAR) against pests. For example, silica nanosphere formulations enhance the ability of pesticides to enter the plant and reach its cell sap, thereby causing systemic effect, which helps control chewing or sucking type insects like aphid (Li et al. 2007). This type of hollow formulation protects pesticides from photo-degradation by sun rays (Panpatte et al. 2016).

Ectopic expression of antigen-specific nanobodies in plants can help reduce crop loss by enhancing resistance against pathogens. Nanobody binding to the critical pathogenic proteins prevents their attachment to cells, reproduction, and movement, consequently disrupting their life cycles. Additionally, nanobodies can neutralize the pathogen toxins directly. Broad bean mottle virus (BBMV) infects *Vicia faba, Cicer arietinum*, and *Pisum sativum*, causing substantial economic loss. Ghannam et al. (2015) isolated three BBMV-specific nanobodies with high affinity, which successfully attenuated its spread and neutralized it when transiently expressed. Grapevine fanleaf virus (GFLV) infects vineyards worldwide. Hemmer et al. (2018) isolated a nanobody (Nb23) which caused strong and specific resistance against GFLV. Nb23 binds to the GFLV capsid surface (Orlov et al. 2020).

24.2.3 Using Nanoparticles to Modulate Plant Growth

Numerous reports show that nanoparticles regulate the growth of plants, predominantly during germination and seedling stages (Kole et al. 2013; Canas et al. 2008; Liu et al. 2009; Li et al. 2007; Khodakovskaya et al. 2009; Lahiani et al. 2015; Zhang et al. 2017; Arora et al. 2012; Gopinath et al. 2014; Ndeh et al. 2017; Syu et al. 2014; Jasim et al. 2017; Pallavi Mehta et al. 2016; Li et al. 2015; Pariona et al. 2017; Jalali et al. 2017; Yuan et al. 2018; Rafique et al. 2014; Rui et al. 2016; Shankramma et al. 2016; Askary et al. 2017; Faizan et al. 2018; Awasthi et al. 2017). The Fe_3O^{4-} nanoparticles (20 mg/L) increase germination in maize, and nanoparticles based on

Cu, Zn, Mn, and Fe oxide (<50 ppm) improve germination and root growth (Li et al. 2015; Liu et al. 2016). In soybean, CuO increases root lignification when applied at 200–500 mg/L (Nair and Chung 2014), and in wheat TiO_2 increases root and shoot length when applied at 60 mg/kg (Rafique et al. 2014). Stem length and total weight of tomato seedlings increased by using multi-walled carbon nanotubes (MWCNTs) (Khodakovskaya et al. 2009). An increase of three-fold root and shoot length has been reported in *Triticum aestivum* using water-soluble CNPs and a 78% increase of cell growth in tobacco by single-walled carbon-nanohorns (SWCN) (Lahiani et al. 2015; Saxena et al. 2014). Carbon nanotubes enhance root growth of *Triticum aestivum* and dry weight of *Zea mays* (Tripathi and Sarkar 2015, Tiwari et al. 2014).

24.2.4 Nanoparticle Toxicity

Nanoparticles in the range of 250–2000 mg/L cause toxicity in plants. It is evident by its negative influence on seed germination, root elongation, shoot elongation, biomass, leaf area, etc. At the anatomical level, cortical and epidermal cells get disintegrated, plasmodesmata get blocked, vacuoles get modified, and cell turgor gets deregulated. At the physiological level, nanoparticles alter stomatal operation, transpiration rate, phytohormone regulation, photosynthetic rate, etc. (Arora et al. 2012; Jacob et al. 2013; Begum and Fugetsu 2012; Tripathi et al. 2017). At the genetic level, nanoparticles damage DNA structure, cause chromosomal aberrations, reduce mitotic index, alter gene expression and chromatin condensation (Ghosh et al. 2010; Kumari et al. 2009; Shen et al. 2010; Khodakovskaya et al. 2009).

24.3 Robotics

24.3.1 Reducing Environmental Footprint

Agriculture, though central to human welfare, causes a negative effect on the environment. It accounts for one-fourth (25%) global greenhouse gas emissions, be it carbon dioxide, methane, or nitrous oxide (Rosenzweig and Hillel 1998). This can be minimized by adopting technologies like drip irrigation, zero tillage, etc., in combination with herbicide-tolerant GM crops, insect-resistant GM crops. This reduces fuel use in on-farm mechanical operations directly, and lower CO_2 emissions (Fawcett and Towery 2002). Between 1996 and 2016, the cumulative permanent reduction in fuel use due to biotech crops is estimated as 29,169 million kg of CO_2, equal to taking 18 million cars off the roads for one year (Brookes and Barfoot 2018). The adoption of no-till farming practices also assists in enhancing the sequestration of carbon (Aalde et al. 2006; Glover et al. 2008). According to Brookes and Barfoot (2018), the additional amount of soil carbon sequestered since 1996 due to biotech crops has been equivalent to 251,390 million tonnes of CO_2 which would otherwise have been released into the global atmosphere. Accurate and precise crop

monitoring using electronics based pollution free robotic technologies will help to further reduce the environmental footprint of agriculture.

24.3.2 Substituting for Labour Shortage

Mechanized tractor marked the beginning of industrial agriculture. These pulled ploughs, hauled loads, towed planters, reapers, cultivators, pickers, combine harvesters, threshers, mowers, balers, etc. This transformed agriculture from a labour-demanding enterprise to a power-intensive production system. This resulted in a continuous labour outflow from the land; for example, in 1900, 41% of the United States workforce was engaged in agriculture, but by 2000, it fell to 2% only (Autor 2014). However, as agricultural production models differ between developing and developed nations with respect to the 'level' of technology usage, the problem in developing nations is more complicated. The technological gap in developing nations is a major cause of lower farm-productivity, despite a considerably higher number of labour engaged in agriculture. Despite this, most of the developing countries face 'seasonal' agricultural labour shortage problem because the majority of youth from villages migrate to urban areas for better income, resulting in labour shortages and delayed agriculture operations during peak seasons. This crisis has got compounded due to the present COVID-19 pandemic due to its negative impact on the supply chain of labour force, owing to restrictions on the movement of seasonal labour across states, sealing of borders, cessation of public transport systems, social distancing, etc.

Despite the greater productivity of modern agriculture, the average age of farmers is rising and, once they retire, it is less likely that younger generations will take their place (Vilsack and Clark 2014). This will cause a great concern about labour shortages in agriculture in the future. In such a situation, robotics and automation in farming can help alleviate labour shortages within both the year-round and the seasonal-labour markets. Additionally, this would release the trapped labour from 'lower' agricultural productivity areas towards more rewarding and better-skilled roles needed for advanced economies.

24.3.3 Precision and Profitability

Robotics combine electrical, electronic, and mechanical engineering with computer sciences to create machines capable of performing complex actions. Future farms will have to use sophisticated technologies based on sensors, robots, cloud computing, artificial intelligence, etc. Unmanned aerial vehicles (UAVs) or drones have emerged in agricultural robotics in recent years (Gogarty and Robinson 2011). These are used to monitor crop growth rates and nutrient deficiencies; map soil and crops; detect weeds, pests, or diseases; detect and prevent water deficiencies; detect nitrogen deficiency; analyse soil characteristics; measure weather parameters; and spray pesticides and fertilizers (Krishna 2016; Zikeli and Gruber 2017). These advances

will not only help in addressing labour availability issues during pandemics like COVID-19, but also let agricultural businesses be more efficient, safe, and profitable.

Unlike industrial robots which operate in controlled environments, the challenge with agricultural robots is that these operate in a continuously changing complex environment like unstructured and unpredictable terrain like muddy soil, dusty atmosphere, strong winds, and different sunlight settings. These need to perform complicated tasks like harvest fruits of variable size, colour, shape, shading, etc. However, by incorporating a human into the robotic system, the human–robot complementarity can increase the performance and robustness. This can further lead to decreased costs and make it economically feasible, a limiting factor for agriculture robotics commercial implementation (Pedersen et al. 2006).

24.3.4 Upcoming Technologies and Reduced Health Risk

Several innovative technologies are being created not only for making farming more precise but also a sustainable business. These game-changing technologies will transform food production, and as seen by many will help large-scale, technology-intensive, modern specialized farms (Bronson 2019; Bronson and Knezevic 2019). Shipping container farms are a rising sector of the agriculture technology landscape. These are fully assembled, vertical hydroponic farming systems built inside standard shipping containers (https://www.freightfarms.com/home/). These farms have automatic nutrient dosing facilities and climate control systems, and are operated using mobile applications. Similarly another company (Plenty) operates indoor vertical farms in warehouses, using infrared cameras and sensors to monitor temperature, humidity, and carbon dioxide in their facilities (Peters 2017).

Multiple sensors are brought together to create devices capable of measuring multiple factors. For example, a unified device combining temperature sensors with sensors that measure barometric pressure and capable of outputting data in a digital format is being created (Charania and Xinrong 2019). Satellite imagery is an indispensable source of data that can help improve agricultural operations and monitor crops. For example, Farmers Edge provides farm management software that collects and analyses data from satellites, weather stations, as well as farm equipment (https://www.farmersedge.ca/). This enables farmers to make data-driven decisions about crop planting, cultivation, and harvesting. Plantix has built up the world's largest database of plant diseases and uses image recognition technology coupled with deep neural networks for the identification of the plant type, possible disease, and pest, or nutrient deficiency. The app also provides information on disease prevention and control measures. Blue River Technology manufactures machines that attach to a tractor and precisely apply herbicides on weeds, while avoiding crops (http://www.bluerivertechnology.com/). FarmBot is an open-source robotics project consisting of Cartesian coordinate machine to automatically plant seeds, control weeds, and irrigate plants (https://farm.bot/). It enables a farmer to use a web-based application to plan the farm, schedule sequences graphically, and

control the machine in real-time. Delicate fruits like apples, plums, pears, and citrus fruits can now be harvested by Israel-based FFRobotics (https://www.ffrobotics.com/). It uses deep learning algorithms to identify the fruit, determine its ripeness state, and extend a linear robotic arm for its harvesting. Spain-based AgroBot has developed the first commercial autonomous robot capable of detecting and harvesting strawberries (http://agrobot.com/).

These game-changing technologies will create farming systems that are safer than conventional farms. Most accidents in which machines and humans interact in agriculture are caused by collisions and human errors (Vasconez et al. 2019). Artificial intelligence-based robotic technologies will mitigate such accidents, especially where humans and robots work cooperatively sharing workspace, and would enable taking preventive actions if humans are involved in risky tasks. Robotic teleoperation combines human alertness with robotic accuracy, repeatability, and power, and makes it possible to remove humans from hazardous operations like spraying plants with chemicals (Roberto et al. 2003). In the near future, the tools and resources for the cultivation of any crop may be allocated with just a push of a button, with only a few human beings needed to manage hundreds of acres sustainably, while being operated entirely by semi-autonomous robots and with minimum possible wastage of natural resources.

24.4 Conclusion and Future Perspective

Nano-encapsulation is an evolving technology for controlled release and better product functionality. It involves packaging of vitamins, antioxidants, probiotics, carotenoids, preservatives, omega fatty acids, proteins, peptides, lipids, carbohydrates at nanoscale with the help of nanocapsules. Nanoparticles can act as delivery vehicles of molecules like nucleotides, proteins, and activators. The advantages of encapsulating ingredients include a longer shelf-life, better stability, consecutive delivery of multiple active ingredients, and pH-triggered controlled release. For example, Mesoporous Silica Nanoparticles system can be loaded with a target gene and its chemical inducer. The ends of MSNs are capped with gold (Au) nanoparticles. Once inside cells, an uncapping trigger is used to cleave the bonds between MSN and Au NPs. This results in the release of the biomolecules, triggering gene expression (Martin-Ortigosa et al. 2014).

Nanoparticle-based formulations are a promising tool for the controlled and targeted release of agrochemicals (fertilizers, insecticides, herbicides, etc.). However, it has been observed that the transformation or aggregation of nanoparticles prior to uptake can limit the effectiveness of nanofertilizers. Therefore, future research has to focus on reducing their phytotoxicity, non-target effects, and better release of mineral ions entrapped in nanoparticles of biodegradable polymer materials. Moreover, the use of nano-encapsulation has raised public concerns about its food safety for human consumption. Nanoparticles possess chemical and physical properties that differ from the normal macro-particles of the same composition and may interact with the living systems in different ways, causing unexpected

toxicity problems. The concerns over such toxicity effects have impacted consumer perceptions and acceptance (Das et al. 2009; Bumbudsanpharoke and Ko 2015). Further, there is no universal guideline developed explicitly for the safety assessment of nanomaterials in food. Recently, EU regulations established that any food ingredient resulting from nanotechnological applications must undergo a safety assessment before being approved for its use (Karthik et al. 2019).

Owing to the challenges posed by lockdowns and social distancing requirements during pandemics like COVID-19, timely availability of farm labour in required numbers has become a major constraint. This can be addressed by 'artificially intelligent' automation of farm operations using robotics and drone-based technologies.

Conflict of Interest The authors declare that they have no conflict of interest.

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Hydroponic and Aeroponic Cultivation of Economically Important Crops for Production of Quality Biomass

25

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Abstract

Nowadays, hydroponic and aeroponic practices are essential for the cultivation of commercially important crops to increase productivity and biomass yield in a lesser time duration. However, the methodological issues concerning the quality plant production in soilless culture are well addressed here, but a very less knowledge is available about the effect of hydroponic and aeroponic practices on plants growth biomass and its bioactive constituents. Therefore, the aim of book chapter is to provide an information about soilless cultivation practices for medicinal, spices and food crops production. The detailed information are represented into four sections (1) Introduction (2) National and international status (3) Cultivation system (4) Plant cultivation and (5) Conclusion and future perspectives.

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Keywords

Soilless cultivation · Electrical conductivity · Nutrient media · Biomass · Bioactives

25.1 Introduction

Worldwide producers and consumers showed enormous interest in more eco-friendly and sustainable solution for quality plants production in the present scenario (Kris-Etherton et al. 2002). Medicinal and aromatic plants are indeed rich sources of health promoting bioactive constitutes which are modulated by various factors viz., light, temperature, humidity, CO_2, genotype selection, ambient conditions and cultivation practices (Aires 2018). For large scale cultivation of plants; herbal, pharmaceutical and food industries are completely dependent on naturally grown plants or conventional agriculture practices. Such practices do not fulfil the desired needs and the issues related to high cost, microbial and dust load, heavy metal, and other contamination in raw material. These fail the standards and norms of the industries for the production of high value products. Based upon above concern, various soilless, modern and sustainable cultivation practices are established for mass production of commercially important plants in a controlled environmental conditions. In horticulture and agriculture sectors, hydroponic and aeroponic cultivation system are greatly advertised for quality produce containing bioactives. These cultivation practices offers a sustainable and reliable platform for the production of variety of economically important crops such as medicinal, spices, aromatic, flowering and vegetables (Thakur et al. 2019; Partap et al. 2020). Hydroponic is a type of soilless cultivation practice in which plant roots are exposed directly to the nutritious liquid and roots are supported by inert substrates such as pebbles, perlite and gravel, etc. (Aires 2018). Aeroponic is a method of growing plants in a nutritious mist environment without using soil or an aggregate medium. Aeroponic cultivation is different from hydroponic techniques because plant roots are not immersed in liquid media, even roots directly uptake the nutrients from the aerosol. In the controlled nurtured environment, these cultivation system gives better plant growth biomass yield, quality and metabolites production as compared to soil cultivated plants (Thakur et al. 2019; Partap et al. 2020).

These cultivation practices are becoming more and more well-known and widely spread around the world. Europe (France, Netherlands and Spain) is considered to be the largest soilless culture market, followed by the USA and Asia-Pacific market (Aires 2018). In hydroponic and aeroponic system, the growers manipulate the concentration of nutrients in the irrigated solution and modulate the production of certain beneficial metabolites in plants (Partap et al. 2020). To fulfil the requirement of standard quality biomass in compliance with the industrial norms, these systems are most promising cultivation practices to enhance plant productivity in a very less time duration (Thakur et al. 2019; Partap et al. 2020). As per available literature, various commercially important crops such as medicinal, spices, vegetables and flowering plants were successfully cultivated in these system. In this chapter, we

explore the up-to-date information on the hydroponic, aeroponic and traditional agro-practices and the effect of these cultivation practices on plant growth biomass and bioactive content accumulation. Apart from these, here also discussed the possibility, their influence, applications, and success rate in the cultivation of commercially important plants.

25.2 National and International Status

The global market size of hydroponic and aeroponic cultivation was valued at USD 126.2 million (year 2017) and is expected to grow to USD 759.4 million (year 2025), with 25.5% of CAGR. The hydroponic and aeroponic based trade of medicinal plant was US dollars 19.95 billion (year 2015), with its value projected to grow at 7% per year. The herbal plant market size is projected globally approximate 72 billion US dollars and will reach 7 trillion US dollars by 2050 with CAGR of 14.88% (Hughes 2016; Thakur et al. 2019). The global market demand for *P. kurroa* raw material is 500 tonnes and production is 375 tonnes per annum. According to National Medicinal Plant Board, India has the estimated annual trade of 1000–2000 MT with price of dried rhizome Rs. 1100–2000 per kg. In India, the market price of dried rhizome and roots of *V. jatamansi* is valued at Rs. 600–1800 per kg, and the estimated annual trade is 1000–2000 metric tonnes (Goraya and Ved 2017). Indian valerian root and rhizomes yields volatile oil upto 0.5–2.12%. The market value of dried roots and rhizomes of *V. jatamansi* depends on quality and degree of processing. The current price of valerian oil is about Rs. 25,000/kg. The world production of the essential oil from parsley is estimated 8.3 tonnes from parsley seed oil, valued at USD 1.16 million, and 4.0 tonnes from parsley herb oil, valued at USD 0.56 million. Real-time market prices of basil is 6.35 USD per kg. According to AYUSH, in India, the estimated annual trade is 200–500 MT with market price of 200–1000 per kg. The global stevia market was valued at USD 370 million in 2017 and is expected to reach USD 640 million by 2023 with a growing 9.4% compound annual growth rate (CAGR) (Table 25.1).

25.3 Cultivation System

A wide range of requirements exists for plants, three main fundamental elements includes (1) water/moisture, (2) nutrients, and (3) oxygen. There are no distinctions between soilless and soil grown plants since in both procedures, nutrients must be dissolved in water before they can be absorbed by plants. The discrepancies lie in the way the plants uses nutrients. In hydroponic and aeroponic system, the nutrient solution provides directly to the roots system in which, it absorb the minerals with water and distributed to different parts of plants. In soil based system, the nutrients bind to the soil particles, pass into the water solution of soil and get absorbed by the roots. While in hydroponic and aeroponic systems, they do not have friction but provide more oxygenous environment to the roots for better growth and development. The environmental control of hydroponic and aeroponic system are equipped

Table 25.1 Estimated annual trade and market price of commercially important plants

Botanical name	Trade name	Part used	Estimated annual trade (MT)	Market price (Rs. per kg)	References
Picrorhiza kurroa	Katuka	Root, rhizome and leaf	1000–2000	1100–2000	National Medicinal Plants Board, Ministry of AYUSH, Government of India
Valeriana jatamansi	Tagar	Root (Rhizome)	1000–2000	600–1800	
Ocimum basilicum	Sweet basil	Leaf, root, Whole plant, fruit (Seed)	200–500	220–1000	
Petroselinum crispum	Parsley	Leaf, root, seed	3.0–3.2	350–600	
Stevia rebaudiana	Meethi Patti	Leaf	<10	2500–3800	

with automatic control monitor for photoperiod, temperature regulation, relative humidity and nutrient solution temperature. It contains reservoirs tanks for the nutrient solution and it is automated refilled with reverse osmosis water or demineralized water. UV lamps or disinfection system are installed in the system for the sterilization of nutrient solution which further provides to plants. Plant cultivation chambers contain dimensions according to the type of cultivation and plants. The chambers consist of a horizontal or vertical platform with holes for plant cultivation at a desired spacing range (Thakur et al. 2019; Partap et al. 2020). For aeroponic system, spray jets with a fine orifice was used for spraying nutrient mist with intermittent spray duration through a pressure accumulator or any suitable pump. Air bubbler or oxygen generated units are used to provide oxygen rich solution to roots in hydroponic and aeroponic chambers. Nutrients solution pH and electrical conductivity can be monitored by EC and pH sensor. The representation of working of model are shown in Fig. 25.1. There are many models of hydroponic system depending on their working strategies. Depending on whether the media is recirculated or is filled after any irrigation cycle, systems may be open or closed. In the closed irrigation systems, the nutrient media is recycled continuously and pH, EC and solubility of the nutrient solution are maintained and adjusted properly; while, in an open irrigation system, the nutrient media is discarded after each cycle of nutrition. Hydroponic system is also classified based on the nutrient solution movement i.e. active and passive. In active type, nutrient media is pumped usually by a high pressure accumulator pump and passive system depends on a wick irrigation system. Other category that the hydroponic with recovery or non-recovery principles i.e. the nutrient solution would be reintroduced or vanished after that. The three essential elements (water, nutrients and oxygen) to success in plant production must be given for all these different hydroponic types. The most widely commercially used soilless systems are classified as wick system, nutrient film technique, ebb and flow, drip system, aeroponic system and deep water culture (Aires 2018).

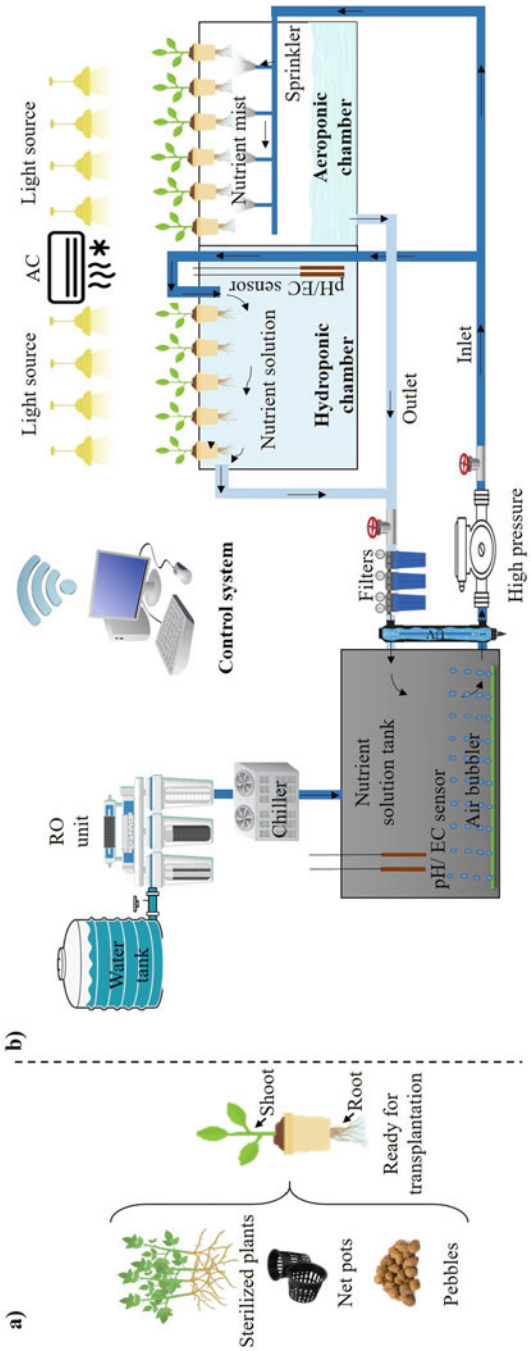

Fig. 25.1 Graphical representation of hydroponic and aeroponic cultivation model (Adapted from Rattan et al. 2022)

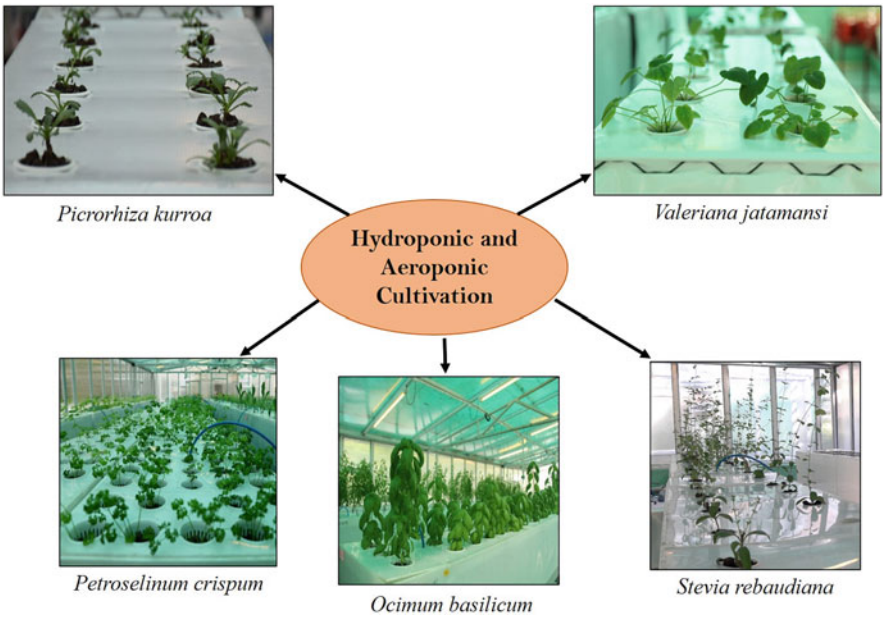

Fig. 25.2 Plants cultivated under hydroponic and aeroponic systems. (**a**) For *Picrorhiza kurroa*: the image adapted from Thakur et al. (2019); (**b**) for *Valeriana jatamansi*: the image adapted from Partap et al. (2020); (**c**) for *Petroselinum crispum*: the image adapted from Rattan et al. (2022)

Details about the successful cultivation of plants and their market potential are represented in Fig. 25.2 and in Table 25.1.

25.4 Plant cultivation

25.4.1 *Picrorhiza kurroa* Royle ex Beth. (Katuka)

Picrorhiza kurroa Royle ex Beth. is a commercially important Himalayan medicinal herb belonging to family Scrophulariaceae and is well known for its iridoid glycosides (anti-cancerous, hepatoprotective and anti-inflammatory properties). The leaf, root and rhizome are naturally enriched with high value picroside bioactives such as picroside-I, II, III and kutkoside. These phytomolecules are used in the formulation of various herbal products. The rising demand of picrosides and over-exploitation of plants led to its endangered status. In wild habitats, plants require 3–4 years to accumulate desired amount of metabolites and vary by various biotic and abiotic ecological factors. Propagation, multiplication, cultivation and conservation of *P. kurroa* is greatly dependent on conventional agro-practices and plant tissue culture techniques. These methods did not fulfil the desired need of herbal industries as per norms and industrial standards. Hence, hydroponic and aeroponic practices offer an alternative platform to reduce the time required for the

accumulation of desired metabolites and year-round biomass production under controlled conditions (Thakur et al. 2019). In this study, the plants were grown under controlled nurtured environment condition such as temperature 25 °C, 16 h light/8 h dark photoperiod, humidity 65%, electrical conductivity 0.5–1.5 mS cm^{-1}, pH 6.8–7.0, chiller temperature 10 °C and photosynthetic photon flux density (PPFD) 150 μmol m^{-2} s^{-1} for 12–14 weeks, respectively. *P. kurroa* plants (sizes 3–3.5 cm) were used for cultivation in hydroponic and aeroponic system (Fig. 25.2). The composition and concentration of the nutrient solution was used as follows; potassium sulphate (70 g), potassium nitrate (300 g), magnesium sulphate (240 g), copper sulphate (0.10 g), ferric-EDTA (10 g), manganese (1.5 g), calcium nitrate (100 g), zinc sulphate (0.30 g) molybdic acid (0.10 g) and boric acid (0.70 g) for 20 litre of nutrient solution (Thakur et al. 2019). As per protocol described by Thakur et al. (2019), at initial stage, nursery plants were disinfected with bavistin 1.0% for 5 min and formalin solution 0.05% for 2 min; while, net pots and pebbles were sterilized by formalin solution (0.2%). After sterilization, plants were anchored in net pots containing pebbles and transplanted to cultivation chamber. The quality of cultivated plants were evaluated by measuring their morphological traits, physiological performance and secondary metabolite content analysis among hydroponic, aeroponic and soil raised plants. Study observed maximum morphological parameters (plant height, leaf length, leaf width and stem diameter), photosynthetic rate, stomatal conductance, transpiration rate, picrosides content under aeroponic system as compared to hydroponic and soil grown plants. While in case of hydroponic cultivated plants, rootlets number/plant, length of rootlets and width of rootlets were achieved the best results. The study revealed that aeroponic system is most suitable and provides alternative and sustainable option for *P. kurroa* quality production required for the preparation of herbal drug to the industries.

25.4.2 *Valeriana jatamansi* Jones. (Tagar)

Valeriana jatamansi Jones. is medicinally important, perennial, rhizomatous herb belonging to family Valerianaceae and is used to cure various diseases (insomnia, hysteria, asthma, leprosy, cholera and disorders in nervous system) (Joseph et al. 2016; Jugran et al. 2019). The underground part, rhizome and roots contain 0.4–0.5% aromatic essential volatile oil and secondary metabolites (Navarrete et al. 2006; Singh et al. 2010; Jugran et al. 2019; Partap et al. 2020). Various herbal industries marketed their herbal formulations and products containing valerenic acid content (Partap et al. 2020). Valerenic acid 3.0 mg/g in *valeriana* plant material has been recommended as quality produce for drugs preparation (Bos et al. 1998). At present, market demand for *Valeriana* roots is entirely depending on its natural habitats and conventional agriculture methods (Table 25.1). Due to the high demand and supply gap, the use of hydroponic and aeroponic cultivation practices provides a key solution for quality produce as per the industrial guidelines (Tabatabaei 2008; Partap et al. 2020). According to recent study published by Partap et al. (2020), aeroponic system offers an alternative, economically viable and sustainable agro-practice for enhanced production of phytochemicals in *V. jatamansi*. Research

highlighted the effect of aeroponic cultivation, soil cultivation, biotic and abiotic elicitor treatment on plant growth biomass, physiology and targeted metabolites alternation in *V. jatamansi* (Fig. 25.2). In the experiment, Hoagland nutrient media basal salt mixture was used as a nutrient recipe for aeroponic cultivation of *V. jatamansi*. The morphological attributes i.e. maximum leaf number, plant height, rootlet length and number were observed in treatment 0.5 mg/L yeast extract; whereas 1.5 mg/L yeast extract and 150μM methyl jasmonate showed maximum leaf width and leaf length, respectively. The photosynthetic rate and stomatal conductance were recorded maximum at treatment levels of 0.5 mg/L and 1.5 mg/L yeast extract respectively, whereas at 150μM methyl jasmonate resulted in maximum transpiration rate. In aeroponic cultivation, at treatment level of 150μM and 100μM methyl jasmonate resulted in maximum content of valerenic acid and hydroxy valerenic acid. Whereas, acetoxy valerenic acid found maximum in root at 150μM methyl jasmonate and leaf of 1.5 mg/L yeast extract. In this study, gas chromatography-mass spectrometry analysis revealed that isovaleric acid (6.72–50.81%), patchouli alcohol (13.48–25.31%) and baldrinal (0.74–25.26%) were the major compounds found in the roots (Partap et al. 2020). The study concluded that besides underground part, aerial parts would also be used as alterenative source for metabolites and volatile oil in *V. jatamansi*. The use of effective concentration of yeast extract and methyl jasmonate in aeroponic cultivation proved as a sustainable agro-practice for metabolite enrichment in *V. jatamansi* plants (Partap et al. 2020). Aeroponic cultivation provides ease to access subsequent biomass harvest without sacrificing the endangered plants to meet the unmet demand of the herbal industries.

25.4.3 *Ocimum basilicum* L. (Sweet Basil)

Basil is an aromatic, medicinal, and spice fresh culinary herb belongs to family Lamiaceae, and also called as basilica and alfavaca. Throughout the world, basil is produced for essential oil production due to high market demand (Table 25.1) (Favorito et al. 2011). Though, there are certain limitations as there are water shortages, low and irregular precipitation, these scenarios urged the need of techniques where these barriers can be resorted. (Silva et al. 2016). Major key factors i.e. salinity hindering basil production and stress lead to morpho-physiological aberrations such as imbalance nutrition, reduced stomatal conductance, minimal photosynthesis and transpiration (Maia et al. 2017). However, the usage of hydroponic systems have become popular for the production of basil in regulated environmental conditions (Walters and Currey 2015). Hydroponic technique has prevailed its existence and consents the plants cultivation independent to salt stress. The system provides close to zero matrix potential, which empowers plants with efficient water and nutrients uptake with minimal energy expenditure as compared to soil condition (Junior et al. 2016). Maia et al. (2017) observed that there is no complications of hydroponic cultivation of basil culture under saline stress. Therefore, it is important to carry out research investigating the hydroponic cultivation of basil under adverse conditions such as salinity, as well as the use of this techniques to reduce the cost of production of this crop, and to evaluate the growth biomass. In order to allow farmers to obtain alternative incomes on rural

properties with low freshwater availability. Santos et al. (2019) found that the reduction in nutrient solution recirculation frequency by 6 h did not lead to any major decreases in water consumption, growth, output of biomass and absolute basil growth rate. The choice of the NFT or DFT hydroponic culture does not cause changes in the growth and production of biomass in basil. Moreover, Walters and Currey (2018) concluded interactions between electrical conductivity of nutrient solution and day light integral on basil growth, morphology or tissue nutrient concentrations. In the spring and the summer, four hydroponic grade samples supplied with potassium iodide (KI) or potassium iodate (KIO_3) were performed with different concentrations in a nutrient solution (0.1–200μM). Plant growth was not affected by either 10μM KI or 100μM KIO_3, while KI levels above 50μM decreased the leaf number, the plants total drought and the height of the plant. In summary, the increased tolerance of iodine of the "Red Rubin" variety was related to the potential, instead of a lower accumulation of higher concentrations of iodine in leaf tissues. This purple cultivar may have an extremely high phenolic content of "Red Rubin." In order to refine their cultivation technique, more studies are required to understand the influence of growing conditions on achievable outcomes and bioactive compounds build-up in basil.

25.4.4 *Petroselinum crispum* Mill. (Parsley)

Petroselinum crispum (Mill.) is a commercially important plant belongs to Apiaceae family. It is usually called as garden parsley in English and bawari in Hindi. *P. crispum* is thought to be initially grown in Sardinia (Mediterranean area) and was cultivated from third century BC. In subtropical and tropical regions, *P. crispum* is light green, annual grass, an erect herb with a heavy branch, which can grow up to 30–100 cm long, smooth and fragrant. Its leaves are enriched with flavonoids, vitamins, ascorbic acid and carotenoids and used as a source of food with good antioxidant capacity (Maodaa et al. 2016). Today, parsley is found in a wide variety of food dishes and commercially utilized in both fresh and dried forms. Its roots and leaves contain essential oils such as myristicin, β-pinene, α-pinene, limonene, ρ-cymene, β-phellandrene, apiol, eugenol, elemicin and γ-terpinene, which are responsible for its antioxidant and medicinal activity (Farzaei et al. 2013). Parsley cultivation has gained considerable global interest in recent years, but scientific literature is still restricted to hydroponic culture (Fig. 25.2). Martins et al. (2019) studied effect of electrical conductivity and brackish water on the growth of parsley under hydroponic culture. It was observed that shoot and root dry mass were increased with increasing electrical conductivity in hydroponic system. The configurations of electrical conductivity was set and resulting in six isosmotic levels ($EC_{ns} = 1.7$ to 6.7 dS m^{-1}) with four types of salt: $CaCl_2$, NaCl, KCl and $MgCl_2$. In the experiment, the amount of consumption of parsley plants was restored by two methods, the urban water source was made a substitute under the first strategy and the respective brackish waters under the second. Moreover, fresh and dry mass decrease was observed if the alternative of the consumed volume was with the brackish waters. The effects of salts on output variables and the dry matter content

of the parsley plants were mitigated by the use of municipal-supply water. The study resulted that hydroponic could be utilized as a promising method for the cultivation of parsley by utilizing waste water for quality biomass production.

25.4.5 *Stevia rebaudiana* Bertoni. (Meethi Patti)

The genus *Stevia* is a bushy shrub native to Paraguay and the genus have about 230 species in which only the species *rebaudiana* and *phlebophylla* produces sweetener compounds. *S. rebaudiana* has commercial importance for its steviol glycosides (SGs) metabolites and huge demand in the markets (Table 25.1). SGs are around 300 times sweeter than sucrose and impart minimally calorie to diet. Various agencies and administration of different countries such as European Food Safety Authority (EFSA), US Food and Drug administration (US FDA), Food Standards Australia New Zealand (FSANZ), and the European Union (EU) have considered the steviol glycosides as a safer sweetener compound for human and authorised to use in food and beverage industries. In recent years, cultivation of *S. rebaudiana* in hydroponic condition is well reported for biomass and secondary metabolite accumulation (Fig. 25.2). Further, growth conditions can be regulated by managing nutrient profile of growth media for improvised production. Bolonhezi et al. (2010) reported increased biomass in hydroponic cultivation system. They supplemented the growth media with 200 mg/L and 300 mg/L of N in hydroponic and observed significantly better biomass accumulation as compared to soil pots supplemented with 20 and 70 kg ha^{-1} of Ossa et al. (2017) comprehensively explained the hydroponic cultivation for *S. rebaudiana* and devised a low cost prototype. For appropriate plant cultivation, irrigation is the foremost factor in which they tested and optimized the irrigation cycle for *Stevia* plants in a hydroponic system. For the cultivation, temperature should be maintained around 25–27 °C, 66% relative humidity with 16:8 h light. Kafle et al. (2017) reported unlike other plants, primary as well as secondary metabolite accumulation reduced in macro and micro nutrient deficient plants. Photosynthetic rate and biomass growth was reported lower in plants grown in N, P, S, Mg or Ca omitted nutrient media. Although K deficiency does not significantly affected photosynthetic rate and various studies reported its effect in field conditions. It is reported that conditions that favour biomass and especially leaf production favour SG production also. But they reported lack of N did not reduce SG concentration. In case of micronutrients, Fe omission does not significantly affected the SG accumulation, but as the biomass is reduced, the overall SG accumulation per plant is recorded to reduce. Shahverdi et al. (2017) used Hogland basal as growth media and observed the effect of NaCl supplementation on SG accumulation, protein and antioxidant activity. They reported significant decrease in root characteristics such as root length, diameter, area, volume, fresh weight and dry weight in NaCl supplemented plants. Antioxidant capacity in saline (0–150 mM) treated stevia plants was also increased. But contrary to that, SG production was increased upto 30 mM supplementation of NaCl and thereafter decreased when the stress became more severe. In a similar study, Debnath et al.

(2018) reported slow and stunted growth in saline treated plants. In case of SG accumulation, there was a considerable reduction in the stevioside and an increase in rebaudioside-A accumulation in salt-stressed plants of stevia. Although decreased plant growth with reduced K^+/Na^+ ratio was also reported in stevia roots and leaves after salt treatment (Zeng et al. 2013). Therefore, extensive research is required for hydroponic and aeroponic cultivation of stevia for better growth and secondary metabolites enrichment.

25.5 Conclusion and Future Perspectives

Hydroponic and aeroponic are being recognized worldwide, offering farmers and consumers opportunities in the field of modern agriculture. This book chapter provided an overview of the role of the hydroponic and aeroponic system for enhancing biomass with major secondary metabolites accumulation in plants. Based on the facts, it would appear that hydroponic and aeroponic may be a necessary activity to produce high quality commercially valuable plants. However, according to above discussion, it is hypothesised that hydroponic is well suitable for the metabolite enriched biomass production in shoot while, aeroponic is most preferable for quality root and rhizome production. In principle, both soilless and soil-based systems need careful monitoring and properly implemented with complete plant needs i.e. soil, water, climate, growers and product safety. Modern soil less agro-practises in agriculture industries are to yield enough plant biomass to satisfy producers and consumers requirements and their interests. Soilless agriculture is intensively used in safe agriculture in contrast with soil-based farming, to promote eco-sustainability and strengthen the agro-industries.

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Amaranth, Buckwheat, and Chenopodium: The "ABC" Nutraceuticals of Northwestern Himalayas

26

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Abstract

The Northwestern Himalayan region is a rich storehouse of nutraceuticals enriched potential crops which have been underutilized and neglected by mankind for a long. The pseudocereals "ABC", namely amaranth (*Amaranthus* sp.), buckwheat (*Fagopyrum* sp.), and chenopodium (*Chenopodium quinoa*) are excellent examples of such nutraceutical superfoods which are generally cultivated marginally in limited areas but can perform a significant role in nutritional security. The phytochemical constituents and unique nutritional profile of these pseudocereals have made them popular worldwide nowadays. They also form suitable alternatives as gluten-free products for celiac patients. The high dietary fiber, well-balanced amino acid content, and health beneficial metabolites make them a popular choice for functional food and biofortification. This chapter presents comprehensive information about the bioactive compounds available in these crops which may possess outstanding biological activities and have nutraceutical potential. The role of these pseudocereals as potential nutritional food sources for the masses is also discussed besides highlighting the on-going national and international biotechnological interventions for the genetic improvement of these crops.

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Keywords

Pseudocereals · Underutilized crops · Northwestern Himalayas · Landraces · Genetic diversity · Genomics

26.1 Introduction

The shrinking agricultural land and stagnating yield of most major crops along with the upsurge in the demand of food due to increasing population necessitate the urgency to look for alternative and nutritive crops. In order to feed the projected nine billion population by 2050, the exploration of multi-purpose and underutilized crops may serve as a suitable alternative to ensure food security. Besides food security, there is an increasing interest in promoting good health and life expectancy worldwide by providing functional foods or nutraceuticals that have potential therapeutic and beneficial properties. Nutraceutical crops are in the focus of agriculturists globally as these crops are rich in natural bioactive chemical compounds that can prevent diseases and promote good health. Amaranth, buckwheat, and chenopodium are excellent examples of such multi-purpose and underutilized nutraceutical crops that besides providing basic nutrition can also provide health benefits to reduce the risks of chronic diseases and promote health. These three crops are not the "true cereals" like wheat but they are rather "pseudocereals." The unique feature of these three crops is that they possess gluten-free grains having distinct morphological, biochemical, and nutritional profiles. The minimum agronomic demands and the capability of these three pseudocereals to survive on marginal land including fragile hill and mountain areas also outshine these grains over others to ensure their contribution towards food production as well as nutritional security to the ever-increasing population. The nutritional components of a crop determine its popularity for the consumption by masses. As more research in the arenas of exploring the underutilized crops that could be targeted to reduce the burden on the staple crop is performed, the availability of data on amaranth, buckwheat, and chenopodium definitely makes them the popular choices. Owing to diverse agro-climatic conditions, Northwestern Himalayas that cover Jammu and Kashmir, Himachal Pradesh, and Uttarakhand is a rich hub of crop diversity. Wide range of diversity in amaranth, buckwheat, and chenopodium is observed in this region. Despite excellent nutraceutical potential and profile, the advancements in molecular genetics and breeding hitherto to increase the bioavailability of bioactive components of these underutilized pseudocereals still need to be paced up. The availability of genomic resources of these pseudocereals would ensure the rapid advancement in the field of molecular markers and their role in molecular breeding and genetic improvement of these crops. The current book chapter highlights the studies undertaken on these neglected crops besides providing an overview of the on-going biotechnological interventions at the national and international level to improve their agricultural traits.

26.2 Amaranth

Though amaranth is one of the New World's ancient crops, it is considered as the third-millennium crop because of its high nutritional value and undemanding cultivation (Pavlik 2012). Native to America, amaranth underwent a remarkable migration to Asia and presently it has become increasingly popular among hill tribes of Northwestern Himalayas including India, Pakistan, Nepal, Tibet, and China. *Amaranthus* is reported to be distributed in wide range of climatic zones including tropical, subtropical, and temperate region (Suma et al. 2002). It is a fast-growing, self-pollinated branched annual dicotyledonous herb, belonging to family *Amaranthaceae*, with about 70 species, 17 of which are edible. Based on the utilization method, the amaranth species are categorized into four main divisions (Rastogi and Shukla 2013): grain amaranth, e.g. *A. caudatus*, *A. hypochondriacus*, *A. edulis,* and *A. cruentus*, vegetable amaranth, e.g. *A. lividus* and *A. tricolor*, weedy amaranth, e.g. *A. powellii*, *A. hybridus,* and *A. quitensis,* and ornamental amaranth, e.g. *A. tricolor*. In India, around 20 species are reported. The height of the plant ranges between 0.3 m and 5.0 m. The plant possesses colorful inflorescence and foliage that may be light green to dark green or red in color. The leaves may be oval, oblong, elliptical, or ovate in shape and stem color differs from green to red, orange or pink-based with green. The plant possesses terminal inflorescence with one male flower per glomerule of 100–250 flowers. Pollen grains are spherical and seeds are small, lenticular, and 1.0–1.5 mm in diameter (Rastogi and Shukla 2013). The seeds display either white, cream, black, or red-brown color (Thapa and Blair 2018). The domestication of grain amaranth is attributed to monoecious habits along with predominant outcrossing (Rastogi and Shukla 2013). Amaranth has wide adaptability to varied soil and agro-climatic conditions besides being tolerant to extreme environmental conditions like heat and drought and resistant to major diseases and pests (Rastogi and Shukla 2013). Wide morphological diversity, fast growth, low production cost, and observed biotic and abiotic stress tolerance make it a reasonably attractive and economically feasible crop for cultivation by the majority of the population. The recommended cultivation practices include shallow seed sowing at depth of 4 mm on medium to heavy textured soil having pH of 5.0–8.0. The optimum temperature requirement of the crop is 25–40 °C.

26.2.1 Nutritional Components

26.2.1.1 Carbohydrates

Reportedly, the carbohydrate content of grain amaranth is lower than that of wheat (Pastor and Acanski 2018). Polysaccharides are the main component of amaranth grain. Starch forms the major constituent of the polysaccharides and varies from 48–74% of dry grain matter in various species. Amylose content in the amaranth starch is low (0.1–11.1%), while the ramified amylopectin (93.6–95.2%) forms the major portion (Martinez-Lopez et al. 2020) from which the glucose elements are gradually released and then absorbed from the intestine, thus making amaranth

suitable for patients with diabetes mellitus type II (Pavlik 2012). Starch is localized in the perisperm cells in the form of uniquely minute crystalline granules, which is about one-tenth the size of corn starch. The granular shape varies from spherical to angular or polygonal. The remarkable attribute of amaranth starch is higher swelling capability, lower solubility and digestibility, greater water uptake, lower susceptibility to amylases, lower amylose content, and exceptionally high amylopectin content as compared to corn starch (Rastogi and Shukla 2013; Venskutonis and Kraujalis 2013; Martinez-Lopez et al. 2020). The functional and physicochemical properties, as well as bioavailability and potential use of amaranth starch in food industry, pharmacology, and cosmetics industry, are imparted by the unique small size, structure, shape, and composition of the starch granule. The nature of starch types, i.e. glutinous and nonglutinous also varies in different species, e.g. seeds of *A. cruentus* have glutinous type, while *A. caudatus* have nonglutinous type. Seeds of *A. hypocondriacus* have both glutinous and nonglutinous type starch. Monosaccharides and disaccharides are generally limited in grain amaranth. Various low molecular weight carbohydrates reported in amaranth include sucrose, fructose, glucose, galactose, inositol, maltose, raffinose, and stachyose (Alvarez-Jubete et al. 2009; Venskutonis and Kraujalis 2013). Sucrose content is reported to be 2–3 times higher in comparison to wheat (Venskutonis and Kraujalis 2013).

26.2.1.2 Proteins

Amaranth seeds are an excellent source of high-quality protein as compared to true cereals. Protein plays an important role in building muscles, supporting neurological functions, helping in digestion, and maintaining hormonal balance. The protein content in various amaranth varieties ranges between 13% and 19% of dry matter (Martinez-Lopez et al. 2020) which is higher as compared to common grain crops. They have biologically complete proteins with all the essential amino acids, namely leucine, isoleucine, valine, methionine, phenylalanine, lysine, threonine, and tryptophan (Pavlik 2012). Moreover, high lysine and tryptophan content and lesser glutamic acid and proline content in amaranth grain make it comparable with animal proteins (Pavlik 2012). A larger proportion of protein (approximately 65%) is available in the embryo as compared to the perisperm, which accounts for only 35% of protein. This also contributes to the high quality of amaranth protein in contrast to other cereal crops which have a larger concentration of proteins mainly in the perisperm (Martinez-Lopez et al. 2020). The amaranth proteins are composed of easily digestible water soluble albumins (40%) and globulins (20%), alkali-soluble glutenins (25–30%), and alcohol soluble prolamins (2–3%) (Arendt and Zannini 2013; Venskutonis and Kraujalis 2013). Gluten which is an important protein in grain crops like wheat, rye, oats, and barley is absent in amaranth seed proteins. This makes it suitable for preparing nutritious food products for people suffering from serious autoimmune celiac disease where the ingestion of gluten leads to damage in the small intestine (Soriano-Garcia and Aguirre-Diaz 2019). The level of amaranth protein constituents is quite close to the level required in balanced human diets (Soriano-Garcia and Aguirre-Diaz 2019).

26.2.1.3 Lipids

Amaranth grain is reported to have higher lipid content as compared to wheat, corn, and rice. Different species of amaranth show a lipid content of around 1.9–13.0% (Orona-Tamayo and Paredes-López 2017). Amaranth lipid is reported to have a higher composition of unsaturated fatty acids. The degree of unsaturation is over 75% (Rastogi and Shukla 2013). Linoleic acid is the major fatty acid in the oil followed by oleic acid and palmitic acid. Amaranthus oil is of significant benefit for patients with cardiovascular diseases (Shin et al. 2004). Tocotrienols and squalene known to affect cholesterol biosynthesis are also reported in amaranth grain (Rodas and Bressani 2009). Squalene is reported to possess antioxidant properties as well.

26.2.1.4 Dietary Fibers

Dietary fiber is well known to have beneficial role in human consumption. It provides protection against the risk of a number of chronic diseases, including type II diabetes, obesity, and cardiovascular disease, with both soluble and insoluble forms contributing to the beneficial effects. They also help normalize bowel movements and maintain bowel health. Total dietary fibers (TDF) content varies among the vegetable amaranth and grain amaranth. It ranges between 6.95% and 9.65% in the vegetable amaranth and between 7.0% and 49.3% in grain amaranth which is more than that in wheat, barley, rye, rice, and corn (Punna and Rao Paruchuri 2004; Alvarez-Jubete et al. 2009; Chauhan et al. 2015).

26.2.1.5 Minerals

Yet another remarkable attribute of amaranth seed is the presence of an abundance of necessary minerals. The chief minerals present in amaranth seeds are calcium, iron, magnesium, manganese, potassium, phosphorus, sulfur, and sodium (Montoya-Rodríguez et al. 2015). *A. caudatus* possesses a considerate amount of iron (0.072–0.174 g/l), calcium (1.3–2.8 g/l), magnesium (2.3–3.3 g/l), and zinc (0.036–0.040 g/l) (Gamel et al. 2005). The amount of iron, calcium, and phosphorus is higher than that reported in maize and rice, while the iron content is comparable to that of wheat (Nascimento et al. 2014). Higher levels of calcium help in the healthy development of bones and preventing osteoporosis (Galan et al. 2013). High level of manganese in amaranth helps in protecting against diet-induced diabetes and boosting the immune function besides controlling cholesterol levels, skin, and bone health. The vegetable amaranth leaves have a comparatively lower amount of minerals over grain types. In the grain amaranth, around 66% of mineral is present in the bran and germ fractions (Rastogi and Shukla 2013).

26.2.1.6 Vitamins

Amaranth has adequate amounts of vitamins and thus could serve as a source to reduce vitamin deficiency. Reportedly, the amaranth has a higher amount of vitamin B_2 and vitamin C than the cereals (Rastogi and Shukla 2013). The other reported vitamins are niacin and pyridoxine. The unique vitamins that are identified in amaranth are isoforms of vitamin E, i.e. tocopherols and tocotrienols. The presence of vitamin E isoforms is considered notable as they are also known to have natural

antioxidant and antihypercholesterolemic atherosclerosis properties and are often correlated to the relative abundance of unsaturated fatty acids (Ozer and Azzi 2000; Martinez-Lopez et al. 2020).

26.2.1.7 Antinutritional Factors

Amaranth has two main antinutritional compounds, oxalates and nitrates, in both vegetable and grain types (Gelinas and Seguin 2007). Amaranth can be made suitable for edible purposes and devoid of these antinutritional factors by boiling the leaves or seeds for 5 min (Rastogi and Shukla 2013; Singh et al. 2020a). The presence of these two antinutritional factors may cause the development of kidney stones by inhibiting the absorption of calcium and zinc (Radek and Savage 2008).

26.2.1.8 Bioactive Components

Since amaranth is overloaded with medicinally beneficial effects, its phytochemical analysis is of the prime focus of many scientific investigations. These investigations established the presence of bioactive constituents in the extract of various species of amaranth. The main bioactive components reported are alkaloids, flavonoids (quercetin, isoquercitin, kaempferol, isorhamnetin, rutin), glycosides, phenolic acids (caffeic acid, vanillic acid, tannic acid, ferulic acid, gallic acid, dihydroxybenzoic acid, coumaric acid), steroids, saponins, terpenoids, tannins, betaine, and carotenoids (Peter and Gandhi 2017; Tang and Tsao 2017; Martinez-Lopez et al. 2020). Besides, these amaranthoside, amaricin, anthocyanins, zeaxanthin, betalains (amaranthin and isoamaranthin), betaxanthin, hydroxycinnamates, and nicotiflorin are also reported metabolites from the amaranth (Peter and Gandhi 2017; Martinez-Lopez et al. 2020). The above-mentioned bioactive constituents reportedly hold diverse pharmacological properties like cytotoxic, anticarcinogenic, antitumor, antioxidant and thus the medicinal properties observed in amaranth could be attributed to them. Besides, a large number of low molecular weight biopeptides exhibiting diverse pharmacological activities such as antiinflammatory, antioxidant, antihypercholesterolemic, antihypertensive, antidiabetic, antitumor, antibacterial, and immunomodulatory have been reported in various *Amaranthus* species (Martinez-Lopez et al. 2020).

26.2.1.9 Biological Activities

The utilization of amaranth extracts for the cure of ailments like stomach diseases, respiratory disorders, and diabetes has been reported in the traditional medicinal system of India, China, Nepal, and Thai (Rastogi and Shukla 2013; Peter and Gandhi 2017). In the past few decades, reports on a large number of biological activities of amaranth have emerged that aid in amassing the popularity of amaranth as a "superfood" worldwide. Some of these are listed in Table 26.1. In the near future, there is need of a comprehensive analysis of the beneficial effects of extracts from different parts of plants of different species of *Amaranthus* along with correlating it with the bioactive constituents. The bioavailability of nutritive components of amaranth and deciphering the pharmacological mechanism of these constituents

Table 26.1 Biological activities of *Amaranthus* species

Biological activity	Plant part/active component/ extract type	References
Anthelmintic	Aqueous extract of *A. spinosus* L. whole plant	Baral et al. (2010)
	Methanolic extracts of *Amaranthus* species	Kumar et al. (2010a)
Antiatherogenic	Hydroalcoholic extracts of *A. caudatus*	Kabiri et al. (2010, 2011)
Antibacterial	Peptides isolated from *A. caudatus* seeds	Broekaert et al. (1992)
	Ethanol extract of stem and leaves of *A. viridis* L.	Malik et al. (2016)
	A. caudatus extract	Mohanty et al. (2018)
Anticarcinogenic	Quercetin and rutin and in *A. caudatus* L.	Kalinova and Dadakova (2009)
	Lunasin-like peptide isolated from seeds of *A. hypochondriacus*	Maldonado-Cervantes et al. (2010)
	Methanolic extract of the young plant of *A. lividus*	Al-Mamun et al. (2016)
Antidiabetic	Methanolic extract of the leaves of *A. spinosus*, *A. caudatus,* and *A. viridis*	Girija et al. (2011), Kumar et al. (2011)
	Grains of *A. hypochondriacus* L.	Jorge et al. (2015)
	Seeds of Amaranth	de la Rosa et al. (2017)
Antifungal	Peptides isolated from *A. caudatus* seeds	Broekaert et al. (1992)
	Amaranth cystatin	Valdes-Rodriguez et al. (2010)
Antihypercholesterolemic	Seeds of *A caudatus*	Plate and Areas (2002), Caselato-Sousa and Amaya-Farfán (2012)
	Methanolic extract of the leaves of *A. spinosus*, *A. caudatus,* and *A. viridis*	Girija et al. (2011)
	Hydroalcoholic extracts of *A. caudatus*	Kabiri et al. (2010, 2011)
	A. caudatus seed or oil	Achigan-Dako et al. (2014), Alemayehu et al. (2015)
Antihyperlipidemic	Methanolic extract of *A. caudatus*	Girija and Lakshman 2011; Girija et al. 2011
Antihypertensive	*A. caudatus* seed or oil	Achigan-Dako et al. (2014), Alemayehu et al. (2015)
Antimalarial	Extracts of *A spinosus* L.	Hilou et al. (2006)
	Ethanolic formulation of *A. spinosus* L	Susantiningsih et al. (2012)

(continued)

Table 26.1 (continued)

Biological activity	Plant part/active component/ extract type	References
Antineoplasic	*A. caudatus* seed	Caselato-Sousa and Amaya-Farfán (2012)
Antioxidant	Bioactive peptides from leaves *A. caudatus*	Bruni et al. (2001)
	Ethanolic extracts of *A. caudatus* seeds	Conforti et al. (2005)
	Methanolic extract of *A. spinosus* L.	Kumar et al. (2010b, c)
	A. cruentus seeds	Soares et al. (2015)
	A. caudatus seed or oil	Achigan-Dako et al. (2014), Alemayehu et al. (2015)
	Petroleum ether, dichloromethane, and methanol extract of *A. tricolor*	Amornrit and Santiyanont (2015)
	Tannins isolated from the leaves of *A. caudatus*	Jo et al. (2015)
	Methanolic extract of *A. caudatus*	Peiretti et al. (2017)
	Squalene from *A. caudatus*	Campos et al. (2018)
Cardioprotective	*A. caudatus* seed or oil	Achigan-Dako et al. (2014), Alemayehu et al. (2015)
Hepatoprotective	Methanolic extract of whole plant except roots of *A. spinosus*	Gul et al. (2011)
	Ethanolic extract of whole plant of *A. spinosus* L	Guria et al. (2014)
Hypoglycemic	*A. caudatus* seed	Caselato-Sousa and Amaya-Farfán (2012)
Immunomodulatory	*A. caudatus* seed or oil	Caselato-Sousa and Amaya-Farfán (2012), Achigan-Dako et al. (2014), Alemayehu et al. (2015),
	Water extract of leaves of *A. spinosus*	Guria et al. (2014)
Neuroprotective	Petroleum ether, dichloromethane, and methanol extracts of leaves of *A. lividus* and *A. tricolor*	Amornrit and Santiyanont (2015)

needs to be undertaken to completely exploit the clinical benefits of this underutilized pseudocereal.

26.2.1.10 Landraces/Cultivars and Commercial Varieties in Northwestern Himalayas

The adaptability of grain amaranth is wide in the Himalayas and it can thrive well in regions of tropical lowlands to 3500 m above sea level (Joshi and Rana 1991; Joshi et al. 2018). In Himachal Pradesh, *A. caudatus* is reportedly cultivated in the areas of Kinnaur, Kullu, and Sirmour (Mohil and Jain 2012). The evaluation of 55 accessions of *Amaranthus* species for their performance in submountain Himalayan region of India has led to the identification of the putative donors for nutritional enrichment (Sood et al. 2018). The identification of genotypes which can perform consistently over a wide range of environmental conditions and show high seed yield stability is highly desirable. Sharma et al. (1998) investigated the seed yield stability of indigenous and exotic genotypes of amaranth (*Amaranthus* sp.) in the Northwestern Himalayas. Various traditional cultivars of amaranth like *chaulai*, *bhavri*, *amaranth, seoul*, *jhin jhin dhan*, *jalli*, *laldhan*, *kalgi*, *kodra,* and *kataili chaulai* are being cultivated by the poor and marginal farmers of different regions of Shimla, Kullu, Chamba, Mandi, Solan, Lahaul and Spiti, and Kinnaur of Himachal Pradesh (Fig. 26.1). Traditional cultivars like *ramdana*, *marchha*, *tota*, *chua*, *choa*, *chun,* and *chauli* are being cultivated in Uttarkashi, Chamoli, Rudraprayag, Dehradun, Tehri Garhwal, Pauri Garhwal, Almora, Bageshwar, Udham Singh Nagar, Haridwar, Nainital, Pithoragarh, and Champawat regions of Uttarakhand. Likewise, the cultivars, *ganhaar*, *seual ganhaar*, *seoul,* and *babri* are popular among the farmers of Kishtwar, Bandipur, Badgam, Doda, Ramban, Leh, Kargil. Udhampur, Shopian, Kathua, Baramulla, and Srinagar regions of Jammu and Kashmir (Genebank Dashboard 2016). As many as 3545 accessions of different landraces/cultivars of amaranth and its wild species including *A. hypocondriacus* (2507), *Amaranthus* sp. (743), *A. caudatus* (96), *A. cruentus* (77), *A. hybridus* (31), *A. tricolor* (25), *A. viridis* (22), *A. gangeticus* (13), *A. caudatum* (10), *A. dubius* (9), *A. retroflexus* (8), *A. albus* (4), *A. cordatus* (3), *A. palmeri* (3), *A. spinosus* (2), *A. fimbriatus* (2), *A. graecizans* (2), *A. flavus* (1), *A. mangostanus* (1), *A. powellii* (1), *A. crispus* (1), *A. caudatus* var. *albiflorus* (1), and *A. caudatus* var. *atropurpurea* (1) have been conserved in the genebank of National Bureau of Plant Genetic Resources (NBPGR), New Delhi and NBPGR Regional Station, Shimla (Genebank dashboard 2016). These accessions are a rich reservoir of genetic diversity with many favorable traits for breeding purposes and development of improved cultivars or varieties. The main objectives of breeding "grain amaranths" involve improvement of traits like increased yield, increased lodging resistance, less seed shattering, timing and uniformity of maturity, reduced leafiness in the green head area, reduced plant height, good seedling vigor, pest resistance/tolerance, larger seeds, good nutritional profile of seed, and tolerance to cold. For "vegetable amaranths," traits like heat-tolerance, improved seedling establishment, and resistance to diseases, insects, and drought are taken care for the breeding purposes and development of improved varieties (Kauffman and Weber 1990). Many high yielding varieties with high antioxidant activity and minimum nitrate and oxalate contents have been released. The released varieties are generally rich in vitamins and minerals like calcium and iron, have excellent cooking quality, and are resistant to white rust. Some varieties of grain

Amaranth at farmer's field in Karsog, Mandi, Himachal Pradesh

Amaranth at farmer's field in Dodra-Kwar, Shimla, Himachal Pradesh

Amaranth at farmer's field in Rampur Bushahr, Shimla, Himachal Pradesh

Amaranth at farmer's field in Chaupal, Shimla, Himachal Pradesh

Fig. 26.1 Amaranth cultivation by farmers of different districts of Himachal Pradesh

amaranth released for plains are GA-1, GA-2, GA-3 BGA-2, Suvarna, and RMA-4. Various improved varieties of amaranth like *Badi Chaulai*, *Chhoti Chaulai*, *Lal Sag*, *Pusa Kiran*, *Pusa Lal Chaulai,* and *Pusa Kirti* have been released by IARI, New Delhi. *Badi Chaulai* (*A. tricolor*) can be cultivated for commercial purposes and is

best suited for summer season. It has a thick tender stem and large leaves. *Chhoti Chaulai* (*A. blitum*) is quick growing with slight dwarf erect plants. Its stem is thinner and leaves are smaller as compared to those of *Badi Chaulai*. *Lal Sag* is a high yielding early variety grown quite popularly in many states of India. It bears small flowers and produces seeds early. The varieties, *Pusa Kiran*, *Pusa Lal Chaulai,* and *Pusa Kirti* are recommended for cultivation all over India and are high yielding varieties. *Pusa Kiran* developed by natural crossing between *A. tricolor* and *A. tristis* is suitable for sowing in rainy season. It has glossy green leaves as well as stems. The color of the upper surface of leaves of *Pusa Lal Chaulai* (*A. tricolor*) is magenta or deep red and that of lower surface is purplish-red. The color of the stem is deep red. The variety, *Pusa Kirti* (*A. blitum*) is best suited for summer season. Its leaves are ovate green in color and broad and the stem is green and tender. Some of the popular released varieties released for Northwestern Himalayas are *Annapurna*, *PRA-1*, *PRA-2*, PRA-3, *Durga*, and *VL Chua 44* (Joshi 1985; Singh 2018). The details of these varieties are mentioned below:

1. *Annapurna*: This variety was developed in 1984 as pure line selection from material collected from Pauri Garhwal, Uttarakhand. It is recommended for mid and high Himalayan region of India. This variety is drought tolerant and widely adapted. The seeds have high protein content (15%). Its average yield is 22.5 q/ha. It has excellent popping quality.
2. *PRA-1*: This variety was released in 1997 for Uttarakhand hills. The seeds of this variety are creamish yellow in color and have bold appearance with 13–14% protein content and 9.2% oil content. Its average yield is 14.5 q/ha.
3. *PRA-2*: This variety was released in 2000 for Northwestern Himalayas. The seeds of this variety have shining cream color and bold appearance with 14–15% protein content and 12% oil content. Its average yield is 14.5 q/ha.
4. *PRA-3*: This variety was released in 2003 for Northwestern Himalayas. Seeds are medium bold, shining, and creamish in color. Its average yield is 16.5 q/ha.
5. *Durga*: This variety released in the year 2006 is recommended for hilly areas of Northwestern Himalayas. This is an early variety and matures in 125 days. Seeds have shining cream color and bold appearance with 14–15% protein content and 12% oil content. Its average yield is 21 q/ha.
6. VL Chua 44: This variety was released in 2006 for cultivation in Uttarakhand, Himachal Pradesh, Jammu and Kashmir, and Northeastern hills. It is a short duration crop and matures in 116 days. It possesses tolerance to the viral disease mosaic mottling. The seeds have 14.1% protein and 12.2% oil content. Its average yield is 10–13 q/ha. This variety involves easy threshing and reduced drudgery.

26.2.2 Biotechnological Interventions

Amaranth is a multistress-tolerant C4 pseudocereal that is widely reported from broad geographical locations. However, its taxonomic characterization is cumbersome due to resemblance among various *Amaranthus* species, broad distribution,

and lower internal transcribed spacer (ITS) divergence. Due to the rich nutritional profile and numerous health benefits of amaranth, the crop has gained tremendous importance leading to several research approaches and resources to study it. Genetic markers like RAPD (random amplified polymorphic DNA), microsatellite markers or SSR (simple sequence repeats), and SNP (single nucleotide polymorphism) have been developed and used vastly to assess polymorphism and genetic variation in various species of *Amaranthus*.

RAPD analysis was efficiently used to classify grain amaranth accessions by species (Transue et al. 1994). The genetic diversity analysis of amaranth assessed by the isozyme and RAPD data analysis revealed close association of grain amaranths with *A. hybridus* than with *A. powellii* or *A. quitensis* (Chan and Sun 1997). The RAPD analysis of six cultivars of *Amaranthus* belonging to different geographical regions revealed low intra- and inter-species polymorphism (Chan and Sun 1997). Microsatellite markers for amaranth were discovered and characterized by Mallory et al. (2008). A step forward, Maughan et al. (2009b) identified SNPs in amaranth accessions. Due to lower mutation rate, SNP marker detection is reliable and popular choice for amaranth genetic diversity analysis. Later on, Maughan et al. (2011) reported the first high-density genetic linkage map by utilizing 41 genotypes of *Amaranthus* that included 11 accessions of *A. caudatus* and ten accessions each of *A. cruentus*, *A. hypochondriacus,* and *A. hybridus*. The data revealed the detection of maximum number of polymorphic SNPs in *A. hybridus* followed by *A. hypochondriacus, A. caudatus,* and *A. cruentus*. The lower level of genetic diversity observed in *A. cruentus*, where only 35 polymorphic SNPs were detected, could be attributed to domestication. The development of SNP markers in this study paved the way for the characterization of amaranth germplasm and for the breeding programs targeting impotant agronomic traits. The genetic variation and population structure analysis of *Amaranthus* species using 11 SSRs detected 122 alleles besides revealing high level of genetic diversity (Suresh et al. 2014). Akin-Idowu et al. (2016) assessed 29 grain amaranth accessions of different geographical regions by exploiting 27 phenotypic characters and 16 RAPD primers. Their study identified the species having distinctive morphological characters and yield that could potentially be targeted for mining gene resources for hybridization purpose.

A step forward, a bacterial artificial chromosome (BAC) library was reported (Maughan et al. 2008). The library having utility for herbicide target genes was constructed using *A. hypochondriacus* cultivar "Plainsman." The frequency of BAC clones which carried inserts derived from chloroplast DNA and mitochondrial DNA was around 6.9%. The utilization of 454 pyrosequencing runs for analyzing the stem as well as biotic and abiotic stress-responsive transcriptome profiles of *A. hypochondriacus* generated the genomic data that shed light on the stress-responsive mechanism of grain amaranth (Delano-Frier et al. 2011). A reference genome of a local landrace of a crop is the pre-requisite for improving the agronomic traits of grain amaranth and transfer of desirable traits to dicot crops. The draft genome of a landrace of grain amaranth *A. hypochondriacus*, *A.hyp_K_white*, which is quite suitable for cultivation in India and use in tilling-based crop improvement, was developed in 2014 (Sunil et al. 2014). Their study revealed the estimated

genome size of 466 Mb, which could code for 24,829 proteins and contained 13.76% of repeat elements. Around 411 linkage SNPs were reported by them. Very recently, Deb et al. (2020) presented the chromosomal level assembly of this landrace. Additionally, they normalized the variants of various accessions, generated from genotyping by sequencing and whole genome sequencing data, that included the important landraces well-adapted in India for development of phylogenetic tree. The data generated in this study serves as a reference for the species of amaranth in South Asian region. It would be helpful for classifying more landraces from various regions of India and elsewhere. Clouse et al. (2016) also sequenced the draft genome of *A. hypochondriacus*. The genome assembly was of 377 Mb and Copia-like elements were found to be the predominant repetitive elements. Moreover, in the same study, they generated a de novo physical map assembly of the *A. hypochondriacus* genome that is crucial for the identification of structural rearrangements at the chromosome level.

Later on, the previously reported assembly of *A. hypochondriacus* was improved by Lightfoot et al. (2017). They also produced a chromosome-scale assembly for the identification of structural rearrangements at chromosome level. Using their assembly, they successfully mapped and identified candidate genes involved in the betalain pigmentation pathway. The study facilitated the understanding of chromosome loss and fusion events in amaranth. Stetter and Schmid (2017) performed the comprehensive analysis of genome size and molecular phylogeny of 35 amaranth species by genotyping through sequencing approach. The analysis suggested that the three cultivated grain species had *A. hybridus* as the common ancestor, whereas South American grain amaranth potentially evolved from *A. quitensis*. Further, the genome size of most species was reported to be around 500 Mbp. The smallest genome size (421 Mbp) was reported to be of palmer amaranth, while the largest genome size (824 Mbp) was that of *A. australis*.

Chaney et al. (2016) reported the size, structure, and gene content of a high-quality chloroplast genome assembly of *A. hypochondriacus* var. "Plainsman". This chloroplast genome assembly has important role in the phylogenetic studies of various members of *Amaranthaceae* family. The developed genome assembly was also helpful in reconstructing the chloroplast genome of more accessions of amaranth species and in mining for the SSRs, SNPs, and insertion/deletion polymorphism (InDels).

26.3 Buckwheat

Buckwheat (genus *Fagopyrum*) belonging to the family *Polygonaceae* is an important nutraceutical pseudocereal. In general, *F. tataricum*, *F. esculentum*, *F. cymosum*, *F. emarginatum*, *F. tataricum var. himalianum,* and *F. giganteum* are the main species found in Northwestern Himalayas (Kumari and Chaudhary 2020). Common buckwheat (*F. esculentum*) and tartary buckwheat (*F. tataricum*) are the two main cultivable species. Common buckwheat is a self-incompatible species cultivated at lower altitudes, has lower yield, and is susceptible to frost injury. On the contrary, the tartary buckwheat has greater potential at higher altitude mountainous areas mainly because of its frost tolerance (Ohnishi 1998; Zhou et al.

2016). Buckwheat is an herb possessing dense fibrous roots (Farooq et al. 2016) and single, erect, and hollow stem that may be branched or unbranched varying in color from green, red to brownish. It may be annual or perennial. The triangular or heart shaped leaves are alternately arranged on the stalk. The inflorescence includes 7–9 blossoms and flowers are arranged in racemes either on short pedicels arising from the leaf axil or at the end of the branches. The flowers may be white, pink, or red in color and comprises 3–5 sepals, 6–9 stamens, and singular pistil. Dimorphic flowers (pin type and thrum type) leading to self-incompatibly are present in common buckwheat. However, tartary buckwheat is capable of self-fertilization as they have homomorphic flowers (Farooq et al. 2016; Woo et al. 2016; Kumari and Chaudhary 2020). The shape of seeds in both the species is triangular. The color of seeds varies and may be gray brown, glossy brown, silver gray, or black. The outer thick seed coat is called hull and the inner portion is called groat. The hull is characteristically harder in tartary species than in common buckwheat.

Buckwheat is a short duration crop (70–90 days) requiring low maintenance and thrives best on moist and cool climate. The optimal buckwheat plant performance is observed on sandy soil which is well drained. However, it grows well on acidic soils also. The sowing period is dependent on agronomic variables like temperature and climatic conditions. At the lower altitudes, the seed sowing is generally done in the month of May to August, while at the higher altitudes, it is during the months of April and May (Kumari and Chaudhary 2020). To avoid delayed seed emergence and to promote synchronous crop maturity, very deep sowing is avoided. The recommended seed sowing depth is 4–5 cm (Farooq et al. 2016). Temperature is an important limiting factor for proper yield (Joshi et al. 2019). The crop is generally not affected by competition from weeds because of its fast germination, fast growth, and large above ground biomass (Jacquemart et al. 2012). Buckwheat pellets are also used to decrease weed and parasite abundance due to allelopathic action (Iqbal et al. 2003, 2006). This crop requires low inputs like fertilizers but proper management for higher yields (Joshi et al. 2019).

In India, buckwheat is cultivated in the mountainous regions of Northwestern Himalayas, Northeastern Himalayas, and parts of Palani and Nilgiri Hills (Rana et al. 2012). In the Northwestern Himalayas, buckwheat is grown as ground cover in the apple orchards for bee cultures that not only yield high-quality honey but also aid in pollination of apple via bee activity (Farooq et al. 2016; Kumari and Chaudhary 2020).

26.3.1 Nutritional Components

26.3.1.1 Carbohydrates

The starch is the major carbohydrate in buckwheat. It is found primarily in the endosperm of the buckwheat grains as an energetic storage component. On dry weight basis, the starch content may be 59% to 70% (Christa and Soral-Śmietana 2008; Zhu 2016). The major portion of starch is made up of amylopectin (Christa and Soral-Śmietana 2008), while amylose content is quite low (20–28%). The shape of starch granules may be spherical, oval, round, or polygonal and the size may vary from 2 μm to 15 μm with a diameter of 6 μm to 7 μm (Zhu 2016). Resistant starch,

which avoids absorption in the small intestine and is fermented in the large intestine by microflora, is high in buckwheat grains. Other water soluble polysaccharides found in the endosperm comprises xylose, mannose, galactose, and glucuronic acid (Suzuki et al. 2020). The main soluble carbohydrates in buckwheat are sucrose and fagopyritols. They are present in low amount mainly in the embryo.

26.3.1.2 Proteins

The buckwheat proteins have higher biological value very close to that of egg protein and much higher than for other cereals. The amino acids in buckwheat proteins are well balanced and rich in limiting amino acids like lysine, methionine, and tryptophan (Przybylski and Gruczyńska 2009; Sytar et al. 2016). The protein content of buckwheat species varies from 11% to 15% and the majority of protein is located in embryo (55%) followed by endosperm (35%) and hull. Water soluble and salt soluble albumins and globulins constitute the main protein fractions (50–60%) in buckwheat seeds followed by alkali-soluble glutelins (11–23.8%) and alcohol soluble prolamines (1–7%) (Christa and Soral-Śmietana 2008; Milisavljević et al. 2004; Joshi et al. 2019). Buckwheat flour possessing essential amino acids and lacking gluten type proteins constitute an important source of dietary proteins for people suffering from the celiac disease (Przybylski and Gruczyńska 2009; Sytar et al. 2016).

26.3.1.3 Lipids

The total lipids content in common buckwheat is reported to vary from 1.5% to 4.0% and in tartary buckwheat from 1.2% to 4.3%. The embryo has the major portion of lipids. Higher concentration of short chain lipids like linoleic acid, oleic acid, and palmitic acid and long chain lipids are found in the buckwheat seeds as compared to true cereals. Further, the nutritional value of buckwheat is better than all other cereals due to the higher proportion of unsaturated fatty acids (74–79%) as compared to the saturated fatty acids (16–25%) (Jacquemart et al. 2012).

26.3.1.4 Minerals

Reportedly, the buckwheat genotypes are rich in minerals like potassium, magnesium, calcium, sodium, nitrogen, phosphorus, iron, manganese, and zinc. Buckwheat is richer in minerals like manganese, zinc, and copper than other cereals (Jacquemart et al. 2012). Buckwheat seeds are also rich in selenium, which is an oligoelement frequently deficient in human nutrition (Cuderman et al. 2010). Selenium is known to enhance resistance to cancer and AIDS (Zheng et al. 2011).

26.3.1.5 Vitamins

Buckwheat grains are known to contain high levels of vitamins B_1, B_2, B_3, B_6, vitamin C, vitamin E, vitamin K, and choline (Przybylski and Gruczyńska 2009; Sytar et al. 2016). Tartary buckwheat reportedly possesses higher amounts of vitamin B_1, B_2, and B_3 and lesser amounts of vitamin E than common buckwheat. Moreover, α-tocopherol is the chief tocopherol in buckwheat and the tartary buckwheat possess higher tocopherol content than that of common buckwheat (Ahmed et al. 2014).

26.3.1.6 Dietary Fibers

The dietary fibers, which avoid hydrolysis by digestive enzymes in the small intestine and is fermented by the microflora in the large intestine, is composed of oligosaccharides, polysaccharides, and other hydrophilic derivatives. TDF in buckwheat is mainly composed of non-starch polysaccharides like hemicellulose, cellulose, pectins, gums, and non-cellulosic polysaccharides. TDF content in buckwheat ranges between 50 mg/g and 110 mg/g and accounts for both soluble fiber (30–70 mg/g) and insoluble fiber (20–40 mg/g) (Ahmed et al. 2014). TDF content in the buckwheat groat is 5%–10%, which is reported to be approximately ten times lesser than that in buckwheat husk (Sytar et al. 2016).

26.3.1.7 Antinutritional Factors

Protease inhibitors, trypsin inhibitors, phytic acid, and tannins are the main antinutritional factors in buckwheat (Jacquemart et al. 2012). Trypsin inhibitors in buckwheat are neither destroyed by heat treatment nor by treatment with acids (Przybylski and Gruczyńska 2009). Besides, low molecular weight legumin-like allergic proteins are also reported in buckwheat (Christa and Soral-Śmietana 2008).

26.3.1.8 Bioactive Components

Buckwheat grain and hull is reported to possess many bioactive components with healing properties and biological activities. These components include flavonoids and flavones (rutin, orientin, vitexin, quercetin, isovitexin, quercitrin, and homoorientin), phenolic acids (chlorogenic acid, hydrobenzoic acids, *p*-anisic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, synergic acid, vanillic acid), condensed tannins (catechins), phytosterols (β-sitosterol), and fagopyritols (Christa and Soral-Śmietana 2008; Bystricka et al. 2011). The flavonoids are reportedly known to possess antitumor and antioxidant capabilities (Jing et al. 2016). Phenolic acids in buckwheat are known to exhibit antioxidant, antitumor, and antibacterial activities (Guo et al. 2011). The fagopyritols are shown to exert antidiabetic activities (Wu et al. 2018). Fagopyritol A1 is also reported to be used in the treatment of polycystic ovarian syndrome (Christa and Soral-Śmietana 2008). Widely spread pigments, anthocyanins are also reported to be present in buckwheat sprouts (Przybylski and Gruczyńska 2009). The other reported bioactive components in buckwheat include triterpenoids, steroids, alkaloids, anthraquinones, coumarins, carbohydrate derivatives, and volatile compounds (Jing et al. 2016). Besides, salicylaldehyde is also reported in buckwheat which imparts the distinct aroma to its grains (Janes and Kreft 2008).

26.3.1.9 Biological Activities

The buckwheat plants are suitably enriched in various phytochemical constituents that impart nutraceutical potential to this pseudocereal. It was used traditionally to treat hypertension, diabetes, periodontitis, and gum bleeding (Campbell 1997; Singh et al. 2020a). The leaves of buckwheat were utilized for the treatment of ulcer, hemostasis, bathing wounds, ulcer, constipation, eye ailments, fatigue, and anemia by traditional healers (Al-Snafi 2017). A large number of pharmacological studies

Table 26.2 Biological activities of Buckwheat species

Biological activity	Plant part/active component/extract type	References
Antiallergic	Extract of *F. esculentum* grain	Kim et al. (2003)
Antibacterial	Ethanolic extract of *F. cymosum*	Dong et al. (2012)
	Extract of F. esculentum hulls	Cabarkapa et al. (2008)
Anticarcinogenic	Extract of *F. cymosum* rhizomes	Chan (2003)
	Protein isolated from water soluble extract of *F. tataricum*	Guo et al. (2010)
Antidiabetic	D-chiro-inositol isolated from tartary buckwheat	Cao et al. (2006)
	Ethanolic and water extracts of *F. esculentum* seed	Han et al. (2008)
Antifatigue	Extract of *F. esculentum*	Kothiyal and Ratan (2011)
Antifungal	Peptide isolated from buckwheat seed	Leung and Ng (2007)
Antigenotoxic	Methanolic extracts of *F. tataricum* and *F. esculentum*	Vogrincic et al. (2013)
Antihypercholesterolemic	Tartary buckwheat sprout powder	Kuwabara et al. (2007)
Antihyperlipidemic	Extracts of *F. esculentum* and *F. tataricum*	Tomotake et al. (2015)
Antihypertensive	Tartary buckwheat flavonoids	Hou et al. (2017)
Antiinflammatory	Ethanolic extract of the sprouts of *F. esculentum*	Ishii et al. (2008)
	Trichloromethane fraction obtained from partitioning of ethanolic extract of *F. dibotrys* roots	Cheng and Pan (2009)
	Ethanolic extract of *F. cymosum* roots	Liu et al. (2012)
Antineoplasic	Ethanolic extract of *F. cymosum* roots	Liu et al. (2012)
Antioxidant	*Ethanolic extract of F. esculentum hulls*	Watanabe et al. (1997)
	Methanolic and hexane extracts of buckwheat seeds	Przybylski et al. (1998)
	Methanolic and acetone extracts of *F. esculentum, F. homotropicum, and F. tataricum*	Sun and Ho (2005), Jiang et al. (2006)
	Ethanolic extract of F. tataricum and F. esculentum	Cao et al. (2008)
Hepatoprotective	Ethanolic extract of *F. esculentum* germinated seeds	Choi et al. (2007)
Neuroprotective	Tartary buckwheat flavones	Hou et al. (2017)

have been carried out worldwide to assess the various therapeutic activities of buckwheat plant and its individual parts. Some of these are listed in Table 26.2. More clinical trials displaying the efficacy and safety are needed to exploit the tremendous potential of buckwheat and its products.

26.3.1.10 Landraces/Cultivars and Commercial Varieties in Northwestern Himalayas

Buckwheat is a multi-purpose crop and holds great promise for increasing production in the hilly regions of Northwestern Himalayas due to early maturity and suitability for cultivation in marginal and degraded lands on sustainable basis (Chaudhary and Sharma 1997). It is imperative to evaluate and document the occurrence and distribution of local landraces and wild species of *Fagopyrum* in Northwestern Himalayas in order to broaden the gene pool for crop improvement programs. In Northwestern Himalayas, buckwheat can be grown in various crop combination in order to increase production. In high hills, only one crop is grown, while in the mid-hills, two crops combination both during winters and summers and in the foothills, three crops combination is witnessed (Joshi 1999; Babu et al. 2018). The development of improved commercial varieties is of prime importance in meeting the nutritional requirements of people. The large number of locally available landraces/cultivars and wild species of buckwheat serve as a rich reservoir of genetic diversity, which can be exploited for the breeding programs to develop improved varieties having advantages of superior quality, earliness, uniformity in production, higher production, and resistance to various abiotic and biotic stresses. Several traditional cultivars of buckwheat like *phaphra*, *ogla*, *oagle*, *chuobroo*, *phophra brash*, *bharesh*, *fafra*, *Jamru*, *phulan*, *kalpa local*, *sangla local*, *phagra*, *faira*, *jatingri*, *gongri*, *chabru*, *chahra*, *chalari*, *ghanghadi*, *phulado*, *bhares*, *kathu*, *safed ghanghri*, *fullan*, *bhagdi*, *dhesh,* and *bhangri* are being cultivated by the resource poor to marginal farmers in different regions of Himachal Pradesh like Kinnaur, Shimla, Kullu, Mandi, Kangra, Chamba, Kinnaur, and Lahaul Spiti (Fig. 26.2). Likewise, several cultivars like *phaphra*, *phafra*, *ogla*, *chawbri*, *ugal*, *phaphar*, *chawari*, *kuttu*, *oogal*, *phapriya*, *palthi*, *phafor*, *bay,* and *kotu* are being cultivated by the farmers of Uttarakashi, Champawat, Chamoli, Nainital, Rudraprayag, Bageshwar, Almora, Pithoragarh, Pauri Garhwal, and Tehri Garhwal regions of Uttarakhand. Cultivars like *trumba*, *chok trumba*, *tromba*, gayhun, *baro*, *barve/vhuti*, *mure/mori,* and *chay* are being cultivated by the local farmers of Leh, Kishtwar, Kupwara, Bandipura, Kargil, Telial, Shopian, and Ladakh regions of Jammu and Kashmir (Genebank Dashboard 2016). A total of 438 accessions of different cultivars of buckwheat including *F. esculentum* (394), *Fagopyrum* sp. (35), *F. himalianum* (8), and *F. emarginatum* (1) have been collected from the diverse agro-ecological regions of Himachal Pradesh, Jammu and Kashmir, and Uttarakhand (Genebank Dashboard 2016) and conserved for long term storage at NBPGR, New Delhi and medium term storage at NBPGR Regional Station, Shimla at −20 °C and 4–10 °C, respectively. Few accessions are also maintained under natural conditions at −7 °C to +20 °C at Udaipur, Sangla, Ranichauri and Vivekanand Parvatiya Krishi Anusandhan Shala (VPKAS), Almora (Joshi 1999). Several varieties of buckwheat suitable for cultivation in Northwestern Himalayas like *Himpriya*, *Himgiri*, *Sangla B1*, *Shimla B1*, *OC-2*, *VL UGAL-7*, *KBB-3*, *PRB-1,* and *PRB 9001–1* have been released by different institutes (Rana et al. 2016; Joshi et al. 2019; Singh et al. 2020b). The details of some of the released varieties are mentioned below:

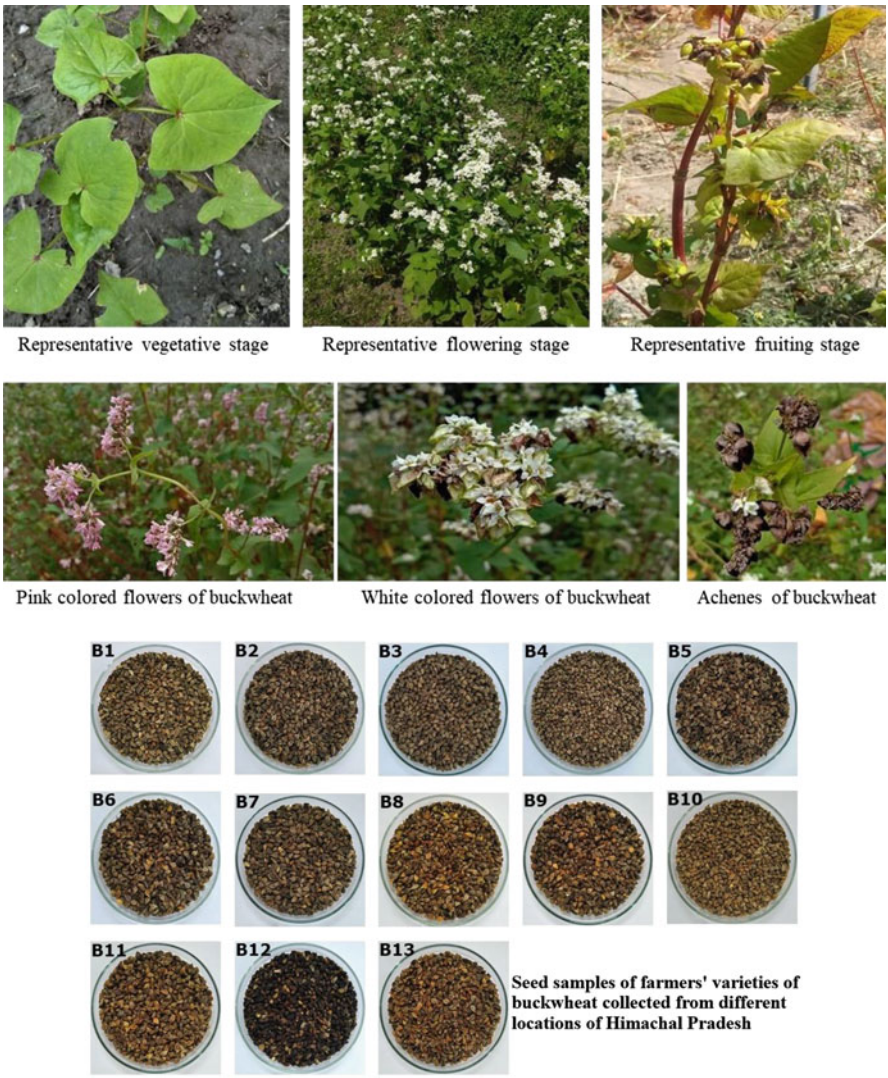

Fig. 26.2 Buckwheat cultivation at farmer's field of Barot, Mandi, Himachal Pradesh and Mountain Agriculture Research and Extension Centre, Sangla, Kinnaur, Himachal Pradesh

1. *Himpriya*: This is the first *tataricum* variety released by NBPGR Regional Station, Shimla in 1991 as a pure line selection from IC13374. The variety is recommended for cultivation in the high Himalayas. The variety matures in 119 days and gives the average yield of 12q/ha. Growth habit is trailing and indeterminate with average plant height of 90 cm. This is free from leaf spot diseases and insect pests. The seed weight is around 7.0 g. The variety is suitable for early as well as normal planting.

2. *Himgiri*: This *tataricum* variety was also released by NBPGR Regional Station, Shimla as a pure line selection from EC321978 in 1991 and is recommended for cultivation in the high Himalayas.
3. *VL UGAL-7*: The variety was released by VPKAS, Almora, Uttarakhand in 1991 through mass selection (Joshi and Rana 1995). This high yielding variety of common buckwheat is recommended for cultivation in mid-hills and foothills and it matures very early. Flower color is white and seeds are black.
4. *PRB-1*: The variety was released and registered by GB Pant University of Agriculture and Technology, Pantnagar, Uttarakhand for high seed yield in 1997 for hilly regions of Uttar Pradesh, Himachal Pradesh, and Northeastern states. It belongs to common buckwheat group and matures in about 102 days. It requires low input under timely and late sown conditions. It gives 12 q/ha seed yield.
5. *Shimla B-1*: This is a *tataricum* variety released in 2005 through pure line selection for mid-hills and high hills of Himachal Pradesh and Uttarakhand. This variety can fit well in double cropping system where buckwheat is grown after potato, cabbage, peas, and hops during mid-August to mid-October. It gives 12.11 q/ha seed yield and matures in about 80 days.
6. *Sangla B-1*: The *tataricum* variety was released for high seed yield in 2005 for mid-hills and high hills of Himachal Pradesh and Uttarakhand as a pure line selection (Rana et al. 2016). It matures in about 106 days. It is resistant to powdery mildew and gives 13 q/ha seed yield.

The influence of Chinese buckwheat on Himalayan buckwheat has been reported (Ohnishi 1993). Joshi and Paroda (1991) carried out the evaluation of a large number of accessions of buckwheat from Himalayan region and reported accession IC13145 as *F. himalianum*. This accession was listed as *F. tataricum* var. *himalianum* by International Plant Genetic Resources Institute later on. Evaluation of landraces of *F. esculentum* and *F. tataricum* led to the identification of landraces resistant to powdery mildew (*Erysiphe polygoni*) (Kapoor and Chaudhary 1994). Under the present scenario of global warming, a lot of fluctuation in climatic conditions is being observed which necessitates the identification of genotypes with high seed yield and seed yield stability. Chaudhary (1996) and Sharma and Chaudhary (1998a, b) investigated the seed yield stability of *F. tataricum* and *F. esculentum* in the dry temperate zone of Northwestern Himalayas. In 2002, the stability analysis of eighteen common buckwheat genotypes for traits like plant height, seed yield, days of maturity, and branches per plant in the rainfed conditions of dry temperate regions of Northwestern Himalayan region revealed suitability of accessions EC323731, VL7(A), and IC18869 for the cultivation in Himachal Pradesh (Dhiman et al. 2002). Likewise, based on the mean performance of the tartary buckwheat, the cultivars *KBB-3* and *Khaldo* were recommended for high scale cultivation in Himachal Pradesh (Dhiman and Chahota 2003). In 2004, the accession IC258233 which is an easy dehulling accession was registered by NBPGR Regional Station, Shimla. The morphological and molecular diversity analysis of 46 buckwheat populations including both common and tartary buckwheat from Northwestern

Himalayas using 23 set of descriptors revealed the occurrence of distinct regional variation for quantitative traits. The RAPD analysis revealed that Uttarakhand accessions exhibit higher inter-population variation than Himachal Pradesh accessions. Moreover, the tartary buckwheat accessions were found to be more diverse than common buckwheat accessions (Senthilkumaran et al. 2008).

26.3.1.11 Biotechnological Interventions

Limited efforts in crop improvement programs on buckwheat as compared to major crops have resulted in the decline of its production. Lately, breeding programs on buckwheat in India are being undertaken to develop improved breeding material under All India Coordinated Research Project (AICRP) on Underutilized and Underexploited crops/All India Coordinated Research Network (AICRN) on potential crops (Hore and Rathi 2002; Singh 2018; Babu et al. 2018). Initially, the breeding programs on buckwheat were mainly based on conventional mass selection for common buckwheat and pure line selection for tartary buckwheat. However, conventional breeding programs in buckwheat are associated with many limitations like unsynchronized flowers, apomixis, low seed availability, an inherited self-incompatibility in common buckwheat, shattering, and sterility (Joshi et al. 2019; Singh et al. 2020b). Biotechnological approaches and tools form a favorable alternative to address molecular genetics and breeding programs. An important pioneer in this direction is the discovery and utilization of molecular markers based on polymorphism of DNA (Cullis 2002). The molecular markers have wide applications in different aspects of plant breeding programs like marker-assisted selection, genetic map construction, gene mapping and cloning, and genetic diversity analysis (Li et al. 2013). RAPD markers analysis unveiled the occurrence of high degree of polymorphism in common and tartary buckwheat (Javornik and Kump 1993). RAPD markers also helped in the analysis of the origin of common buckwheat (Murai and Ohnishi 1996). Later on, AFLP marker analysis was also used to study the origin of cultivated common buckwheat (Konishi et al. 2005). The SDS PAGE and RAPD profiles of different accessions of Himalayan buckwheat revealed greater closeness of common buckwheat with tartary buckwheat in comparison to that with cymosum buckwheat (Rout and Chrungoo 2007). Utilization of RAPD, AFLP, and SSR markers contributed substantially towards detecting polymorphism and genetic variation in buckwheat (Iwata et al. 2005; Konishi and Ohnishi 2007; Senthilkumaran et al. 2008; Ma et al. 2009; Song et al. 2011). Even sequence-related amplified polymorphism (SRAP) markers have been used to detect genetic variation in tartary buckwheat (Li et al. 2009). Despite the wide genetic diversity observed, the lack of organized cultivation and changing cropping pattern has led to depletion of buckwheat species (Rodriguez et al. 2020).

The reports on linking various molecular markers with specific traits also exist. Nagano et al. (2001) found *Sh* allele specific AFLP markers with tight linkage to the buckwheat homostylar locus. Earlier Aii et al. (1998, 1999) reported the linking of RAPD markers with homostylar locus and *Sh* gene. The homostylar locus is related to self-compatibility in the buckwheat and thus the developed markers could further be utilized for the mapping of *Sh* allele. Likewise, five AFLP markers linked to

non-seed shattering *sht1* locus and shattering habit (Matsui et al. 2004) and sequence-tagged site (STS) markers linked to *dwarf* E gene (Yasui et al. 2008) in buckwheat were also identified. Recently, the development of 18 gene-specific STS markers linked to specific phenotypic traits in buckwheat has been reported (Archak et al. 2017). These markers hold the potential for assisting in marker-aided selection in buckwheat. By utilizing genome wide markers, AFLP technique has also contributed towards the construction of first chromosome map of common buckwheat (Yasui et al. 2004). Later on, the linkage map of common buckwheat was also developed by employing the SSR and AFLP markers (Konishi and Ohnishi 2006). These maps helped in the identification of quantitative trait loci (QTLs) related to stem length (Yabe et al. 2014) and photoperiod sensitivity (Hara et al. 2011).

The role of expressed sequence tags (ESTs) for rapid development of molecular markers in buckwheat (Yasui et al. 2008) and BAC libraries for rapid enrichment of genetic resources of buckwheat (Chauhan et al. 2010; Rana et al. 2016) cannot be ignored. Mass discovery of SNPs in tartary buckwheat using transcriptome sequencing was also reported (Suzuki et al. 2013). Availability of well-assembled reference genomic resources of buckwheat has also helped in genomics assisted breeding programs. Nagano et al. (2000) analyzed the genome size of *F. esculentum* and *F. tartaricum* and reported the genome size of around 1340 Mbp for common buckwheat and around 540 Mbp for tartary buckwheat. The genome size variation observed during this study could account for the interspecific crossing barrier among various species. Moreover, the comparative analysis of chloroplast genome sequences revealed that the chloroplast genome of tartary buckwheat is shorter than that of the common buckwheat. The two species show resemblance in the gene content, orientation, and order with slight structural variations at tandem and palindromic repeat frequencies and junction areas (Cho et al. 2015). Later on, two more chloroplast genome sequences have also been developed (Liu et al. 2016; Wang et al. 2017).

In order to accelerate the molecular breeding programs of buckwheat, a draft assembly of common buckwheat genome was developed and a Buckwheat Genome DataBase was created. The developed genome with size of 1200 Mbp, comprising of 387,594 scaffolds, was utilized to identify a novel candidate gene for heteromorphic self-incompatibility of buckwheat (Yasui et al. 2016b). In 2017, the integrated DNA and RNA sequence analysis was used to develop a genome of tartary buckwheat. The assembly was further improved by utilizing the data from the large Hi-C sequencing data, DNA insert fosmid library, and BioNano genome maps. The genome size was 489.3 Mbp and it facilitated the identification of genes associated with biosynthesis and regulation of rutin, aluminum stress resistance, and abiotic stress responses. Interestingly, the data pointed out that the expansion of gene families associated with membrane transport, signal transduction, and gene regulation contribute towards abiotic stress tolerance trait in buckwheat (Zhang et al. 2017a). The genome sequences would assist in mapping of numerous markers leading to development of more saturated maps (Joshi et al. 2020).

The next generation sequencing technology has also been employed to understand the molecular response of buckwheat to various cues. The candidate genes

regulating various biological processes can be characterized using transcriptome-based gene expression profiling (Joshi et al. 2020). The comparative RNA sequence analysis of flowers and inflorescences of common buckwheat and tartary buckwheat revealed the differentially expressed genes (DEGs) that included retrotransposon and disaccharide metabolic genes (Logacheva et al. 2011). In common buckwheat, the upregulation of transporter genes and operation of multiple transcription factors (TFs) has been conveyed in aluminum toxicity responsive transcriptome of leaf and root tissue (Chen et al. 2017b; Xu et al. 2017). Wu et al. (2017) analyzed salinity responsive transcriptome of *F. tataricum* seedlings. The analysis revealed upregulation of 455 DEGs under stress which included genes related to metabolic processes and abiotic stress related TFs. Later on, Lu et al. (2018) analyzed the salinity responsive transcriptome of common buckwheat. Comparative analysis revealed 385 DEGs that included the genes related to metabolic processes, signaling, growth, and cellular processes. The salinity responsive genes obtained from these studies could be mined and exploited further to develop salt-tolerant cultivars in near future.

Very recently, Wang et al. (2020) analyzed the lead stress-responsive transcriptome of tartary buckwheat that revealed the upregulation of genes associated with cell wall binding, transport, lipid, and energy metabolism. It is an interesting study as buckwheat has been reported as a phytoremediator plant for lead in previous studies (Honda et al. 2007). The study concluded that lead ions are accumulated in the leaf vacuoles. In separate set of studies, the comprehensive global transcriptome analysis of rice tartary buckwheat and common buckwheat at pre-filling, filling, and mature stage highlighted the transcriptome dynamics during seed development and nutrient accumulation of buckwheat seeds. In rice tartary buckwheat, the RNA sequence analysis revealed 108 specifically and 11,676 DEGs (Huang et al. 2017). In common buckwheat, upregulation of genes associated with calcium signal transduction, hormone signal transduction, seed size, and starch biosynthesis was observed (Li et al. 2019). Based on the transcriptome data, Gao et al. (2017) suggested the association of *FLS1* transcript with rutin accumulation during the seed filling stage.

26.4 Chenopodium

The genus *Chenopodium* belonging to family *Amaranthaceae* subfamily *Chenopodiaceae* comprises 250 species which have been cultivated since time immemorial as a leafy vegetable, e.g. *C. album* or as a grain crop, e.g. *C. quinoa* commonly known as quinoa. The latter belongs to the group of crops collectively named as "*pseudocereal*." It has remained highly neglected and underutilized and often belittled as "poor people's plant." Owing to its high nutritional value and versatility and tolerance to harsh weather, climate, and soil conditions, it has also been named as "golden grain." Nowadays, quinoa cultivation has regained tremendous interest. It is a gynomonoecious annual herb bearing alternately arranged leaves of variable colors on erect stem. The leaves may be green, red, yellow, or purple.

Variation in color is observed due to the accumulation of betacyanins. The Indian cultivars reach up to the height of 1–2 m and generally display extensive branching, large sized leaves, and well-developed tap-root system (Bhargava et al. 2006). The inflorescence is a panicle and has hermaphrodite and unisexual female flowers. The fruit may be conical, cylindrical to ellipsoidal in shape and is an achene. Seeds are of varying size and may be black, red, or white in color (Jaikishun et al. 2019).

Quinoa is mainly self-pollinating crop and associated with breeding problems as it is difficult to manually emasculate the small and tightly clustered flowers. Well drained seed bed with 25–50 cm row spacing and 1–2 cm seed sowing depth is ideal for quinoa cultivation. It can also perform well on marginal areas (Jacobsen and Stolen 1993; Jacobsen 2003). Water management is a crucial determinant for quinoa yield and reportedly drip irrigation is appropriate system to boost its agronomic traits and yield (Ramesh et al. 2019). Although quinoa, in general, displays multi-stress tolerance, the temperature beyond 35 °C during flowering stage adversely affects its seed setting and yield (Jacobsen et al. 2005). In India, quinoa cultivation was initiated by the state governments of Andhra Pradesh, Rajasthan, and Uttarakhand. Quinoa belonging to the Bolivian variety was cultivated in the districts of Hyderabad and Anantapur under the "Anantha project" in 2013. Thereafter, quinoa was also introduced in Bhilwara and Chittorgarh districts of Rajasthan during 2015–2016. In order to promote this crop among farmers and boost their income, quinoa seeds mini-kits were distributed to farmers of thirteen districts of Rajasthan in the financial year 2017–2018 (Dhaka and Prasad 2020). Two accessions of quinoa were introduced in the mid-hills of the Northwestern Himalayas in 2016 by CSIR-Institute of Himalayan Bioresource Technology, Palampur, Himachal Pradesh. The cultivars showed optimal performance with balanced nutritional profile indicating towards the suitability of quinoa cultivation in the Northwestern Himalayas (Rathore et al. 2016).

26.4.1 Nutritional Components

26.4.1.1 Carbohydrates

Quinoa seeds are rich in carbohydrates content. On dry weight basis, the carbohydrate content varies from 49% to 68% (Angeli et al. 2020). The predominant carbohydrate in quinoa seeds is starch. Quinoa starch comprises small annular polygonal granules that are less than 3 μm in size (Bhargava et al. 2006). Attributes like small size, low temperature of gelatinization, higher maximum viscosity, greater swelling power, and water absorption capacity make the quinoa starch ideal candidate for specialized applications in cosmetics industry and food industry (Filho et al. 2017; Angeli et al. 2020). Quinoa starch has much lower amylose content (3–22%) than corn and wheat (Navruz-Varli and Sanlier 2016). The sugars in quinoa are present in low amounts (3%) and include maltose, galactose, ribose, fructose, and glucose (James 2009).

26.4.1.2 Proteins

The protein content of quinoa seeds may vary from 9.1% to 16.5% with an average of about 15%. The protein content in quinoa seeds is more than that in cereals like rice, oats, barley, and wheat. Moreover, the protein content is dependent on the cultivar and the geographical region (Nowak et al. 2016). The main location of protein in the seed is the embryo. The quinoa seeds possess high-quality proteins rich in essential amino acids like lysine, isoleucine, histidine, cysteine, phenylalanine, tyrosine, tryptophan, and methionine and provides a protein value comparable to casein in milk (Bhargava et al. 2005; Navruz-Varli and Sanlier 2016; Filho et al. 2017; Angeli et al. 2020). The presence of balanced composition of amino acids and the absence of gluten type proteins in quinoa seeds makes this crop ideal for human consumption. Water soluble albumins (35%) and globulins (37%) represent the main storage protein in the seeds of the quinoa. Alcohol soluble prolamins are present in low concentrations.

26.4.1.3 Lipids

The lipid content in quinoa seeds is higher than that in maize and other cereals and varies between 2.0% and 9.5%. The quality of quinoa oil is quite superior as it is rich in essential fatty acids like oleic acid, linoleic acid, and linolenic acid. Further, around 88% of total fatty acids is accounted for by the healthy unsaturated fatty acids. Palmitic acid which is a saturated fatty acid constitutes 10% of the total fatty acids in quinoa oil. The quantity and quality of lipids of quinoa seeds have made quinoa an alternative oily seed (Navruz-Varli and Sanlier 2016; Angeli et al. 2020).

26.4.1.4 Minerals

The mineral content in quinoa seeds is reported to be at concentrations much higher than that in most grain crops. Quinoa seeds are rich in calcium, iron, copper, magnesium, zinc, potassium, and phosphorus (Angeli et al. 2020). Further, the mineral concentrations in quinoa vary considerably when cultivated in different soil types.

26.4.1.5 Vitamins

The quinoa seeds are reported to have appreciable amounts of β-carotene (0.39 mg/100 g), thiamin (0.40 mg/100 g), riboflavin (0.39 mg/100 g), niacin (1.06 mg/100 g), panthothenic acid (0.61 mg/100 g), pyridoxine (0.20 mg/100 g), folic acid (23.5–78.1μg/100 g), ascorbic acid (4.0–16.4 mg/100 g), α-tocopherol (2.6–5.4 mg/100 g), and biotin (7.1μg/100 g) (Bhargava et al. 2006; Vega-Galvez et al. 2010). Concentrations of many of these vitamins have been reported to be higher in quinoa than typical cereal grains.

26.4.1.6 Dietary Fibers

The total dietary fibers in quinoa seeds are comparable to that found in cereals and ranges between 7.0% and 9.7%. About 80% of quinoa fibers is insoluble which helps to regulate lipid metabolism, reduce nutrient diffusion through the small intestine,

promote satiety, and increase cholesterol and lipid excretion (Ahamed et al. 1998; Navruz-Varli and Sanlier 2016).

26.4.1.7 Antinutritional Factors

The main antinutritional factors present in quinoa seeds are saponins, tannins, phytic acid, protease inhibitors, and prolamins. Saponins, which are basically glycosidic triterpenoids, are bitter in taste and influence the palatability and color of the products. On dry weight basis, the saponins content in quinoa seeds is lesser in sweet genotypes (0.2–0.4 g/kg) than in bitter genotypes (4.7–11.3 g/kg). The hulls of quinoa seeds accommodate around 34% of the total saponins which can be removed either by washing and rubbing in cold water or dehulling (Ahamed et al. 1998; Bhargava et al. 2006). The tannins content in raw quinoa seeds has been reported to be around 0.5%. Tannins disrupt digestion by forming complexes with digestive enzymes and dietary proteins. The content of phytic acid in quinoa seeds ranged between 10.5 mg/g and 13.5 mg/g. It is present primarily in the outer layers of quinoa seeds and the endosperm. It reduces the availability of minerals like zinc, magnesium, iron, and calcium in diet by forming complexes with them. Less than 50 ppm of protease inhibitors were detected in quinoa seeds. Trypsin inhibitor is found in much lower concentration than that in other grains. Celiac-toxic prolamins have undesirable effects in patients with celiac disease.

26.4.1.8 Bioactive Components

The presence of numerous bioactive components in quinoa has helped it gain international attention as a "superfood." Quinoa seeds are rich in phenolic compounds that are known to exert antioxidant, anticarcinogenic, antitumor, antiinflammatory, allelopathic, and antifeedant activities (Tang et al. 2016; Lin et al. 2019). Phenolic compounds may exist either as phenolic acids or polyphenols. The phenolic acids are simple single-ringed structures present either in free form or associated in the cell wall with pectins. Several phenolic acids including their derivatives like ferulic acid, caffeic acid, *p*-coumaric acid, ferulic acid 4-O-glucoside, benzoic acid, protocatechuic acid 4-O-glucoside, 1-O-galloyl-β-D-glucose, vanillic acid glucosyl ester, vanillic glucoside, vanillic acid, and vanillin have been reported to be present in quinoa seeds (Dini et al. 2004; Gorinstein et al. 2008; Gomez-Caravaca et al. 2011). Phenolic acids detected in quinoa leaves include ferulic acid, sinapinic acid, chlorogenic acid, *p*-coumaric acid, *o*-coumaric acid, gallic acid, vanillic acid, syryngic acid, *p*-hydroxybenzoic acid, and benzoic acid (Gawlik-Dziki et al. 2013). Polyphenols are multi-ringed structures and divided into several subgroups, the flavonoids being the most widely explored. Flavanoids include both flavanol glycosides and isoflavones. Flavanol glycosides are the main phenolics in quinoa seeds and leaves. More than a dozen different flavanol glycosides like kaempferol 3-O-(2,6-di-O-α-L-rhamnopyronosyl)-β-D-galactopyranoside, kaempferol 3-O-[β-D-apiofuranosyl (1→2)-α-L-rhamnopyranosyl (1→6)]-β-D-galactopyranoside, kaempferol 3-O-β-D-glucuronic acid, kaempferol 3-O-[β-D-apiofuranosyl(1→2)]-β-D-galactopyranoside, quercetin 3-O-glucoside, quercetin 3-O-β-D-apiofuranosyl-(1→2)-O-[α-L-rhamnopyranosyl-(1→6)]-3-D-galactopyranoside-3′,4′-dimethyl ether, quercetin

3-O-(2,6-di-α-L-rhamnopyranosyl)-β-D-galactopyranoside, quercetin 3-O-(2,6-di-O-α-rhamnopyranosyl)-β-glucopyranoside, quercetin glucuronide, quercetin 3-O-[β-D-apiofuranosyl(1→2)]-β-D-galactopyranoside, and quercetin 3-O-(2-O-β-apiofuranosyl-6-O-α-rhamnopyranosyl)-β-galactopyranoside (De Simone et al. 1990; Dini et al. 2004; Hirose et al. 2010; Gomez-Caravaca et al. 2011) have been identified in quinoa seeds. Flavanol glycosides, namely rutin, kaempferol, quercetin, and isorhamnetin are reported to be present in quinoa leaves (Gawlik-Dziki et al. 2013). Isoflavones were described for the first time in quinoa by Lutz et al. (2013).

Betalains, which are nitrogen-containing aromatic indole derivatives synthesized from tyrosine, are responsible for the yellow, black, or red colors of quinoa plants. They are structurally and biosynthetically distinct from phenolics but exhibit several medicinal properties identical to phenolic compounds. Quinoa is also reported to contain a higher amount of betaine (3.93–6.00 mg/g) as compared to cereals and other pseudocereal crops. Betaine and its precursor, choline, are reported to play an important role in homocysteine regulation as well as in the prevention and treatment of obesity, diabetes, and cardiovascular diseases. Quinoa seeds are also reported to contain the maximum concentrations of phytoecdysteroids which are polyhydroxylated steroids. These are reported to have a range of biological activities like antidiabetic, anti-depressive, antioxidant, growth-promoting, hepatoprotective, hypocholesterolemic, immunomodulatory, neuroprotective, and wound healing activities (Zhu et al. 2001; Kumpun et al. 2011). Phytosterols, which are lipophilic compounds structurally similar to cholesterol, are also reported to be present in quinoa seeds. The main phytosterols reported are brassicasterol, campesterol, β-sitosterol, and stigmasterol (Ryan et al. 2007). Phytosterols have an important contribution in lowering cholesterol in humans by virtue of their structural similarity with cholesterol. They compete with cholesterol for intestinal absorption besides reducing production of atherogenic lipoprotein in the liver and intestines (Navruz-Varli and Sanlier 2016).

26.4.1.9 Biological Activities

Due to tremendous nutraceutical properties of quinoa, United Nations named the year 2013 as the "International year of Quinoa." There are numerous folklores claims of use of seeds and leaves of quinoa in the treatment and prevention of a wide variety of ailments like bruises, abscesses, sprains, dislocations, fractures, internal hemorrhaging, throat and stomach ailments, skin conditions, urinary tract infections, and helminthic infections (Bhargava et al. 2006; FAO 2011; Kumpun et al. 2011; Singh et al. 2020a). Quinoa holds great potential in the development of various food products, supplements, and cosmetics to target different health related areas like malnutrition, weight loss, celiac disease, sports performance and fitness enhancement, diabetes, obesity, hypertension, hyperlipidemia, post-menopause, and skin and hair care. Though the number of animal and human clinical trials on the therapeutic potential and efficacy of quinoa health products are severely inadequate, numerous studies have indicated beneficial effects associated with quinoa consumptions. Some of these are listed in Table 26.3.

Table 26.3 Biological activities of *Chenopodium quinoa*

Biological activity	Plant part/active component/extract type	References
Antibacterial	Quinoa starch biofilms containing gold nanoparticles	Pagno et al. (2015)
	Acetone, ethanolic, ethyl acetate, hexane, methanolic, and water extracts of quinoa seeds	Bhaduri (2016)
Anticarcinogenic	Acetone, ethanolic, ethyl acetate, hexane, methanolic and water extracts of quinoa seeds	Bhaduri (2016)
	Bioactive polysaccharide from *C. quinoa*	Hu et al. (2017)
	Bioactive peptides derived from quinoa proteins	Vilcacundo et al. (2018)
	Oleanolic acid in the seed bran and hederagenin analogues in the leaves of quinoa	Lin et al. (2019)
Antidiabetic	Quinoa seeds	Pasko et al. (2010)
	Phytoecdysteroids	Graf et al. (2014)
Antifungal	Saponins in the seeds of quinoa	Stuardo and San Martín (2008)
Antihypercholesterolemic	Quinoa protein isolate	Takao et al. (2005)
Antiinflammatory	Saponins from quinoa seeds	Yao et al. (2014)
Antioxidant	Polysaccharides extracted from aqueous and alkali extracts of quinoa	Yao et al. (2014)
	Water, methanol and ethanol extracts of quinoa	Bhaduri (2016)
	Water and ethanol extracts of quinoa leaves	Chen et al. (2017)
	Bioactive polysaccharide from *C. quinoa*	Hu et al. (2017)
	Ethanolic extract of quinoa seeds	Park et al. (2017)
Dermatological care	Quinoa seed oils	Msika (2012)
Immunomodulatory	Polysaccharides extracted from aqueous and alkali extracts of quinoa	Yao et al. (2014)
	Bioactive polysaccharide from *C. quinoa*	Hu et al. (2017)

26.4.1.10 Landraces/Cultivars and Commercial Varieties in Northwestern Himalayas

The production of good quality high protein grain under ecological extremities makes quinoa imperative for the agricultural diversification in the high altitudes of the Himalayas (Bhargava et al. 2005). In Northwestern Himalayas, quinoa is grown in mountainous villages of different regions of Shimla, Kullu, Lahaul and Spiti, Kinnaur, and Mandi districts in Himachal Pradesh, Leh and Kargil in Jammu and Kashmir, and Pithoragarh in Uttarakhand (Genebank Dashboard 2016) (Fig. 26.3). The crop is grown in multi-cropping system usually along with maize, rice, millet, potatoes, sesame, taro, amaranth, and beans. The time of sowing varies from April at higher elevations to May and June at the lower elevations of the Himalayas and harvesting is done in the months of September to October (Partap et al. 1998).

Quinoa at farmer's field in Leh, Ladakh, Jammu and Kashmir

Quinoa at farmer's field in Karsog, Mandi, Himachal Pradesh

Quinoa at farmer's field in Lag valley, Kullu, Himachal Pradesh

Quinoa at Pithoragarh, Uttarakhand

Fig. 26.3 *Chenopodium quinoa* cultivation by farmers of Leh, Ladakh, Jammu and Kashmir; Karsog, Mandi, Himachal Pradesh; Lag valley, Kullu, Himachal Pradesh and Pithoragarh, Uttarakhand

Fig. 26.4 Commercial cultivation of quinoa at village Hundar, Nubra tehsil, Leh, Ladakh

Among the three "ABC" pharmaceutical crops of the Northwestern Himalayas, amaranth and quinoa are lesser studied as compared to buckwheat. Recently, an agriculture graduate from Sher-e-Kashmir University of Science and Technology (Jammu), Mr. Tsewang Nurbu, has started commercial cultivation of quinoa at village Hundar (34.576 N 77.495E) of Nubra tehsil in the Leh district of Ladakh (Fig. 26.4). Fifty nine accessions of *Chenopodium* species including eight accessions of *C. quinoa* locally cultivated by the traditional farmers of Northwestern Himalayas have been collected and conserved at NBPGR, New Delhi and NBPGR Regional Station, Shimla (Genebank Dashboard 2016). The traditional landraces/cultivars and wild species serve as a bioresource of high genetic diversity, which can be utilized for various breeding programs in order to develop new varieties with high grain yield and large seed size having high-quality nutritional components and low antinutritional factors. An improved variety, *Pusa Bathua-1* was developed and notified in 1998 by IARI, New Delhi. The variety is recommended for cultivation

in National Capital Region (NCR) and yields about 300 q/ha. The plants grow up to a height of 2.25 m and have tender purplish green leaves and stem. The variety is salt-tolerant and reported to possess 60% more vitamin C and 10% more β-carotene as compared to local varieties. Another improved variety, *Pusa Green*, has been developed in 2016 and released for commercial cultivation in 2018 by Indian Agriculture Research Institute, New Delhi. The variety suitable for Northwestern Himalayas is *Him Bathua-1* which was released in 2013 for high hills and dry temperate region of Himachal Pradesh and adjoining areas. The crop matures in approximately 111 days and the average yield is 9–10 q/ha. The grains are black in color.

26.4.1.11 Biotechnological Interventions

Considering the nutritional profile of quinoa, attempts have been made for the molecular approaches for breeding and crop improvement of quinoa. The genomics studies particularly targeted the genetic diversity and the evolutionary history. The main traits for breeding efforts included the seed size, color, yield, shattering, early maturity, lodging, abiotic and biotic stress tolerance, and low saponin content.

The phenotypic, biochemical, and molecular markers have been utilized for the analysis of genetic diversity and phylogenetic relationship among cultivated quinoa species and wild relatives. Ruas et al. (1999) reported low intraspecific variation within the accessions of *C. quinoa* by utilizing RAPD markers. Rojas (2003) classified 1512 accessions preserved in Bolivia germplasm into seven groups by utilizing phenological stages, morphological traits, agronomic traits, and harvest index. The genetic diversity analysis of 29 germplasm lines that included 27 lines of *C. quinoa* and 2 lines of *C. berlandieri* subsp. *nuttalliae* for 12 morphological traits and 7 quality traits for the two test seasons revealed gigantic genetic variability. The studied parameters clustered the quinoa lines separately from the 2 lines of *C. berlandieri* subsp. *nuttalliae* (Bhargava et al. 2007). del Castillo et al. (2007) reported the hierarchical structure of the genetic diversity present in the eight quinoa fields containing both cultivated and weed quinoa lines representing *Altiplano* and *inter-Andean* valleys of Bolivia. Based on RAPD analysis, the study revealed occurrence of high intra-population variation for the autogamous species.

Mason et al. (2005) reported 208 polymorphic microsatellite markers for quinoa which included 91 dinucleotides, 110 trinucleotides, and 7 tetranucleotides or larger. Jarvis et al. (2008) reported 216 new polymorphic SSR markers and developed the first genetic map of quinoa based on sequence-tagged SSR markers. Christensen et al. (2007) reported fluorescence-tagged SSRs for characterizing the genetic variation of 152 quinoa accessions. The analysis revealed the segregation of lowland and highland accessions into two discrete clusters. The genetic diversity of 59 quinoa accessions that included 28 *Altiplano* and 31 coastal *Chilean* quinoa accessions revealed the segregation of quinoa accessions into two distinct groups, namely the north (*Andean highlands*) and south (*lowland/coastal*) group. The development of seven sets of fluorescent multiplexed microsatellite PCR markers was also reported (Fuentes et al. 2009). The molecular diversity analysis of 14 coastal accessions of quinoa by exploiting AFLP markers also revealed the existence of wide genetic

variability (Rodríguez and Isla Rodriguez and Isla 2009). Zhang et al. (2017b) resequenced 11 diverse quinoa germplasms to mine the genomic variation in quinoa through whole genome resequencing. The assembled sequences revealed wide genomic variations and identified SNPs and InDels. Eighty five InDels developed along with 62 SSR markers were used for genotyping 129 quinoa accessions.

The linkage maps aid in QTL mapping that play an important role in the marker-assisted selection (MAS) in order to accelerate the breeding process. Maughan et al. (2004) developed the first genetic linkage map of quinoa based on 230 AFLP, 19 SSR, and 6 RAPD molecular markers. This linkage map spans 1020 cM and comprises 35 linkage groups with an average marker density of 4.0 cM per marker. Thereafter, Jarvis et al. (2008) presented the SSR-based linkage map of quinoa that spans 913 cM and contains 275 markers (200 SSRs) and 38 linkage groups. Maughan et al. (2012) utilized two recombinant inbred line (RIL) populations and developed a linkage map that spans 1404 cM and consists of 451 SNP loci and 29 linkage groups. Jarvis et al. (2017) developed a high-density linkage map that comprises 6403 markers and 18 linkage groups and spans 2034 cM. The role of ESTs and BAC libraries for gene discovery in quinoa also cannot be overlooked. The BAC library led to identification and characterization of seed storage proteins, namely 11S globulin and 2S albumin, and *Salt Overly Sensitive* 1 (*SOS1*) gene in quinoa (Balzotti et al. 2008; Maughan et al. 2009a). Raney et al. (2014) utilized the RNA-Seq to mine the putative genes involved in the drought response in quinoa. Morales et al. (2017) reported the drought responsive transcriptome of drought tolerant *C. quinoa* genotype R49 (Salares ecotype) to mine the genes associated with drought tolerance. Data revealed upregulation of plasma membrane related genes and transporter genes. Among 1579 over-represented genes, 19% were unknown. Observed minor upregulation of ABA genes indicates operation of the ABA-independent mechanisms to acclimatize to water deficit.

The annual pseudocereal quinoa is allotetraploid having the estimated genome size of 1.00–1.59 pg (Kolano et al. 2012). The molecular analysis of quinoa is limited by the genetic heterogeneity due to outcrossing and its genome complexity derived from allotetraploidy. Yasui et al. (2016a) established the inbred and standard quinoa accession "*Kd*" for molecular analysis. They developed the draft genome sequence of "*Kd*" by using an optimized combination of high-throughput next generation sequencing. The de novo assembly comprises 25,000 scaffolds with the N50 length of 86 Kbp. Further, they created a free-access Quinoa Genome Data-Base. The assembly is valuable to mine the genes associated with the important agronomic traits and to understand the genome evolution. Using an inbred line of the quinoa cultivar *Real*, Zou et al. (2017) reported the generation of a high-quality genome assembly. The genome contains high repetitive sequences (64.5%), 54,438 genes, and 192 microRNA. The enriched expression of genes involved in ion and nutrients transportation, ABA homeostasis, and signaling seemed to provide the halophytic characteristic to the quinoa and justify for the salinity and drought tolerance. Very recently, the transcriptome profiling was exploited to identify the TFs and the key homologs governing abscisic acid (ABA) mediated seed dormancy and germination regulation in *C. quinoa*. Around 1066 (ABA)-repressed and

392 ABA-induced DEGs were found to be associated with regulation of seed germination. The study revealed the tight synchronization of 18 TFs and 53 key homologs involved in the process of dormancy and germination that could be exploited for molecular breeding of quinoa elites with pre-harvest sprouting tolerance in future (Wu et al. 2020).

26.5 National and International Status

The accumulating research information on the nutritional quality and phytochemical profile of amaranth, buckwheat, and chenopodium (quinoa) clearly affirms the role of these crops in global food and nutritional security and their wide potential as important nutraceuticals. However, the vast natural resource available in the form of these pseudocereals has not been utilized and nurtured to the full extent for the betterment of the global society due to various constraints that include agronomic, social, technological, and economic factors (Bekkering and Tian 2019; Pirzadah and Malik 2020). This necessitates extensive and multi-interdisciplinary research at national and international level on various aspects of these crops so that their proper and sustainable utilization could be made. The role of these underutilized pseudocereals in biofortification of cereal crops has been vastly emphasized. The importance of these crops in creating nutritional synergy with the major cereals and helping in combating hidden hunger crisis has also been laid down by various researchers (Bekkering and Tian 2019; Pirzadah and Malik 2020). However, limited efforts have been made for the genetic improvement of these crops using molecular approaches. Such molecular approaches have mainly been used for the studies on genetic diversity and evolutionary history. Nowadays more emphasis is being laid down to explore the development of genetically improved varieties of these nutraceuticals by genomics assisted breeding methods worldwide (Yabe and Iwata 2020). Several genomic resources including molecular markers, linkage maps, and QTLs are being explored for speeding up the breeding programs (Rodriguez et al. 2020). Various transcriptome sequencing studies are being carried out at national and international level to understand the molecular basis of various biotic and abiotic stress tolerance shown by these hardy pseudocereals (Li et al. 2019; Joshi et al. 2020; Wang et al. 2020; Wu et al. 2020). Such studies are serving as pioneer in stress-responsive and tissue specific gene discovery as well. Likewise, genome sequence information, ESTs, and BAC libraries are also playing pivotal role in gene discovery and genetic improvement of these crops (Deb et al. 2020; Rodriguez et al. 2020). International collaborations and funding for extensive research programs on the genetic improvement of amaranth, buckwheat. and chenopodium (quinoa) will undoubtedly pave way to sustainable utilization of vast natural resources available in the form of these pseudocereal nutraceuticals.

26.6 Future Prospective and Conclusion

Amaranth, buckwheat, and chenopodium (quinoa)—The "ABC" nutraceuticals of Northwestern Himalayas are gifted with unique nutritional profiles and have rightly been designated as "superfoods." The optimal performance of these pseudocereals in the marginal areas and tolerance to harsh environmental conditions like frost, salinity, and drought is added boon and indicative of robust resistance mechanisms operating in these species. They represent a rich reservoir of genetic diversity and can be utilized for mining of genes of agronomically intended traits. There is an urgent need for the collection and documentation of locally available cultivars to broaden the gene pool which can be harnessed further for the nutritional enrichment via traditional and molecular breeding. To breed for nutritionally rich varieties, the identification of nutrient rich donor and the development of potential genotypes are crucial. The documentation of more and more cultivars of Northwestern Himalayas in amalgamation with advancement in biotechnological tools is anticipated to contribute towards the sustainable utilization of the genetic resources of these pseudocereals to meet the food and nutritional security in changing environments in the coming years. These underutilized resilient pseudocereals show high degree of acceptability in the traditional Himalayan agro-ecosystems and can definitely serve as the potential candidate for crop diversification. However, extensive exploration programs, screening of germplasm for the agronomic traits, cultivar improvement in terms of wider adaptability, seed size, yield, early maturation, and short growing period will definitely boost farming community to undertake their cultivation. The biotechnological interventions including the use of transcriptomics and genomics to remove undesirable traits such as bitter taste, low digestibility, antinutritional factors for increasing the palatability further, and identification and isolation of therapeutically important compounds will also contribute significantly towards utilization of these nutraceuticals on mass scale. Authentication of nutraceutical and therapeutic utilization of the bioactive components of these crops need to be done using more appropriate in vivo model systems and clinical tests. Efficient post-harvest technologies including biofortification and value-addition need to be generated so as to tap the diverse industrial and commercial potential of these crops.

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Application of Plant Growth Promoting Rhizobacteria (PGPR) in Crop Productivity Improvement and Sustainable Agriculture

27

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Abstract

The world population is increasing amazingly, which exerts continuous pressure on farmers to enhance crop productivity and to achieve these targets, a large quantity of chemical fertilizers is being used in the agriculture system. The continual application of chemical fertilizers results in considerably declining microbial activity, nutritional imbalance, and a drop in the population of beneficial microbes in the soil. At present, global climate change is a significant problem for agriculture and considered abiotic stress. The application of bacterial inoculation, specifically the plant growth-promoting rhizobacteria (PGPR), is an effective and eco-friendly technique to improve plant health under normal and stressful conditions. This chapter provides the detailed impact of global climate change, and environmental stress on crop plants thus compromises crop yield. The PGPR employ various mechanisms for plant growth promotion comprising uptake of essential nutrients, regulation and modulation of phytohormones, and production of biocontrol metabolites such as antibiotics, siderophores, and volatile organic compounds. Moreover, the role of PGPR in the reprogramming of host plant transcriptome under various environmental stresses is also efficiently known. The application of PGPR is noted to improve the growth and yield of various crops such as cereals, legumes, oil seed and vegetables. The PGPR have a vast perspective in agriculture especially, concerning global food security, climate change resilience, and sustainability of the agriculture system.

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Keywords

Plant growth promoting rhizobacteria (PGPR) · Transcriptome · Abiotic stress · Plant productivity

27.1 Introduction

The world population is increasing at an alarming rate, and it is projected to rise around 9.8 billion by the year 2050 (United Nations Organization 2017). This prediction poses a critical question for farmers to increase agriculture productivity. To relieve the pressure of enhanced crop productivity, the extensive use of chemical fertilizer in agriculture came into view. Moreover, the intensified uses of agrochemicals have drastically affected the soil's indigenous properties, decreased soil fertility, and posed several environmental problems (Backer et al. 2018). The continual application of chemical fertilizers results in a considerable decline in soil pH and microbial activity, nutritional imbalance, along with a drop in the population of beneficial microbes and their micro-ecological environment (Lin et al. 2019). Therefore, the sustainability of agricultural systems is a critical concern worldwide. The application of bacterial inoculation, specifically the plant growth-promoting rhizobacteria (PGPR) is effective and eco-friendly to improve plant health under normal and stressful conditions. The PGPR are primary performers in contributing to the fertility of soils (Bargaz et al. 2018). They are indigenous to soil and plant rhizosphere and are actively indulged in soil nutrient cycling and breakdown of complex organic matter into simpler forms. Similarly, the implementation of PGPR is also attaining immense consideration as an effective strategy to combat the effect of various abiotic and biotic stresses (Gontia-Mishra et al. 2020). These microbes have enormous potential and emerged as a promising tool for sustainable agriculture. Numerous reports supported the confirmatory role of soil microbes in enhancing plant growth and registered a significant increase in crop yield (Pérez-Montaño et al. 2014; Prasad et al. 2019). This chapter provides the detailed impact of global climate change and environmental stress on crop plants, thus compromises crop yield. The later section of the chapter highlights the mechanism of action employed by the PGPR for plant growth promotion. Furthermore, the role of PGPR in the reprogramming of host plant transcriptome under various environmental stresses is also discussed. Moreover, the chapter also summarizes the application of PGPR for the improvement of various crops such as cereals, legumes, oilseeds, and vegetables.

27.2 Impact of Climate Change and Environmental Stress on Yield of Crop Plants

Climate is the average meteorological conditions at a specific part of the earth for a long time and changes in these conditions are constant. These changes lead to modify or reform the ecosystem of the earth. However, the swiftness of the changes in the climate begins to fasten in recent years or decades. Due to human-made factors like CO_2 emission and deforestation, temperature and drought are increasing day by day on earth. The greenhouse gas emissions have impacted the increase of 0.9 °C average temperature of the atmosphere since the last century and estimated to be 1.5 °C or even more by the year 2050 (Arora 2019). Growing crops in the soil is a crucial activity of agriculture and involve various environmental factors. These factors in specific amount or range are very crucial for maximum production. At present, changes in environment are now a significant problem for agriculture and considered abiotic stress. These abiotic stresses are responsible for decreasing the area of tillable soil (Zahran 1999). With these abiotic stresses, food security is a challenge of the next century with the world's continuously growing population. Drought and high temperature and irrigation with polluted water enhance salinity in soil (Vincent et al. 2006). Soil salinity is also a global problem leading to limited plant growth and yield, reduced nodulation in legumes and organic matter in soil due to enhanced salt uptake by the plants (Van Hoorn et al. 2001; Hashem et al. 2016; Manchanda and Garg 2008; Waskiewicz et al. 2013).

Climate inconsistency significantly influences plant physiology by various means. Several plant stresses have been induced and enhanced due to rapid climate inconsistency and environmental extremes. There are two severe stress factors, drought and high temperature, which have a significant influence on cereal yields and the process of photosynthesis is regulated by a key enzyme *Rubisco*, which gets disordered if the temperature rises from 35 °C; this rests the photosynthetic process (Fahad et al. 2017). The reproductive phase of plant development gets affected by climate change, water deficit, and temperature extremes. Water stress severely disturbs the flower initiation and inflorescence in cereals (Winkel et al. 1997). The plant responses to stress are reliant on the tissue or organ affected by the stress. In roots, the response at the transcriptional level is tissue- or cell-specific and depends on the type of stress (Dinneny et al. 2008).

The hydrologic cycle gets triggered by altering rainfall, warmer climate, magnitude, and timing of run-off. Warm air results in an increase in evaporation of surface moisture, as warm air, holds more moisture. Climate change has a straight impression on crop evapotranspiration (ET). Thus, variation in climate will disturb the groundwater recharge, soil moisture, flood or drought occurrance, and finally groundwater level in different areas (Huntington 2003; Eckhardt and Ulbrich 2003). Climate change proportionally will affect the water cycle (Xu et al. 2007), rise in sea level will surge the risk of permanent or seasonal saline invasion into groundwater and rivers. This rise in salinity directly impacts on water quality and its potential use in domestic, agricultural, and industrial purposes. Climate change effects on agriculture (Gautam and Sharma 2012), like production patterns of

different crops, get severely affected by higher temperatures and changing precipitation. Increased level of carbon dioxide in the atmosphere severely affects agricultural productivity (Kumar and Gautam 2014). In this situation, food security is crucial worldwide because sufficient and nutritional food - two major challenges ahead of all agriculturist under variable climate (Kang et al. 2009). Numerous latest studies have concluded that the expected rise in temperature in the coming years will disproportionately affect agriculture in the planet's lower latitudes. In such a case, agriculture will require improved supervision of natural resources like water, land, and genetic resources to make it more resilient (Kumar and Gautam 2014).

27.3 PGPR and Their Mechanisms of Action

The rhizosphere is an area of soil in the proximity of plant roots; hence, the dynamic and nutrient-rich ecological niche is mostly favoured by the soil microorganisms for interaction with plants (Hiltner 1904). Plant growth-promoting rhizobacteria (PGPR) are a cluster of microorganisms that reside in the rhizosphere and stimulate plant growth. The PGPR has envisaged several plant growth promotion mechanisms and their successful colonization in the rhizosphere (Goswami and Deka 2020). The PGPR have numerous ways by which they can promote and enhance plant growth, such as facilitating the assimilation and uptake of essential nutrients (N_2 fixation, solubilization of insoluble mineral nutrients viz. P, K, Zn, Si, Fe) and production, regulation, and modulation phytohormones (Rashid et al. 2016). Besides, PGPR are also equipped to control the attack of phytopathogenic soil-borne fungi on host plant producing secondary metabolites such as antibiotics, siderophores, volatile organic compounds (VOCs), and enzymes (Glick 2012). These indirect mechanisms of PGPR also benefit plant growth and health. The detailed mechanisms of action utilized by PGPR to stimulate plant growth will be discussed in this section.

27.3.1 Phytohormone Production

Phytohormones play a diverse role in sustaining plant growth and development throughout their life cycle. Auxins, cytokinins, abscisic acid (ABA), ethylene, and gibberellic acid are the chief hormones utilized by plants to properly function (Backer et al. 2018). The microorganisms residing in the rhizosphere are equipped with the ability to produce or moderate phytohormones under in vitro conditions and modulate the phytohormone levels in plants, especially in response to environmental stress (Glick 2012). Notably, plant hormones produced by rhizobacteria do not have a relevant function in bacterial cells; hence it is anticipated that it must have evolved to develop plant-microbe interaction (Patten and Glick 2002). The application of indole acetic acid (IAA) or synthetic auxins to plants leads to enormous changes in plant growth and development (Zhao 2010). The PGPR that produces IAA in the rhizosphere mediate typically the formation of primary root elongation and the development of lateral roots (Vacheron et al. 2013). The microbial produced IAA

modifies the root architecture, which expedites the accessibility of mineral nutrients and improved uptake of water by the associated plants (Cox et al. 2018; Meena et al. 2020). Besides, the IAA producing PGPR have established a significant role in regulating stress-related genes (Korver et al. 2018) and initiating auxin-responsive genes signalling in plants, resulting in improved plant growth under stressed conditions (Gontia-Mishra et al. 2016a; Jochum et al. 2019). Cytokinin is another essential plant hormone that functions in cell division, apical dominance, and initiation of root hairs formation, but unlike auxins it influences root elongation and impedes lateral root formation (Akhtar et al. 2020). The PGPR are endowed with the capability to produce cytokinin which may mitigate various functions in associated plants, including shoot growth and root elongation. This plant hormone also plays a crucial part in inducing the defence response of plants against phytopathogens and pest insects (Grosskinsky et al. 2016; Akhtar et al. 2020). Apart from this, cytokinins also develop resistance towards various abiotic stresses (Liu et al. 2013; Tsukanova et al. 2017). Some of the PGPR were reported to produce gibberellin, resulting in enhanced growth of shoots and yield increase in plants (Joo et al. 2005; Kang et al. 2014). ABA is essential for controlling numerous plant processes such as seed development and its response to various environmental stresses such as drought, salt, and cold. Hence, ABA is considered as plants' stress hormone (Porcel et al. 2014). Several PGPR are reported to modulate endogenous levels of ABA, which resulted in alleviating abiotic stresses in host plants (Porcel et al. 2014; Kousar et al. 2020).

27.3.2 Mineral Acquisition [Nitrogen (N_2) Fixation, Solubilization of Phosphorus (P), Potassium (K), Zinc (Zn), and Silicon (Si)]

In order to achieve proper growth of plants, there is a prerequisite of the essential macronutrients and micronutrient in adequate quantities. Nitrogen is the major component of plants' building block proteins, enzymes, chlorophyll, and vitamins. Usually, the soil is deficient in nitrogen content which is fulfilled mainly by chemical fertilizers. The use of chemical fertilizers is associated with several environmental issues; hence the application of N_2 fixing bacteria is a better possibility for agriculture. The biological nitrogen fixation (BNF) transforms atmospheric N_2 into a usable form such as NH_3, which is assimilated by plants (Mus et al. 2016). The legume crops form a symbiotic association with N_2 fixing rhizobium to fulfil their nitrogen requirement when grown under nutrient-deprived soils (Wang et al. 2018). The biological nitrogen fixed in the agriculture systems due to legume–rhizobia interaction shares approximately 80% of total biologically fixed N in the soil. The application of *Rhizobium* spp. with legume crops is becoming the first choice of bio-fertilizer applied by farmers to increase the nitrogen content of plants. Harnessing the atmospheric nitrogen for cereal is a challenging task. BNF is possibly an alternative supplementing nitrogen for cereal crop production (Rosenblueth et al. 2018). Hence, the application of free-living nitrogen bacteria such as *Azospirillum* sp., *Azotobacter* sp., *Azoarcus* sp., and cyanobacteria as bio-fertilizer for cereal crops

is a feasible and sustainable option to chemical fertilizers (Bloch et al. 2020). Thus, there is a vast demand for free-living diazotrophs as bio-fertilizers in cereal cropping agriculture systems.

Phosphorus is an essential macronutrient required by the plant for proper plant growth and development (Alori et al. 2017). The distribution of phosphorus in soil is adequate, but its tendency to readily convert into insoluble forms poses the problem of unavailability (Richardson 2001). To overcome the problem of soil phosphorus deficiency usually, phosphorus fertilizers are applied. Nevertheless, the superphosphates fertilizers applied to soil is often converted into insoluble forms by making complexes with Al^{3+} and Fe^{3+} in acidic soils, whereas Ca^{2+} in calcareous soils (Sharma et al. 2013). Many PGPR have the unique ability to solubilizing insoluble P to soluble form and are considered as phosphate solubilizing bacteria (PSB). When present in the rhizosphere, PSB can increase the availability of P in the rhizosphere (Jha et al. 2012). Several soil fungi and bacteria can solubilise phosphorus in vitro and enhances its mobilization into the plants (Alori et al. 2017; Gontia-Mishra et al. 2017a). The most common solubilization mechanisn of inorganic P present in association with Ca, Fe, or Al are solubilized through the production of organic acids excreted by PSBs (Backer et al. 2018). The organic phosphorus in the form of phytate/phytic acid in the soil usually remains unutilized by plants. However, certain PGPR and soil fungi can utilize the phytate as a sole source of phosphorus by the action of phytase activity (Singh and Satyanarayana 2011; Gontia-Mishra et al. 2013). Hence, the application of phytase producing microbes to soil help in increasing phosphorus in the soil, in turn, better acquisition of phosphorus by the plant (Ramírez and Kloepper 2010). Besides, PGPR, arbuscular mycorrhizal (AM) symbiosis, with the roots of higher plants is also associated with increasing the plant phosphorus acquisition (Campos et al. 2018). Thus, the application of soil microbes for efficient management of soil phosphorus is required to improve its uptake by plants.

In addition to nitrogen and phosphorus, the deficiency of other vital nutrients, such as K and Zn, also limit crop yields. Several strains of Zn and K solubilizing and mobilizing bacteria have been reported to promote the accelerated Zn and K uptake and further increased yield of several crops (Ramesh et al. 2014; Gontia-Mishra et al. 2017b; Dong et al. 2019). Similarly, silicon has an essential function in plants for improving their ability to overcome biotic and abiotic stresses (Ma and Yamaji 2006). Plant roots absorb silicon as monosilicic acid. The silicate solubilizing bacteria (SSB) solubilize the insoluble silicon to soluble form and increase its mobility in the rhizosphere for plant uptake (Bist et al. 2020). Hence, the application of PGPR has a significant influence on mineral acquisition in plants.

27.3.3 1-Aminocyclopropane-1-Carboxylic Acid (ACC) Deaminase Enzyme Activity

The crop plants are exposed to various abiotic and biotic stresses during their life span (Gontia-Mishra et al. 2014). Ethylene is regarded as a stress hormone because

of its production in response to different environmental stresses and exposure to phytopathogens (Glick et al. 1999). The ethylene generated due to stress response has numerous inhibitory effects on auxin transport, retards root growth and initiates plant senescence (Pandey and Gupta 2019). Increased concentration of ethylene hampers root elongation and consequently affects the overall plant growth. 1-aminocyclopropane-1-carboxylic acid (ACC) is a precursor for the synthesis of ethylene in plants and in due course, some of the ACC is secreted by the plants as root exudates into the rhizosphere (Glick 2014). Several reports suggest that PGPR which possessess the ACC deaminase enzyme activity, can disintegrate ACC into ammonia and α-ketobutyrate, reducing ethylene concentration in plants under stressful conditions (Sapre et al. 2019a). Hence, the PGPR possessing ACC deaminase activity indirectly promotes plant growth by lowering ethylene concentration and enhancing root growth (Glick 2014). The PGPR endowed with ACC deaminase producing activity exhibit several promising effects on plants by protecting them against abiotic and biotic stresses (Gontia-Mishra et al. 2020). Several reports affirm the positive response of PGPR with ACC deaminase activity in ameliorating adverse environmental conditions such as salinity (Jha et al. 2012; Pandey and Gupta 2019), drought (Gontia-Mishra et al. 2016a, b; Danish et al. 2020), heavy metal (Zafar-ul-Hye et al. 2020), temperature (Mukhtar et al. 2020) stresses in several crops. Moreover, the PGPR with ACC deaminase activity have also registered their positive influence in combating biotic stresses, particularly phytopathogen attacks (Hao et al. 2011).

27.3.4 Exopolysaccharide (EPS)

The EPSs produced by soil microbes are biosynthetic polymers predominantly comprising carbohydrates (Freitas et al. 2009). It is noted that bacterial EPS are possibly associated with several processes such as pathogenesis, symbiosis, biofilm formation, and stress resistance (Parikh and Madamwar 2006). PGPR also have a unique ability to produce EPS when exposed to abiotic stress, which aids them in developing biofilm for their persistence in the plant rhizosphere. Microbial EPS can regulate and influence numerous functions in a microbial system such as cell-to-cell communication via quorum-sensing, survival under confrontational stressed conditions, and hosts tissue colonization (Gontia-Mishra et al. 2020). The EPS producing PGPR can efficiently colonize the root surface and make a resilient plant-microbe association, especially under adversative conditions (Ali et al. 2014). The EPS produced by soil microbes consists of different anionic functional groups (sulfhydryl, carboxyl, hydroxyl, and sulfonate), which form complex with the Na^+ ions and other metallic ions (Cu^{2+}, As^{3+}, Pb^{2+}, Hg^{2+}) in saline (Qurashi and Sabri 2012) and heavy metal contaminated soils, respectively (Gupta and Diwan 2017; Zeng et al. 2020). This way the microbial EPS renders the uptake of sodium ions and metals ions into the plant cells thereby enhancing their salinity and metal tolerance. Moreover, under drought stress, the EPS secreted by the PGPR interact with the soil particles, initiate soil aggregates and benefit plants by retaining the soil

moisture and enhance the water holding capacity of the soil (Sandhya et al. 2009; Costa et al. 2018). Hence, EPS producing PGPR increases the water and nutrient uptake by plants ensuring better plant growth under water-deficient condition. Thus, the EPS production by microbe shields the associated plant against several environmental stresses such as heavy metal toxicity, drought, salinity, and others (Sapre et al. 2019b). The application of EPS producing PGPR on plants influences the fitness and survival of plants under stressful conditions.

27.3.5 Volatile Organic Compounds (VOCs)

The plants produce volatile compounds from root exudates; likewise, soil microbes also secrete VOCs for interaction with other microbes and plant (Schenkel et al. 2015). The VOCs are emitted by several microorganisms, including bacteria and fungi (Kanchiswamy et al. 2015). The VOCs are characterized as a complex mixture of low molecular weight organic compounds, which has a markedly high vapour pressure enabling them to diffuse into the surroundings (Schulz-Bohm et al. 2018). Microbial VOCs have been recognized as an effector/signalling molecule to participate in inter-and intra-species interaction and cell-to-cell communication (Asari et al. 2016). They also function as chemical messengers that convey information regarding the molecular basis of microbial interaction with plants (Kanchiswamy et al. 2015). The main role of VOCs is to comprehend in guarding associated plants against phytopathogens by stimulating induced systemic resistance (ISR) in plants (Panpatte et al. 2017). The VOCs producing microbes can be effectively used as biocontrol agents. Several *Trichoderma* spp. are known to release different VOCs, which possibly play a pivotal role in controlling plant-pathogens and as priming agents that induce plant defence (Guo et al. 2019). PGPR strain which emits VOCs, can also modulate plant growth and improve the root architecture (Gutierrez-Luna et al. 2010). Besides inducing ISR in plants against phytopathogens, the role of VOCs is also noted in improving various abiotic stresses. The VOCs producing bacterial strains *Bacillus amyloliquefaciens* GB03 (Zhang et al. 2008) and *Bacillus thuringiensis* (Timmusk et al. 2014) improved the salinity tolerance in Arabidopsis and drought tolerance in wheat seedlings, respectively. These reports reveal a prominent role of VOCs in stimulating plant growth under biotic and abiotic stresses, although the exact mechanism of how they interact with plants is still unknown.

27.4 Re-Programming of Host Plant Transcriptome by PGPR Inoculation under Various Environmental Stresses

The use of PGPRs as biocontrol agents to alleviate crops disease is efficiently recognized. Nonetheless, it is also a potential targets for inducing abiotic stress tolerance in crop plants. The phytopathogen and symbiotic microbes with associated plants modulate transcriptional regulation of genes related defence and development activities, but scanty information is gained regarding their effect of PGPR on plant

gene expression (Drogue et al. 2014). The inoculation of PGPR to the crop plants under stressed environmental conditions has a powerful effect that switches the signalling of several stress-responsive genes. With the advent of recent techniques such as microarrays and RNA-seq, which have conveniently comprehended the molecular mechanism studies involved by plant-microbe interaction under abiotic stresses (Gontia-Mishra et al. 2020). It is recently noted that PGPR inoculation can prominently modulate the transcriptome of the inoculated plants and control the expression of several genes (Rekha et al. 2018). The interaction of *Azospirillum* with rice revealed the upregulation of genes involved in response to stress and plant defence under non-stressed conditions (Drogue et al. 2014). In a similar study, the interaction of *Bacillus subtilis* with rice demonstrated the upregulation of genes related to the transport of nutrients and stress modulation (Xie et al. 2015).

In order to ascertain the transcriptional regulation of plants under abiotic stresses but inoculated with PGPRs, few transcriptomic studies have been reported. The PGPR inoculation enhances the abiotic stress tolerance by downregulating the stress-responsive genes relating to ABA and ethylene pathways. In a transcriptomic study, a halotolerant bacteria, *Arthrobacter nitroguajacolicus,* was inoculated to wheat plants under saline condition, demonstrating the upregulation of stress-responsive genes, especially the Na^+ influx transporter/antiporter genes (Safdarian et al. 2019). In another study, *Bacillus amyloliquefaciens*-SN13 and rice ineraction showed the modulation in expression of photosynthesis and stress-responsive genes (Chauhan et al. 2019). It was also noted that inoculation of *Pseudomonas* sp. in *Arabidopsis* under salt stress validated the changes in the transcript levels of ABA and jasmonic acid signalling pathways.

In a transcriptome experiment, the interaction of *Gluconacetobacter diazotrophicus* with sugarcane plants induced drought tolerance by controlling the drought-responsive genes compared to the un-inoculated plants under similar condition. The PGPR inoculated plants survived the prolonged drought due to the activation of ABA-dependent, auxin and ethylene mediated signalling genes and modulated the transcript levels of DRE/CRT-binding protein (DREB) (Vargas et al. 2014). In a similar study, the inoculation of *Pseudomonas putida* strain FBKV2 in maize exhibited via transcriptome analysis ameliorated the drought stress. The transcript profiling suggested that inoculation of *P. putida* in plants downregulated the antioxidant enzyme genes and phytohormone signalling (ethylene, ABA, auxin) pathways. However, PGPR inoculation caused the upregulation of genes related to heat shock proteins and late embryogenesis abundant (LEA) proteins, suggesting a mechanism of alleviated drought stress tolerance in maize plants (SkZ et al. 2018). The transcriptome study of the symbiotic relationship of metal-resistant *Sinorhizobium meliloti* with *Medicago lupulina* revealed the upregulation of the metal transporter, thus aiding in bioremediation of Cu and Zn contaminated soils (Lu et al. 2017). The *Bacillus velezensis* act as a biocontrol agent for *Fusarium oxysporum,* causing *Fusarium* wilt in watermelon. The transcriptome experiments demonstrated the modulation of genes involved in the mitogen-activated protein kinase (MAPK) signalling pathway and phytohormone signalling, which indicate the role of *B. velezensis* in enhanced plant disease resistance (Li et al.

2019). Hence, it could be recommended that PGPRs induce the stress-responsive pathway in plants under abiotic and abiotic stresses, thereby reduces the generation of stress-inducing molecules.

27.5 Success Stories Entailing the Effect of PGPR Inoculation in Crop Productivity Enhancement

There are enormous reports on the beneficial effects either directly or indirectly on plant health by interacting with PGPR strains under various biotic and abiotic stresses. Various PGPRs have supported most cereals, pulses, vegetables, fruits, medicinal and many other crops to stand under stress and maintain or improve their productivity. The research outcomes of PGPR's interaction with various crops enhanced the popularity of microbial stimulants (Thakore 2006; Berg 2009). Several PGPRs advised for specific crops are available in various formulations commercially (Lucy et al. 2004). The effect of different PGPR strains on various groups of crops for alleviation of biotic and abiotic stresses is summarized in Table 27.1.

27.5.1 Cereals

Cereals are the primary food source for the population of the whole world. Reduction in the production of cereal crops is threatening for food security and required sustainable solution without damaging environmental factors. PGPR interaction with different cereal crops has positively impacted the alleviation of drought, salinity, heavy metal toxicity, and biotic stress (Table 27.1). In maize, drought tolerance with improved plant growth and nutrient uptake was successfully achieved by inoculation with *Cupriavidus necator* and *Pseudomonas fluorescens* PGPR strains (Pereira et al. 2020). In contrast, Kuan et al. (2016) observed higher nitrogen fixation, resulting in enhanced plant growth with interactions of PGPR strains such as *Klebsiella*, *Acinetobacter,* and *Bacillus*. Rice is a vital cereal staple crop feeding a large world population. This crop is also affected by several diseases and abiotic factors like drought, salinity, and heavy metal toxicity. PGPR strain *Klebsiella pneumonia* helped the rice plants under cadmium toxicity by improving the biochemical status and antioxidant enzyme activity (Pramanik et al. 2017). Similarly, wheat growth also improved under salinity (Singh and Jha 2016), drought (Gontia-Mishra et al. 2016a), drought and salinity tolerance (Gontia-Mishra et al. 2017a), and mercury toxicity (Gontia-Mishra et al. 2016b) using different PGPR strains. PGPRs are also helpful for oat production in soil contaminated by petroleum (Liu et al. 2015) and containing excess salts (Sapre et al. 2018). The above reports emphasized the modulation of biochemical and physiological parameters and significant improvement in plant growth and yield encouraged by PGPR strains. These results also suggested the reduced application of chemical fertilizers for crops.

Table 27.1 The PGPR strains and their impact on biotic and abiotic stresses in different crop plants

PGPR strains	Crop	Effect on plant	Reference
Cereal crops			
Cupriavidus necator, and *Pseudomonas fluorescens*	Maize	Improved plant growth as well as nutrient uptake efficiency under drought	Pereira et al. (2020)
Pseudomonas aeruginosa, P. fluorescens, and *Bacillus subtilis*	Rice	All the PGPR strains showed significant plant growth compared to un-inoculated plants	Karnwal (2017)
Klebsiella sp., *Klebsiella pneumonia*, *Acinetobacter* sp., and *Bacillus pumilus*	Maize	Study demonstrated higher N content in maize plants and suggested reduced requirement of N fertilizers	Kuan et al. (2016)
Paraburkholderia tropica and *Herbaspirillum frisingense*	Sorghum	Enhanced root and shoot biomass under phosphate deficient condition	Kuramae et al. (2020)
Klebsiella pneumonia	Rice	Positive impact on antioxidant enzyme activity and biochemical parameters of plant under cadmium stress	Pramanik et al. (2017)
Enterobacter sp.	Wheat	Improved plant growth and biochemical status of plant under salinity	Singh and Jha (2016)
Pseudomonas aeruginosa and *Serratia marcescens*	Oat	Plant growth in petroleum-contaminated soil	Liu et al. (2015)
Pseudomonas aeruginosa, Ralstonia pickettii, and *Klebsiella pneumoniae*	Rice	ZSB enhanced and promoted the growth of rice seedlings	Gontia-Mishra et al. (2017b)
Klebsiella sp., *Enterobacter ludwigi,* and *Flavobacterium* sp.	Wheat	Drought tolerance was enhanced by improving physiological and biochemical status	Gontia-Mishra et al. (2016a)
Citrobacter sp. and *Empedobacter cloacae*	Wheat	Plant growth increased under drought and salinity	Gontia-Mishra et al. (2017a)
Enterobacter cloacae, Enterobacter ludwigii, and *Klebsiella pneumoniae*	Wheat	Mercury toxicity was alleviated using PGPR strains	Gontia-Mishra et al. (2016b)
Klebsiella sp.	Oat	PGPR stimulated salt tolerance in seedling stage	Sapre et al. (2018)
Pulses crops			
Pseudomonas putida, *Pseudomonas fluorescens,* and *Serratia ficaria*	Lentil	Specificity between lentil and PGPR strain was revealed under salinity to improve plant growth	Muscolo et al. (2019)
Pseudomonas plecoglossicida, Brevibacterium antiquum,	Chickpea and pigeonpea	Several minerals were found enhanced in grains of inoculated samples	Gopalakrishnan et al. (2016)

(continued)

Table 27.1 (continued)

PGPR strains	Crop	Effect on plant	Reference
Bacillus altitudinis, Enterobacter ludwigii, E. ludwigii, Acinetobacter tandoii, and *Pseudomonas monteilii*			
Ochrobactrum ciceri and *Mesorhizobium cicero*	Chickpea	Higher nodules, yield, and harvest index were observed	Imran et al. (2015)
Enterobacter cloacae and *Bacillus drentensis*	Mung bean	PGPR strains along with silicon improved physiology, growth, and yield	Mahmood et al. (2016)
Bradyrhizobium	Pigeonpea	PGPR strains with biochar as carrier proved to improve performance of pigenpea	Araujo et al. (2020)
Pseudomonas aeruginosa and *Bacillus subtilis*	Mung bean	Both strains enhanced the growth parameters of mung bean	Kumari et al. (2018)
Cedecea davisae	Chickpea	Root growth and nitrogenise activity was improved	Mazumdar et al. (2020)
Rhizobium leguminosarum bv *viciae* And *Pseudomonas fluorescens*	Lentil	PGPR strain along with *Rhizobium* exhibited enhanced nodulation and grain yield	Singh et al. (2018)
Rhizobium leguminosarum bv. Viciae	Pea	ACC deaminase containing strain has improved drought and cadmium toxicity tolerance.	Belimov et al. (2019)
Variovorax paradoxus	Pea	Plant growth and photosynthetic activity was improved	Wang et al. (2016)
Oil crops			
Pseudomonas putida, Burkholderia cepacia, Burkholderia sp.	Mustard	Yield of mustard enhanced	Dutta et al. (2017)
Bradyrhizobium sp. and *Azospirillum brasilense*	Peanut	PGPR interaction modified exudates and improved drought	Adriana et al. (2019)
Bacillus licheniformis and *Pseudomonas plecoglossicida*	Sunflower	Biofilm production by PGPR strains stimulated salinity tolerance	Yasmeen et al. (2020)
Pseudomonas fluorescens	Mustard	PGPR suppressed mustard blight in pot and field condition.	Gupta et al. (2020)

(continued)

Table 27.1 (continued)

PGPR strains	Crop	Effect on plant	Reference
Bacillus megatorium and *Bacillus subtilis*	Safflower	Improved seed and oil yield in PGPR inoculated plants	Ekin (2020)
Bradyrhizobium japonicum and *Pseudomonas putida*	Soybean	Changes in root architecture by PGPR interaction improved nitrogen and phosphorus uptake under salt stress	Egamberdieva et al. (2017)
Bradyrhizobium japonicum and *Stenotrophomonas rhizophila*	Soybean	Plant growth and nutrient abortion enhanced under salinity in hydroponic condition	Egamberdieva et al. (2016)
Azotobacter sp. and *Azospirillum* sp.	Sesame	Improvement in yield and their components and oil quality occurred with PGPR interactions	Shakeri et al. (2016)
Azotobacter vinelandii and *Azospirillum brasilense*	Safflower	PGPR strains exhibited better growth in reduced NP fertilizers and improved oil quality traits required for green fuel production	Nosheen et al. (2018)
Stenotrophomonas maltophilia	Peanut	Under N_2 starving condition, plants with PGPR showed improved growth and stress tolerance capacity.	Alexander et al. (2019)
Acinetobacter pittii	Canola	PGPR strain together with humic acid showed significant yield enhancement	Ahmad et al. (2016)
Horticultural plants			
Bacillus amyloliquefaciens and *Bacillus pumilus*	Tomato	PGPR interaction improved plant growth and nutritional uptake by tomato in calcareous soils	Fan et al. (2017)
Bacillus amyloliquefaciens	Chilli	PGPR interaction improved plant growth along with reducing anthracnose disease	Gowtham et al. (2018)
Trichoderma spp. and *Bacillus subtilis*	Muskmelon	Biological control of root was exhibited in laboratory conditions	Anjum et al. (2019)
Pseudomonas stutzeri, Bacillus subtilis, Stenotrophomonas maltophilia, and *Bacillus amyloliquefaciens*	Cucumber	PGPR interaction added multiple beneficial traits in plants along with improved resistance towards *Phytophthora* crown rot disease	Islam et al. (2016)

(continued)

Table 27.1 (continued)

PGPR strains	Crop	Effect on plant	Reference
Pseudomonas fluorescens and *Azospirillum brasilense*	Tomato	Enhanced yield parameters as well as fruit quality was observed under field condition	Perez-Rodriguez et al. (2020)
Kocuria sp., *Alcaligenes* sp., and *Pseudomonas* sp.	Strawberry	PGPR strains ameliorated effect of salinity and lime in soil	Arikan et al. (2020)
Paenibacillus polymyxa and *Sinomonas atrocyanea*	Watermelon	PGPR strain effective in management of bacterial fruit blotch	Adhikari et al. (2017)
Paenibacillus polymyxa	Watermelon	PGPR enhanced growth by reshaping root proteins	Yaoyao et al. (2017)
Brevundimonas spp.	Potato	PGPR strain colonize in root and enhance plant growth	Naqqash et al. (2020)
Bacillus subtilis	Potato	Potato plants showed improved tolerance and reduced oxidative stress and antioxidant enzymes activities	Batool et al. (2020)
Bacillus megatorium and *Bacillus subtilis*	Potato	Productivity of potato has been enhanced using PGPR along with humic acid	Ekin 2019
Bacillus methylotrophicus, Bacillus amyloliquefaciens subsp. plantarum, Bacillus cereus, Pseudomonas brassicacearum subsp. Brassicacearum, Pseudomonas veronii, Paenibacillus polymyxa, and *Ensifer adhaerens*	Watermelon	Resistance towards *Fusarium* wilt and growth promotion has been observed	Yang 2019
Medicinal crops			
Pseudomonas aeruginosa and *Bacillus subtilis*	Turmeric	PGPR strains were found promising against rhizome root rot disease	Chenniappan et al. (2019)
Dietzia natronolimnaea	*Ocimum basilicum*	Plant growth enhanced under low fertility soil and salinity	Bharti et al. (2016)
Pseudomonas fluorescens	Turmeric	Plant growth as well as disease resistance were enhanced	Prabhukarthikeyan et al. (2018)
Pseudomonas plecoglossicida, Bacillus flexus, Acinetobacter calcoaceticus, and *Bacillus safensis*	*Bacopa monnieri*	PGPR strains induced higher production of bacoside A yield	Pankaj et al. (2020)

(continued)

Table 27.1 (continued)

PGPR strains	Crop	Effect on plant	Reference
Acinetobacter sp. and *Bacillus sp.*	*Phyllanthus amarus*	PGPR strains improved plant growth and antioxidant activity	Joe et al. (2016)
Bacillus licheniformis	*Withania somnifera*	PGPR strain coupled with AM fungi improved growth and yield and reduced fertilizer requirement	Anuroopa et al. (2017)
Pseudomonas fluorescens	Lemon grass	Higher biomass and yield of lemon grass when PGPR and AM fungi interacted	Kumar et al. (2020)

27.5.2 Pulses

Pulses are an essential protein source for the vegetarian population of the world and important for agriculture due to the natural nitrogen fixation in root nodules by *Rhizobium,* which increases soil fertility. Apart from the *Rhizobium*, various other PGPRs are native to the rhizosphere and internal tissues of roots (root endophytes) and other plant parts. These PGPRs are crucial for direct and indirect plant growth promotion in normal conditions as well as under stressed environment. Various reports have administered different PGPR strains in pulse crops to obtain tolerance against environmental stress conditions. Chickpea and pigeon pea are the main pulse crops of India. The growth of these crops has been improved by various strains of PGPRs reported by several researchers (Imran et al. 2015; Gopalakrishnan et al. 2016; Araujo et al. 2020; Mazumdar et al. 2020). Similar studies in mungbean (Mahmood et al. 2016; Kumari et al. 2018), lentil (Singh et al. 2018; Muscolo et al. 2019) and pea (Wang et al. 2016; Belimov et al. 2019) also revealed the advantage of using PGPR strains for crop production. Some studies also reported silicon (Mahmood et al. 2016) and biochar (Araujo et al. 2020), and PGPR for better crop performance. Application of PGPRs in pulses induced a higher number of nodules and improved nitrogenase activity as well as advanced uptake of nutrients by plants.

27.5.3 Oil Crops

Edible or cooking oil is an important food component used to prepare several food products. The oil is extracted from seeds of various crops like soybean, sunflower, peanut, mustard, etc. A large area of agricultural land is used to produce these crops and fulfil the demand for oil for household and industrial purposes. Numerous PGPR strains are employed to improve the plant growth of mustard (Dutta et al. 2017), oil yield in safflower (Ekin 2020), and oil quality in sesame (Shakeri et al. 2016). Some

reports also suggested the application of PGPRs for alleviation of salinity in soybean (Egamberdieva et al. 2017) and sunflower (Yasmeen et al. 2020), drought in peanut (Adriana et al. 2019) and blight in mustard (Gupta et al. 2020). Nosheen et al. (2018) and Alexander et al. (2019) reported that safflower and peanut exhibited higher plant growth in nitrogen starving conditions and suggested reduced requirement of NP fertilizers to minimize the cost of cultivation and risk environment.

27.5.4 Horticultural Crops

Horticultural crops include various vegetables, fruits, flowers, and other ornamental plants. Vegetables and fruits are the most commonly grown crops by farmers. The increasing demand for these farm products can be fulfilled by improving the productivity of these crops with better nutritional value. Perez-Rodriguez et al. (2020) showed an increased yield of tomato with better fruit quality by using *Pseudomonas fluorescens* and *Azospirillum brasilense* PGPR strains. Similarly, higher nutrient absorption and fruit production in tomato under calcareous soils induced by *Bacillus* spp. (Fan et al. 2017). Using the PGPR strains, potato production has been enhanced under normal conditions (Ekin 2019) and in a stressed environment (Batool et al. 2020). Biocontrol of diseases in various crops was also procured administering PGPR strains. In muskmelon, management of root rot was achieved by *Trichoderma* spp. and *Bacillus subtilis* (Anjum et al. 2019), whereas bacterial fruit blotch of watermelon was controlled using *Paenibacillus polymyxa* and *Sinomonas atrocyanea* (Adhikari et al. 2017). PGPR strains are also helpful in reshaping of root proteins in watermelon (Yaoyao et al. 2017) and tolerance towards salinity and lime of soil in strawberry (Arikan et al. 2020).

27.5.5 Medicinal Crops

Medicinal plants are useful in preparing various formulations to treat of various human and animal diseases (Tripathi et al. 2016). The PGPR strains administered in these crops showed improvement of plant growth, yield, and active component or secondary metabolites in several crops. A higher amount of bacoside A was observed when PGPR strains *Pseudomonas* sp., *Bacillus* spp., and *Acinetobacter* sp. interacted with *Bacopa monnieri* (Pankaj et al. 2020). PGPR strains are also helpful in rhizome rot disease management in turmeric (Chenniappan et al. 2019). PGPR isolate *Dietzia natronolimnaea* improved *Ocimum basilicum* growth in low fertility soil and alleviated salinity (Bharti et al. 2016).

27.5.6 International and National Status in Adoption of Biofertilizers in Agriculture System

After noticing the ill effects and ill practice of used chemical fertilizers in crop plants, several governments, especially in agriculture, are opting and encouraging the use of biofertilizers in the agriculture system. The governments are also spreading awareness among the farmers to adopt this technology. The global bio-fertilizer market is valued at 1–49 billion US dollars in 2019, which is expected to reach 3.28 billion USD by 2027 (www.fortunebusinessinsights.com). The nitrogen-fixing bacteria (bio-fertilizer) are in high demand due to their application in cereals, pulses, and oil crops. *Rhizobium* and *Azospirillum* are the choicest nitrogen-fixing biofertilizers applied in agriculture system. Recently, phosphate solubilizing bacteria (PSB) and other biofertilizers such as cyanobacteria, mycorrhiza, *Trichoderma,* etc. are also being utilized to enhance crop production. Seed treatment and soil application are the two popular methods of biofertilizers applications.

In India, organic farming has become extensive over the past few years increasing the demand for the bio-fertilizer. Biofertilizer production in India has risen from 37,000 tonnes in 2010 to 1,39,000 tonnes in 2016 (Praveen and Singh 2019). Indian and various state governments have promoted biofertilizer and its market at the user-farmer level (Mazid and Khan 2014). The maximum production capacity of biofertilizers is in Tamil Nadu, followed by Madhya Pradesh, Uttar Pradesh, Gujarat, and Maharashtra (Singh et al. 2014). The demand is highest for the nitrogen fixing biofertilizers such as *Rhizobium*, *Azotobacter*, *Azospirillum,* and blue-green algae, followed by PSB and others.

27.5.7 Present Challenges and Future Prospects

Due to global climate change, several environmental stresses have also increased exponentially, affecting crop productivity. In the past decades, the focus of agriculture was to increase crop productivity, which encouraged the extensive application of chemical fertilizers and pesticides. The excessive utilization of hazardous chemicals in agriculture disrupts the ecological balance and decreases soil fertility. Moreover, in recent times, the focus of agriculture is sharply shifted to sustainable agriculture, which allows adequate to minimum use of chemical fertilizers and reassures the utilization of bio-fertilizers to provide the nutrients to the soil. Plants under exposure to abiotic and biotic stress stimuli initially try to adapt, but simultaneous interaction with the PGPR extends their survival rate. The application of PGPR in the agriculture system offers an economically and environmentally sound alternative to chemical fertilizers that could be administered as nutrient suppliers and stress busters including biotic and abiotic stresses. It is well-known that root exudates from plant direct the rhizobacterial population of soil, a recent concept of rhizo-engineering that depends on the partitioning of chemical molecules, thus it is an appropriate option for regulating rhizospheric microbiome and plant-microbe interaction. In the current scenario, substantial efforts have to be concentrated on

PGPR selection with multifaceted plant growth promotion traits and application in field trials to demonstrate their efficiency under environmental conditions. Another feasible strategy is developing effectual consortia of PGPR strains over single inoculation to enhance plant growth. It is also significant to dissect the impact of PGPR-host interaction under non-stressed and stressed conditions on modulation of transcript expression, physiological and metabolic changes to provide an overview of the underlying mechanism of plant-microbe interaction. Furthermore, to decipher regulatory networks involved in response to stress under plant-microbe interaction the latest techniques such as functional metagenomics, transcriptomics, and metabolomics are extensively implied. The shelf life of the biofertilizers is also a critical issue in agriculture application. The most critical challenge faced in application PGPR is its colonization efficiency which is negatively influenced owning to the competition with native microbial communities of the soil. Furthermore, research must be engrossed in rhizosphere biology to gain insights into the molecular pathways responsible for symbiotic interaction to attain the best rhizobacterial communities. Hence it could be concluded that the PGPR have a vast perspective for the advantage of agriculture concerning global food security, climate change resilience, and sustainability of the agriculture system.

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Mushroom Biology and Advances

28

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Abstract

Mushrooms remained as a delicacy in human diet since time immemorial and probably predate any historical account. The therapeutic value of mushrooms has also been recognized by many of the early civilizations of Greeks, Egyptians, Romans, Chinese, and Mexican, etc., and often used them in religious ceremonies. It is now well recognized that mushrooms are not only rich in proteins but also contain vitamins and minerals, whereas lack in fats and have low carbohydrates. Furthermore, they also have high medicinal attributes like immunomodulating, antiviral, antitumor, antioxidants, and hepatoprotective properties. The importance of mushrooms is their ability to secrete an array of extracellular enzymes to convert various agro-waste materials into high-value food and valuable myco-medicinals. Therefore, mushrooms, with their abundant diversity constitute a cost-effective means, both of supplementing the nutrition of humankind and in alleviating the sufferings. The present communication deals with the biotechnology of the cultivated mushroom species with special reference to the uses of mushroom for humankind, its biology and breeding and use of molecular tools and techniques in identification, phylogenetics, and breeding improved varieties of cultivated mushrooms. Molecular markers like RAPDs, RFLPs, AFLPs, SSRs, SCAR, ESTs, microarrays, etc. permit study of any morphological, physiological or developmental process (through profiling and mapping) in which genetic variants exist with a minimum of prior information. Using genomics techniques, all the genes in an organism can be identified and genomes can be sequenced in their entirety. Moreover, using microarray and proteomic techniques activated or deactivated genes during development or in response to an environmental change can be located. Also, the molecular markers

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are important tools for tagging quality traits in a particular mushroom strain or variety, which can subsequently be transferred into the existing variety for the development of an improved strain with the desired quality trait. Electroporation and biolistic approaches have recently been used for the successful delivery of transforming DNA into a mushroom genome. Recently, gene transfer using protoplasts fusion is a non-conventional method used to breakdown the natural barrier of gene exchange encountered in conventional breeding systems. Apart from above-mentioned techniques, marker-assisted selection (MAS), back-crossing strategy, and use of quantitative trait loci (QTLs) have been advocated as innovative approaches for mushroom breeding.

Keywords

Mushrooms · Nutrition · Therapeutic uses · Genetics · Breeding · Global status

28.1 Introduction

Mushrooms are most amazing and fascinating creation of Mother Nature. Their appearance in nature on various surfaces, climate, time, shape, structure, type, color, etc. makes them unique from all other organisms. The mushroom emergence is linked with various beliefs and notions such as their association with thunderstorm and lightning. Various ancient literatures also mentioned the importance of mushrooms like in Vedas (Wasson and Ingalls 1971). As per fossil record, gilled mushroom appeared around 113–120 million years ago (Heads et al. 2017). Mushrooms are fungi that are enriched in diversity in terms on shape, size, color, etc. However, classic umbrella shape cap called pileus and stem (stipe) and base as volva are found in many mushrooms. The term "mushroom" may have been derived from the French word *mousseron* in reference to moss (mousse). Mushroom is a macrofungus with fleshy spore-bearing fruiting body, may be either epigeous or hypogeus including morels, gilled fungi, boletus, polypore's, jelly fungi, cup fungi, bracket fungi, puff balls, earth stars, thelephores, stink horns, bird's nest fungi, etc. However, based on edibility and usage, mushrooms are also classified as edible, medicinal mushroom, and poisonous. If we see the current trade of mushroom, it is around 63 billion US dollars and accounts for 40 mt of global production. Its cultivation is being practiced in more than 100 countries and its production is increasing at an annual rate of 6–7% (Kumar et al. 2013; Singh 2011). Compared to other outdoor horticulture crops, it is the crop with high cropping intensity with superior profits. In India, this crop has gained momentum in the last three decades. The journey of mushroom cultivation and the related research in India started during mid-60s and the recent production in the country has crossed 2.1 lakh tones. The enhancement in production is mainly due to the improved agronomic practices, advancement in breeding lines, integrated pest and disease management, better post-harvest management, etc. Mushrooms are famous saprophytic and utilize variety of wastes such as of dead woods, agriculture waste, decaying, soil, dung,

Fig. 28.1 Various mushrooms (**a**) *Morchella sp.* (**b**) *Panus lecometei* (**c**) *Flammulina velutipes*

humus, leaves, log, etc. It can also grow on gardens, forest, fields, etc. Mushrooms are also parasites or in symbiotic association with living plants like plant roots as mycorrhizas. For example, *Ganoderma spp.* that can grow at the expense of the host plant and may ultimately kill them such as palm trees. On the other hand, microrrhizal mushrooms show a mutualistic association with the plant roots and provide them some nutrients and in return get the supply of sugar from the plants. There are more than 14,000 mushroom species known to exist and 31 genera are regarded as prime edible mushroom (Hawksworth 2001). This chapter is an attempt to review the recent advancement in biology and biotechnology of the cultivated mushroom species with special reference to the use of molecular tools and techniques in identification, phylogenetic, and breeding improved varieties of cultivated mushrooms. Also the most recent cultivation techniques of various mushroom species are included in the chapter as case studies (Fig 28.1). The chapter is divided into the following subheads for better understanding.

1. Human usage and importance of mushroom
2. Reproduction in mushrooms and mushroom breeding
3. Use of molecular tools in mushroom biology

28.2 Human Usage and Importance of Mushroom

Mushrooms from ancient times are used in the human diet and other therapeutic purposes. The early civilizations of Greeks, Chinese, Egyptians, Romans, Mexican, etc., had recognized the value of mushroom as in their religious ceremonies. Greeks combatant considered mushroom as strength food, Romans regarded as "food of Gods," whereas "Elixir of life" words were told by Chinese. In Indian biography, the inebriant mushroom is actually known as "somrus." South Americans worshipped mushroom as divine and called them as "teo-nonacte (Flesh of the Gods)." Mushroom has great importance and attracted the attention of humankind as their rich source of proteins and other supplements where also can convert various agro wastes materials into high-value food and myco-medicinals by secreting extracellular

enzymes as well as other secondary metabolites. For this reason, the mushroom has cost-effective approach where it is supplementing the nutrition of mankind and assuaging the sufferings.

28.2.1 Mushroom as Food

Worldwide fast demographic growth, mushrooms have become attractive due to low-cost protein source as nutraceutical as well as a functional food and becoming more important in our diet. Mushroom has potential nutritional components such as phosphorus, iron, and vitamins, including thiamine, riboflavin, ascorbic acid, ergosterol, and niacin which may cover dietetic requirements related to high protein (presence of all nine essential amino acids), low-fat/energy contents (Barros et al. 2008), and functional food (presence of dietary fiber, beta glucans) (Feeney et al. 2014). Morels, *Agaricus bisporus*, *Lentinula edodes*, *Pleurotus* spp., *Grifola frondosa, Hericium erinaceus*, *Flammulina velutipes,* etc. are the most cultivated mushroom throughout the world (Elena Valverde et al. 2015). In worldwide, mushrooms are healthy foods due to its nutritional properties (proteins, minerals, vitamins, low-fat content, essential amino acids as well as low calories). As compared to other protein-enriched foods like corn, soybeans or beans, the mushroom has many important nutritional properties (Table 28.1).

28.2.2 Mushroom as Therapeutic Use

The major attribute of mushrooms is their therapeutic use, which has been the main focus of researchers around the world. The momentous pharmacological and physiological properties of mushrooms are immunomodulation, antithrombotic, hypercholesterolemia, antidiabetes, antiviral, prevention from hypertension, maintenance of homeostasis and regulation of biorhythm (Kumar 2015), cure and prevention of various diseases, and improvement from life-threatening diseases such as cancer and heart diseases (Rathee et al. 2012). Various examples of mushrooms have been reported as their therapeutic effect in human (Table 28.2).

28.2.3 Mushroom as Cosmetics

Mushroom is used in cosmetics can in two ways, i.e. as cosmeceuticals and as nutricosmetics. Cosmeceuticals are applied on skin or meant to external application, while nutricosmetics are used oral for similar benefits (Wu et al. 2016). Due to the presence of antioxidant, anti-inflammatory, anti-tyrosinase, anti-hyaluronidase, anti-collagenase, anti-elastase, anti-microbial activities make the mushroom a choice in cosmetic industries. The high antioxidant properties of mushroom inhibit reactive oxygen species generation in cells and thus reduce cell damage. The high antioxidant activity also suppresses expression of matrix metalloproteinase 1 (MMP-1) which is

Table 28.1 Different edible mushrooms, their nutritional proximate analysis and uses

Mushroom species	[a]Nutritional proximate analysis ((g 100 g − 1 dry matter), and energy (kcal kg − 1 fresh matter) of unprocessed cultivated mushrooms)	Uses
Agaricus bisporus (Button mushroom, White mushroom)	CP- 27.1, CF- 4.3, A-10.1, CHO-58.5, E-293 (Mattila et al. 2002)	Soups, salads, sausages, pickles, vegetarian dishes, jerky
Pleurotus spp. (Oyster mushroom/Dhingri mushroom/Phoenix mushroom)	CP- 23.9, CF-2.2, A- 7.6, CHO- 66.3, E-434 (Reis et al. 2012)	Soups, Stir fry, sausages, pickles
Lentinula edodes (Shiitake mushroom)	CP- 20.5, CF- 6.3, A-5.3, CHO-67.9, E-747 (Philippoussis et al. 2007)	Soups, salads, dumplings, rissoles, pie
Calocybe indica (Milky white mushroom)	CP- 36.7, CF- 1.3, A- 8.0, CHO- 54.0, E-467	Curries, soups, and stews
Morchella sp. (Sponge mushroom/morels)	CP- 11.5, CF- 2.6, A- 11.3, CHO-74.6, E-349	Sautéed, soup, salads, jerky, dried
Grifola frondosa (Maitake mushroom)	CP- 13.4, CF- 5.6, A- 4.9, CHO- 76.1, E-0 (Cohen et al. 2002); (Cohen et al. 2014)	Soups, sauces, powder, stewed
Agrocybe sp. (Poplar mushroom/chestnut mushroom/Velvet pioppino)	CP- 18.83, CF- 0.20, A- 1.22, CHO-63.3, E-240.08 (Barros et al. 2007)	Muffins, powder, soups, gravies
Macrocybe sp. (Giant mushroom)	CP- 31.04, CF- 1.27, A- 4.53, CHO-52.01, E-336.81 (Das and Saha 2017)	Candies, soups, Sautéed
Hypsizygus tessulatus (beech mushroom)	CP- 19.6, CF-4.1, A-7.8, CHO- 68.5, E-0 (Lee et al. 2008)	Salted cracker; biscuits, powder, soups
Stropharia rugosoannulata (garden giant/burgundy mushroom)	CP- 12.25, CF-2.74, A-2.15, CHO-73.0, E-0 (Ho et al. 2020)	Chips, soups, sausages
Hericium sp. (Monkey's head, lion's mane, and bear's head)	CP- 15.5, CF-5.4, A-7.2, CHO- 71.9, E-0 (Reis et al. 2012)	Fries, powder, salads, vegetarian dishes
Flammulina velutipes (Enoki, velvet shank)	CP- 20.9, CF-8.9, A-6.9, CHO- 64.2, E-454 (Reis et al. 2012)	Soups, boiled and salads
Sparassis (*cauliflower mushroom*)	CP- 13.4, CF-2.0, A-1.8, CHO- 21.5, E— (Kimura 2013)	Beverages, Sautéed, syrup, seasonings
Tuber sp.	CP- 19.1, CF-2.3, A-7.6, CHO- 71.0, E-0 (Yan et al. 2017)	Fermented food product, soups, grilled
Panus/Lentinus strigosus (*Panus rudis fries*)	CP- 18.9, CF-2.45, A-5.1, CHO-58.82, E-286.2 (Sales-Campos et al. 2013)	Pie, tea, soups

[a]*CP* crude protein, *CF* crude fat, *A* ash, *CHO* carbohydrates, *E* energy Kcal

Table 28.2 Therapeutic use of mushroom

Some examples of mushrooms	Activity reported	Active compounds	References
Agrocybe aegerita	Antioxidant, hypoglycemic	β-Glucans, ubiquitin like protein	(Rathee et al. 2012)
Agaricus bisporus	Hypocholesterolemic	Fibers, lectins	(Saglam et al. 2018)
Grifola frondosa	Antioxidant, hypotensive, hypoglycemic	Ergosterol	(K. Kumar 2015)
Ganoderma lucidum	Hypoglycemic, antioxidant and antitumor, antiviral Antiallergic, anti-inflammatory, inhibit the biosynthesis of cholesterol, antioxidative, antihepatotoxic, and free radical scavenging effects.	Glucans, triterpenes (ganoderiol, ganodermanontriol, ganoderic acids, ganosporeric acid A), ganopoly, the polysaccharide-containing preparation, Ganoderan A and B	(Ajith and Janardhanan 2007)
Hericium erinaceus	Antioxidant, ameliorative effect in Alzheimer's dementia	Phenol-analogous compounds, lectins, laccase	(Rathee et al. 2012)
Lentinula edodes	Anti-microbial, antioxidant, Hypocholesterolemic, immunotherapy	Lentinan, oxalic acid, ethanolic mycelial extracts	(Soković et al. 2018)
Pleurotus sp.	Hypocholesterolemic, antioxidant and antitumor	Dietary fibers, methanolic and ethanolic extracts, lectin, laccase	(Soković et al. 2018)
Volvariella volvacea	Antioxidant, Hypocholesterolemic	Exopolysaccharides	(Rathee et al. 2012)

responsible for collagen degradation that result in aged skin effect (Masaki 2010). Various mushrooms such as *Grifola*, *Pleurotus eryngii*, *Flammulina* spp. do possess anti-inflammatory properties for skin. The extract and polysaccharides of these are used for dermatological problems (Choi et al. 2013; Wu et al. 2010). Similarly, anti-tyrosinase activity of mushrooms such as *Pleurotus* sp. (Alam et al. 2012) *Trametes*, *Inonotus* (Yan et al. 2014), etc. have ability to inhibit excessive melanogeneis hence effective for hyperpigmentation disorders of skin.

28.2.4 Mushroom as a Biosremediation Agent

In order to have a sustainable environment, the word remediation applies to the total or partial removal of pollutants from contaminated areas. To remove the pollutants and improve environmental health, various physical and chemical remediation technologies are being developed. Bioremediation is a process in which biological agents such as microbes, plants, or any other living things (Kulshreshtha et al. 2014) and word "mycoremediation" is a process in which sequestration of polluted contaminants by using fungi (mushroom) (Saglam et al. 2018). Mushrooms, a

basidiomycetes class of fungi have ability to secrete potential extracellular enzymes such as laccase, cellulases, lignin peroxidase (LiP), versatile peroxidase (VP), pectinases, manganese peroxidase (MnP), xylanases, phenoloxidases that help to degrade contaminated pollutants. The role of various enzymes in the degradation process has been studied by many scientists; degradation products produced by them and conditions affecting the degradation process (Kulshreshtha et al. 2014), but as safety point of view, various studies show the pros and cons of mushroom cultivation on wastes and further utilization of food because mushroom is food product as well as mycoremediation tool (Barkat Md Gulzar et al. 2020).

Biodegradation, biosorption, and bioconversion are three processes that including the mycoremediation process, where

Biodegradation is an onsite enhancement process by which complex organic molecules in the form of contaminants and hydrocarbons such as nitrotoluenes, pentachlorophenol, PAHs are degraded into its simpler forms by the action of enzymes secreted by mushroom under in vitro conditions (Barkat Md Gulzar et al. 2020). Degradation of polymeric compounds like plastics by using different types of mushrooms has also been documented in recent years (Saglam et al. 2018).

Biosorption is considered to be an alternative to the remediation of industrial effluents and the recovery of metals present in effluent by biomass of live or dried mushroom which often exhibits a marked resistance towards metals and other adverse conditions (Gavrilescu 2004). Mushroom mycelium and spent mushroom compost can be used as biosorbents where adsorption, ion exchange, and covalent binding processes are involved. Biosorption techniques are now becoming very popular for the removal of pollutants due to the high uptake capacity and very cost-effective source of the raw material.

Bisoconversion- Nowadays, there is research on the conversion of industrial or agro-industrial sludge into other useful types. The most significant product of bioconversion is mushroom (Mohd Hanafi et al. 2018). Lignocellulosic waste, i.e. produced from the bioconversion industry can be used for mushroom cultivation. The bioconversion of these wastes into mushroom (protein-rich carpophores) is one of the major applications where it helps in solving pollution problems and on other hand provides mushroom (da Luz et al. 2013).

28.2.5 Mushroom SMS as Manure

Spent mushroom substrate (SMS) is the leftover residue (compost in case of button mushroom and agricultural substrate in case of oyster mushroom) after the termination of the complete mushroom crop, after which mushroom cultivation becomes unremunerative and represents the composted substrate (Mohd Hanafi et al. 2018). SMS is regarded as a nutritionally rich component, which contains a sufficient amount of NPK ratio before and after decomposition. In button fresh SMS manure, N-P-K value is present in the ratio of 1.9–0.4–2.4% and 1.9–0.6–1.0 after decomposition for 8–16 months. However, SMS of button mushroom is nutritionally good as compared to oyster, milky, and paddy straw mushroom (Gobbi et al. 2016) and

recyclable to prepare manure out of it. Button SMS manure can also be used in disease management in crop plants, and reclamation of the abandoned site, whereas SMS from oyster mushrooms can be used to form vermicomposting, biogas production, and feed for animals (Othman et al. 2020). The SMS of a button, oyster, paddy straw, and shiitake mushrooms can be used for bioremediation of chemically polluted sites.

28.2.6 Mushroom as Nanoparticle Producers

Nanotechnology which has emerged as novel science with an enormous application is also remaining untouched by mushrooms (Khandel and Shahi 2018). Mushrooms are known to synthesize nanoparticles. The synthesis is done by both extracellular and intercellular methods (carbon nanoparticles) and inorganic nanoparticles (metal and non-metal NPs) (Kalia and Kaur 2018). These mushrooms offer a huge potential for synthesis of nanoparticles due to the presence of innumerable bioactive compounds such as polysaccharides, proteins to complex terpenes, flavonoids, vitamins, and minerals as well health beneficial effects such as nutraceuticals benefit, immunomodulatory, and immune-stimulating properties (Alam et al. 2012). Mushroom derived nanoparticles exhibit unique features such as high stability, extended shelf-life, water solubility, and good dispersion properties. In recent few years, the focus of nanoparticle research has intensified on exploring the possibilities of NP synthesis by utilizing various genera of edible and medicinal mushrooms (Raudabaugh et al. 2013).

28.2.7 Mushroom as Packaging Material

In recent studies, mushrooms were used as a packaging material by using bio-pulping and bio bleaching industrial processes because mushroom has rich amount of fibers, amino acids, threonine, and tyrosine. The mycelial mushroom-based material can be used for packaging as a sustainable alternative for polystyrene foam. Mushroom helps to create biodegradable, heat/fire resistant, low energy material, and energy-absorbing packaging material, i.e. called myco-bond. This myco-bond is made up from mushroom mycelium and agricultural waste means when mycelium of mushrooms (like fiber) invades any agro-waste (corn or oat husks, wood fiber, cotton hulls, etc.), then mushrooms could digest it and grow into the shape of mold where it utilizes the carbohydrate present in the agro-waste. After a sufficient amount of mycelium growth, heat the material to stop the flowering spores and the fruiting stage is stopped here. No spores or allergens concerns with this overall process. According to its material properties, it can be an excellent substitute for polystyrene and Styrofoam.

28.3 Reproduction in Mushrooms and Mushroom Breeding

Genetic variation is important for any organism to evolve. Reproduction is an efficient means for generating genetic variation. The mushroom life cycle is categorized into two major phases, i.e. vegetative phase and reproductive phase. Vegetative phase includes its mycelium which converts into spore-bearing body in reproductive phase. If the fungus fertilizes within the same haploid fungus mycelium, then it is homothallic while heterothallic requires different haploid compatible mycelium for mating. Heterothallism is governed by genetic factors or mating genes that may be unifactorial by bifactorial. The homothallic fungus spore germinates to give rise a fertile mycelium and forms fruit bodies. The homothalism can be primary homothallic (uninucleate single spore produce fertile mycelium and fruit bodies) and secondary homothallic (binucleate single spore produce fertile mycelium and fruit bodies) for example *A. bisporus*. In heterothallism, a single spore germinates to give rise monokaryotic mycelia and fuse compatible monokaryons to produce dikaryotic mycelia with binucleate cells with clamp connections (Table 28.3).

In mating, if only single mating gene is involved that is called unifactorial mating system or bipolar incompatibility system (gene A) for example *Pholiota nameko*. However, if mating is governed by two unlinked genes (genes A and B), then it is called a bifactorial mating system or tetrapolar incompatibility system, for example, *Pleurotus* sp. (Singh and Kamal 2017). The sexuality mechanism in the Basidiomycetes fungus is controlled by mating-type genes called *MAT-A* and *MAT-B* mechanism of mating-type genes. *MAT-A* gene and *MAT-B* also known as *HD* (homeodomain) and *PR* (pheromone receptor) loci, respectively. The *Hd1* and *Hd2* genes form an HD1 and HD2 protein that form a heterodimeric transcription factor and paves the path for sexual development in mushrooms. On the other hand, *PR* locus helps in the identification of compatibility by a pheromone peptide that binds to the extracellular domain of a pheromone receptor and activating and triggering the PR pathway (Wang et al. 2016). The developmental sequence from mating to sporulation follows the sequence as given in Fig. 28.2.

Table 28.3 Sexual behavior of different mushrooms

Species	Life cycle
Agaricus bisporus	Secondary homothallic, unifactorial
Agaricus bitorquis	Heterothallic, unifactorial
Auricularia auricula	Heterothallic, unifactorial
Flammulina velutipes	Heterothallic, bifactorial
Lentinula edodes	Heterothallic, bifactorial
Pleurotus ostreatus	Heterothallic, bifactorial
Volvariella volvacea	Secondary homothallic, unifactorial

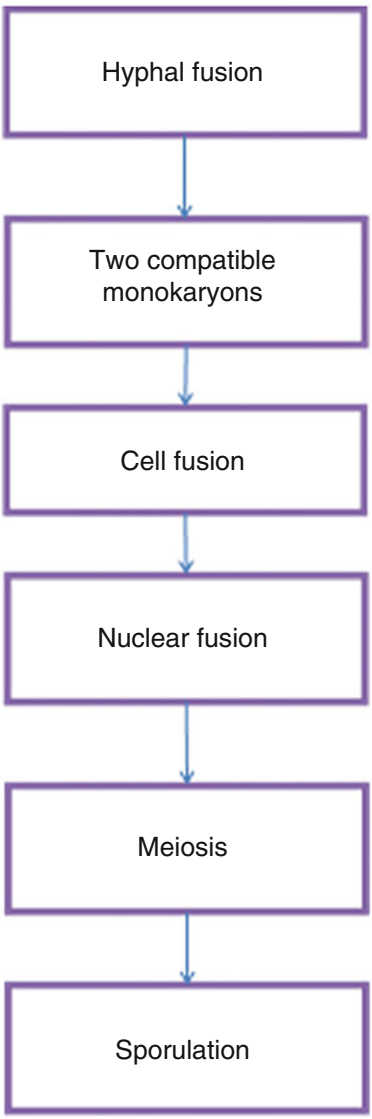

Fig. 28.2 Mating of two monokaryons to sporulation

28.3.1 Mushroom Breeding and Genetic Improvement

Mushrooms genetic improvement is different from plants and animals. The breeding in mushroom depends on the basis of life cycle, genetic variability, and breeding strategies. The genetic makeup of strains is important for obtaining good yield in commercial cultivation. Along with high yield and quality, most of the mushroom strains yield is also dependent on the indoor environment. The high genotype × environment interaction is seen in mushroom strains. When we talk about genetic

improvement across the globe, the first hybrid Horst U1 in *Agaricus bisporus* was developed and commercialized by Darlington in 1981 (Fritsche 1981) followed by its single spore selection U3 that started the genetic improvement for commercial mushrooms.

In India, *A. bisporus* started in 1997 and three strains of *A. bisporus* (NCS-100, NCS-101, and NCH-102) in 1997 was released for commercial use. The various techniques used for the breeding of mushroom are discussed below;

(a) *Introduction*—Introduction is the shortest method of genetic improvement for any crop. Introduction followed by selection is sometimes used for particular geographical regions. In India, from the early 1960s, various exotic strains of *A. bisporus* were introduced after evaluation under different geographical locations, various substrate formulations and growing conditions (Singh and Kamal 2017), for example, Hybrid Horst U1 and U3 line.

(b) *Selection*—The selection is another approach in which promising genotypes are retained while undesirable or off-types are eliminated ones within a strain.
- *Single spore selection*—In *A. bisporus,* secondary homothallism gives an advantage to select self-fertile spore for genetic improvement thus sometimes omitting the need of artificial hybridization.
- *Single spore selection for hybridization*—In heterothallic mushroom species such as *Pleurotus*, selection of parents and single spore isolates (SSIs) is required for getting a promising $F_{1s.}$ Selection of parents can be done based on the useful agronomic traits, while the selection of SSIs is done based on cultural studies, enzyme studies and tolerance studies on various biotic and abiotic stress. In *A. bisporous,* S-11 is an example of selection selected for commercial cultivation.
- *Multispore selection*—The numerous spores collected from the selected fruiting body are germinated in culture plates which ultimately hybridized. The dikaryotic mycelium is selected by natural selection in this process (Barh et al. 2019). Sometimes this selection process may be utilized for the rejuvenation of old and degenerating cultures. However, genetic information to the final mycelia will remain unknown and genetic gain through selection from MSIs will be limited (Fritsche 1981).

(a) *Hybridization or heterosis*—Trait of any crop is genetically improved by phenotypic selection and hybridization. The hybridization is generally done using the dual culture technique (Kumara and Edirimanna 2009). The actively growing mycelia of single spore cultures are kept around 1 cm apart from the center of a petri dish of media like potato dextrose agar/malt extract agar, etc. The fused mycelia showing profuse hyphal growth in point of contact is taken and transferred to new petri-plate and are checked for clamp connections in heterothallic fungi and for phenotypic fruiting in secondary homothallic fungus. Cross breeding in the species having secondary homothallism is comparatively difficult as it needs isolation and identification of non-fertile SSIs (single spore isolates) for mating. The work on hybridization in button mushroom was started in 1976 at the Mushroom Experimental Station in Horst, The Netherlands,

where the crosses between white strains and off-white strains were made. The breeding program in India started in 1984 released the first hybrids NCH 102 in 1997. Similarly in 2014, two browning resistant hybrids of button mushrooms were developed, namely NBS-1 and NBS-5, by traditional breeding (Singh and Kamal 2017).

(b) *Mutation breeding*—Mutation breeding is used to create genetic variation and is being utilized in many crops for quality traits. Mutation breeding creates variation by nucleotide variation (changes in nucleotide sequence), chromosomal variation (change in chromosome structure by breakage of and rejoining), and genomic variation (changes in number of chromosomes by aneuploidy) (Donini and Sonnino 1998). This breeding technique can utilize both physical and chemical mutagens. Mutation breeding in mushrooms can be done on protoplasts, spores or hyphal fragments. However, poor spore germination is major challenge after mutagen treatment. In *Pleurotus,* in most cases, UV mutagenesis is used for mutation breeding. With the advancement in protoplast fusion and its regeneration protocol, mutation breeding has gained a momentum. This approach in mushroom is utilized for trait-specific breeding such as sporelessness trait strain*,* high-temperature tolerant traits strain, development of white strain, etc.

28.3.2 Use of Molecular Tools in Mushroom Biology

With the advancement in mushroom science and biotechnology tools, the researchers can identify, cloning and insert the desirable gene(s) into the genome of the target organism. The development of transgenic and gene editing tools helped in stable integration and constitutive expression of engineered gene(s) in the mushroom. Gene transfer approaches such as electroporation and biolistic approaches are used to produce desirable changes in quality and production of commercial mushrooms. Various other techniques such as marker-assisted selection (MAS), quantitative trait loci (QTLs) mapping, linkage mapping, back-crossing strategy are used for mushroom breeding.

28.3.3 Gene Transfer Approaches

Gene transfer approaches also called transformation can be executed by four different techniques of transformation. These are polyethylene glycol/$CaCl_2$ (Honda et al. 2000), electroporation, practical bombardment (Sunagawa and Magae 2002), and by the use of *Agrobacterium* (Sharma and Kuhad 2010). In 1998, filamentous fungi transformation was done using *Agrobacterium tumefaciens* (De Groot et al. 1998). In 2000, Chen and his research team developed efficient *Agrobacterium*-based transformation that offered higher effective efficiency compared to previous protocols (Chen et al. 2000). Later in 2001, transformation of vegetative mycelium was reported by Mikosch et al. (2001). Pelkmans et al. (2016) studied Cys2His2 zinc

finger protein gene *c2h2* role in mushroom found in *Schizophyllum commune* in *Agaricus bisporus* using *Agrobacterium*-mediated transformation. Expression analysis showed that C2H2 has an effect on mushroom formation timing (Pelkmans et al. 2016). Similarly, in *P. ostreatus*, bialaphos resistance gene from *Streptomyces hygroscopicus* and β-glucuronidase (GUS) from *Escherichia coli* were expressed and co-transformed using *ras* gene promoter and *priA* gene terminator from *Lentinus edodes*(Yana et al. 1996).

28.3.4 Molecular Markers in Mushrooms

Molecular markers in mushroom breeding have helped in many ways. It is also due to the fact mushroom morphology of many mushroom looks quite similar or is look alike. In mushrooms, molecular markers mainly are used in i) species identification; ii) diversity studies; iii) tagging of traits; iv) DNA fingerprinting; v) marker-assisted selection; vi) QTL and linkage mapping; and vii) hybridization studies.

28.3.5 Species Identification

Species identification is one of the common problems in mushroom identification. Less knowledge of mushroom taxonomy makes harder to identify the mushroom precisely. DNA barcoding is the most reliable technique for taxonomical identification as this technique is free from the influence of agro climatic factors. ITS (internal transcribed spacers, ITS1 and ITS2) sequences which are flanked region between the genes of 18S and 28S rRNA, and the 5.8S rRNA gene are the most commonly used in phylogenetic analysis of fungi for interspecific and intraspecific comparisons (Shnyreva and Shnyreva 2015). ITS is also marker of choice due to the high copy number with higher degree of variation (Das and Deb 2015). Various markers of barcoding in mushrooms are used and are described below:

1. *CO1—It is a* "Folmer" region at the 5′ end of the mitochondrial cytochrome c oxidase 1 (*CO*1) gene has been used for barcoding and is officially first animal DNA barcode (Hebert et al. 2003, 2004; Kress and Erickson 2008). The length of DNA barcode region of *CO*1 varies from 642 bp to 1200 bp and length of *CO*1 gene is around 1548 bp to 22 kb. Mitochondrial DNA is frequently used for DNA barcoding as it undergoes little recombination than nuclear DNA. Although, major disadvantage of this barcode is slow evolution rate of *CO*1 sequence which makes it inefficient for species delineation in lower organism (Mcfadden et al. 2011). In fungus *CO*1 was not found successful because of the frequent occurrence of mobile introns in the barcode region (Hamari et al. 2002). Vialle et al. also suggested that the *CO*1 is not suitable for mushrooms barcoding as it contains frequent large intronic regions (Dentinger et al. 2011). In oomycetes fungus, it is reported that no introns are present in *CO*1 gene, therefore, it is used

for barcoding in oomycetes but the result was not appreciating in edible mushrooms like *Agaricus* and *Pleurotus.*

2. *ITS- (*Internal Transcribed Spacer): It is the fragment of DNA present in eukaryotic rRNA coding gene which consists of two transcribed spacer fragment between LSU and SSU including 5.8 s rDNA. Therefore, the *"ITS* region*"* indicated the complete ITS1–5.8S–ITS2 rDNA. These ribosomal RNA genes (rDNA) of the fungus are mainly located on a single chromosome and are present as repeated subunits of a tandem array of transcribed and non-transcribed stretches of DNA, which appeared highly conserved (Wipf et al. 1999). ITS is mostly used in fungi for species level identification *(*Bridge et al. 2005*)*. The ITS barcode length ranges for agaricomycotina from 397 bp to 668 bp with mean hovering around 589 bp. Schoch et al. (2012) reported that ITS marker will be recognized as formal markers for barcoding fungi in future but till now it is a common trend to include more than one unlinked markers to investigate any phylogenetic analysis (Khaund and Joshi 2014; Schoch et al. 2012), (Fig. 28.3).
3. *LSU (Large subunit rDNA)*— This is the 28S large subunit of rDNA. The gene length is around 3.2 kb gene with 1 kb of D1/D2 region. The D1/D2 regions of LSU are used for characterizing yeast species (Scorzetti et al. 2002). LSU is informative marker used in barcoding for identification of species. The discriminating power of LSU is comparatively less than the ITS. LSU, when combined with ITS, is used for DNA barcoding for species. Combinations of both ITS and LSU sequences are also applied in environmental sampling, where tandem amplification can allow simultaneous species identification with ITS and phylogenetic analysis with LSU (Klaubauf et al. 2010), (Fig. 28.3).
4. *SSU (Small subunit rDNA)*—It is 18S smaller subunit nuclear ribosomal gene (SSU) and harbors the homology with 16S region of some bacterial species. The gene size is also large around 1.8 kb but smaller than LSU. In general, the SSU found to have had the lowest species identification success rate as compared to other barcoding systems in fungi (Fig. 28.3), but it is used in the phylogenetic identification and known for species level barcoding of nematodes (Harris and Crandall 2000).
5. *RPB1*—It is the protein coding gene present in the large subunit of RNA polymerase II. RPB1 primers were developed for the Assembling the Fungal Tree of Life (AFToL) project (McLaughlin et al. 2009). These primers possess the potential to perform barcoding for fungus. The largest subunit of this protein RPB1 is also of the candidate of interest because it is the active binding site for toxin α-amanitin, present in *Amanita*, *Galerina,* and other mushroom species (Brandon Matheny et al. 2002).
6. *IGS*—The intergenic spacer (IGS) is also one of the important barcoding regions used for the species discrimination of fungus and cultivated edible mushrooms (Avin et al. 2014). The two noncoding fragments that are IGS1 and IGS2 are suited between the conserved sequences of 25S, 5S, and 18S. IGS1 is normally shorter than IGS2 and can be easily used for discriminating strains of the uncultured species. However, only a limited number of studies have focused on

Fig. 28.3 Diagrammatic representation of various rDNA component used for barcodes

IGS region as a systematic tool for species delimitation of the commonly cultivated edible mushrooms. (Fig. 28.3).

28.3.6 Other Marker Usage

Mushroom genetic diversity is important for allelic diversity with domestication. In many crops, it is seen that diversity caused a reduction in the genetic diversity in genotypes, which narrowed down the genetic base against biotic and abiotic stresses. It is also an important consideration for strain development. Many diversity studies are conducted on various mushrooms with the specific objectives. Markers are also used in identification of mushroom strains and studies of DNA fingerprinting. In various inheritance studies, RFLP markers are used. Genome mapping by molecular markers, a synthesis of concepts from classical genetics with tools from molecular biology, is an exciting new research area in life science. Many of the concepts associated with genome mapping are more than a century old; however, recent technical advances have afforded description of the structure and function of living organisms in unprecedented details. Molecular markers permit study of any morphological, physiological or developmental process (through profiling and mapping) in which genetic variants exist with a minimum of prior information. Molecular tools enable the biologists to establish specific DNA markers at defined places along each chromosome. DNA markers can then be used to delineate when one has reached or passed by a particular gene of interest. There are different techniques to be used as DNA markers for studying DNA at different levels of magnification.

Molecular markers are heritable differences in nucleotide sequences of DNA from two individuals, which follow a simple Mendelian pattern of inheritance. The application of molecular markers in mushroom breeding has hastened the development of genetically improved strains and hybrid. The markers used in mushrooms are Allozyme markers, Restriction Fragment Length Polymorphism (RFLP), Randomly Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Inter Simple Sequence Repeats (ISSR), Simple Sequence Repeats (SSR), Sequence related amplified polymorphism (SRAP), Sequence characterized amplified region (SCAR), Expressed sequence tag- simple sequence repeats (EST-SSR), and single nucleotide polymorphism (SNP), etc. Various markers used in mushroom for various purposes are provided in Table 28.4.

Table 28.4 Molecular marker studies in mushrooms

Sl. No.	Marker	Purpose	Mushroom	References
1.	RFLP	• Identification of homokaryons • Confirmation of hybrids	*A.bisporus*	(Castle et al. 1988)
2.	mtDNA-RFLPs	• Inheritance studies	*A.bitorquis*	(Hintz et al. 1988)
3.	RFLPs	• Inheritance studies	*A.brunnescens*	(Summerbell et al. 1989)
4.	RFLPs	• Genetic relations between species	*A. brunnescens A. bitorquis*, *A. campestris*	
5.	RAPD	• Genetic relationship between strains	*P.eryngii*	(Ro et al. 2007)
6.	RAPD	• Genetic discrimination of strains	*A.bisporus*	(Moore et al. 2001)
7.	RAPD	• Genetic discrimination of strains	*A.bisporus*	(Khush et al. 1992)
8.	RAPD	• Genetic differentiation between two species	*Auricularia auricula* *A. polytricha*	(Yan et al. 2004)
9.	RAPD	• Genetic diversity studies	*Pleurotus eyngii*	(Ravash et al. 2009)
10.	AFLP	• Genetic diversity studies	*Lentinula edodes*	(Terashima et al. 2002a)
11.	AFLP	• DNA fingerprinting	*Pleurotus eryngii*	(Urbanelli et al. 2007)
12.	AFLP	• Linkage map development for sporeless trait	*Pleurotus pulmonarius*	(Okuda et al. 2009)
13.	AFLP	• Genetic diversity studies	*Pleurotus sp.*	(Otieno et al. 2015)
14.	AFLP	• Linkage map development	*Lentinula edodes*	(Terashima et al. 2002b)
15.	ISSR	• Genetic diversity studies	*Auricularia polytricha.*	(Du et al. 2011)
16.	ISSR	• Genetic diversity studies	*Auricularia auricula*	(Tang et al. 2010)
17.	ISSR	• Identification of homokaryons	*A.bisporus*	(Nazrul and Yin-Bing 2010)
18.	ISSR	• Genetic diversity studies	*Lentinula edodes*	(J. Liu et al. 2015)
19.	ISSR	• Genetic diversity studies	*Pleurotus pulmonarius*	(Yin et al. 2014)
20.	SSR	• Marker-assisted breeding	*F. velutipes*	(Lu et al. 2015)
21.	SSR	• Strain identification	*Auricularia auricula-judae*	(Zhang et al. 2012)

(continued)

Table 28.4 (continued)

Sl. No.	Marker	Purpose	Mushroom	References
22.	SSR	• Genetic diversity studies	*A.bisporus*	(An et al. 2019)
23.	SSR	• Genetic diversity studies	*F. velutipes*	Liu et al. 2018)
24.	SSR	• Genetic diversity studies	*F. velutipes*	(Wang et al. 2018)
25.	SCAR	• Strain specific markers for identification	*F. velutipes*	(Su et al. 2008b)
26.	SCAR	• Strain specific markers for identification	*Ganoderma lucidum*	(Su et al. 2008a, b)
27.	SCAR	• Strain specific markers for identification	*Hypsizygus marmoreus*	(Lee et al. 2012)
28.	SCAR	• Strain specific markers for identification of β-glucan in strains	*Pleurotus eryngii*	(Kim et al. 2015)
29.	EST-SSR	• To understand molecular mechanism of cold-induced fruiting in *F. velutipes*	*F. velutipes*	(Wu et al. 2018)
30.	EST-SSR	• Genetic diversity and population structure studies	*Leucocalocybe mongolica*	(Lu et al. 2018)
31.	EST-SSR	• For outcrossing studies	*Pleurotus tuoliensis*	(Dai et al. 2017)
32.	SRAP	• Genetic diversity studies	*Auricularia auricula*	(Tang et al. 2010)
33.	SRAP	• Genetic diversity studies	*Lentinula edodes*	(Liu et al. 2015)

28.3.7 Gene Editing Tools

Genome editing and gene editing tools are recent revolution and have enormous applications. With advent of Clustered Regularly Interspaced Short Palindromic Repeats - associated protein 9 (CRISPER-cas9) systems, various studies were conducted to know the gene function in mushroom and understand the biological mechanism in mushrooms. This is one of the most famous genome editing tools. The Cas9 induces double stranded breaks by guide RNA (gRNA), that will activate HR and/or NHEJ for repairing of breaks and assist further genome editing. The CRISPR-Cas9 system has recently been successfully applied to many filamentous fungi (Qin et al. 2017). In *Flammulina velutipes,* two histidine kinase genes *HK1* and *HK2* were edited using CRISPR/Cas9 system to study the fruiting body development in *F. velutipes* (Ouyang et al. 2018). Similarly in button mushroom, browning resistance strains were developed by cleaving one gene, out of six that encodes polyphenol oxidase (PPO) reducing the enzyme activity by 30% (Waltz 2016).

28.3.8 World Mushroom Production Scenario

Mushrooms have been collected and consumed in different parts of the world, but their cultivation at commercial scale started only after World War II. Few mushrooms like *A. bisporus*, *L. edodes*, *Auricularia* spp., *Pleurotus* spp., *F. velutipes*, *V. volvacea* contribute about 90% of the world mushroom production. Even though man started growing crops more than 10,000 years ago, the cultivation of mushrooms is a relatively new phenomenon. There are reports of success in cultivation of mushrooms like *Auricularia* (600 AD), *Flammulina* (800–900 AD), *Lentinula* (1000–1100 AD), and *V.volvacea* (1700 AD) in China, *Agaricus bisporus* in France in 1650, and *Pleurotus* in 1900 in the USA. All these six species contribute maximum to world mushroom production today.

According to FAO, mushroom production has increased from 0.5 million ton in 1960 to 10.38 MT by 2014. According to other agencies, the mushroom production has increased from 0.17 MT to 34.8 MT in 2013. FAOstat does not cover all the mushrooms cultivated in Asia and has been exemplified by comparing data of FAO and MAFF, Japan where FAOstat includes data of only fresh shiitake production, which at present is only about 14% percent of total mushroom production of Japan. Despite rapid overall increase in mushroom production, the relative contribution of button mushroom has been declining mainly due to rapid increase in production of wood rot fungi like shiitake, *Auricularia* and *Pleurotus* species. Considering growth curve to be sigmoid and relatively less increase in coming years, the current estimated mushroom production should be around 40 MT. It can be seen that there is about 200 times increase in 58 years over 1960 value and about 40 times increase in last 40 years. Production was 0.17 MT in 1960 and it took 18 years for the production to reach 1 MT. It touched 5 MT in 1994, 10 MT in 2000, 20 MT in 2006 and crossed 30 MT in 2012. China is the largest producer of world mushrooms. Presently, the mushroom production for 2014 in China is reported as 34.67 MT.

Shiitake has been an important mushroom and its production has been increasing steadily and at present is the number one contributor. The other four species (*Pleurotus* spp., *Auricularia* spp., *F. velutipes,* and *V. volvacea*) contributed less in the initial years, but there has been sudden jump in their production in this century. *Pleurotus* includes many species and there have been changes in contribution of different species of *Pleurotus*. The major contributor in this growth has been by China that at present accounts for more than 87% of the world mushroom production. In 2015, *L. edodes*, *Auricularia* spp., *Pleurotus* spp., *A. bisporus,* and *F. velutipes* contributed 22, 18, 17, 10, and 7.5%, respectively, to the total world mushroom production (Fig. 28.4).

28.3.9 Indian Mushroom Production Scenario

Despite diverse agro-climate, abundant agro wastes, low-cost labor force, and a rich biodiversity, India has witnessed a lukewarm response in its growth in mushroom production till year 2000. At present, the total mushroom production in India is

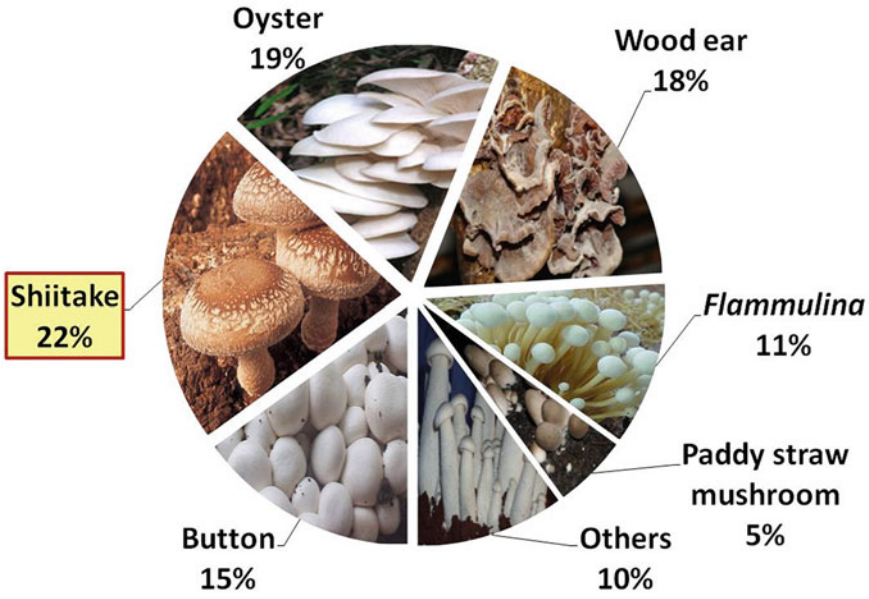

Fig. 28.4 Contribution of different mushrooms in total world production

approximately 0.21 million tons. From 2010 to 2018, the mushroom industry in India has registered an average growth rate of 4.3% per annum. In India, white button mushroom share is about 73% followed by oyster mushroom (16%), paddy straw mushroom (7%), and milky mushroom (3%) during 2018. Per capita consumption of mushrooms in India is also meager (<100 grams per year). Mushroom industry in India has focused on white button mushroom, which is a highly sophisticated and capital-intensive activity.

28.3.10 The Relative Contribution of Different Mushroom Species

In India, there are five mushroom species, viz., white button mushroom (*A. bisporus*), oyster (*Pleurotus* spp.), paddy straw (*V. volvacea*), milky (*C. indica*), and shiitake (*L. edodes*), which are in commercial cultivation. Even though, cultivation technologies of many exotic were standardized, the commercial markets are still dominated by *A. bisporus*, *Pleurotus* spp., and *V. volvacea*. These three mushrooms are contributing about 96% of total mushroom produced in India (Fig. 28.5). Milky mushroom (*C. indica*) is an indigenous mushroom, but its commercial cultivation is restricted to south Indian states only and contributing up to 3% to the total mushroom production. Production of paddy straw mushroom became more popular in the states of Odisha and Chhattisgarh and its production was registered at 7% to the total mushroom production. Cultivation of shiitake has so far not been exploited at commercial scale in India. Few of the growers in Uttharakhand

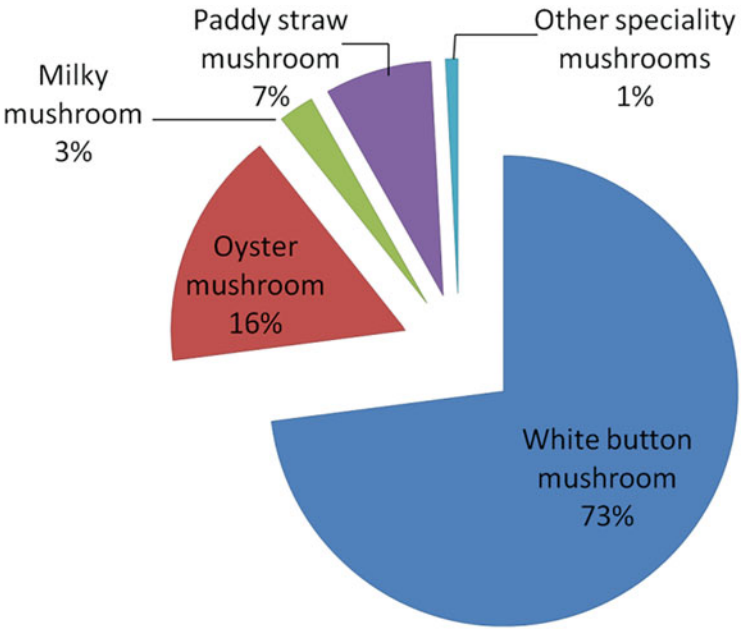

Fig. 28.5 Relative contribution of different mushroom species in total production

and Himachal Pradesh successfully cultivated the shiitake mushroom. The markets are still dominating by the dried mushrooms imported from China and Taiwan. In North-Eastern states, Uttharakhand and Chhattisgarh states, oyster mushroom cultivation is emerging as one of the leading cottage industry.

28.3.11 Growth of Mushroom Production in India

There has been significant increase in the production of mushrooms in the last few years, especially of the oyster and paddy straw mushrooms in India. The country's production in 2010 was 1.00 lakh metric tons, of which button mushroom accounted for 89% of the total production, followed by oyster (6%), milky (1%), and others (4%). Punjab, Uttrakhand, Haryana, Uttar Pradesh, and Tamil Nadu states were the leading producers of the mushroom in the country at the time of 2010. The present production status revealed that Maharashtra and Odisha are emerging as the leading states in mushroom production. By considering the present production data, mushroom industry in India recorded an average annual growth rate of 4.3%. During this period, the productivity has raised from 20% to 24.5% by the releasing of improved strains in commercial edible mushroom (Fig. 28.6).

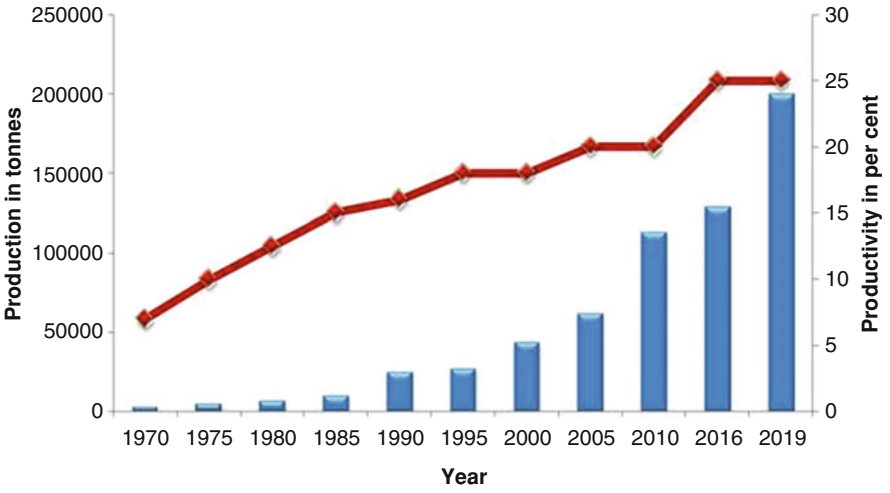

Fig. 28.6 Production and productivity of mushrooms in India

28.3.12 Contribution of Different States in Mushroom Production

There are two main types of mushroom growers in India, those who are growing white button mushroom round the year under controlled conditions and seasonal growers who are growing button mushrooms during the winter season. Seasonal button mushroom cultivation is popular in north and western part of India. Farmers cultivate oyster, paddy straw, and milky mushroom seasonally in low-cost structures. Oyster is popular in Bihar, Chhattisgarh, Uttarakhand, and North-Eastern states. Paddy straw mushroom is popular in Orissa and Chhattisgarh, whereas milky mushroom is popular in Tamil Nadu. The state wise production data of mushroom in India is shown in Fig. 28.7.

28.4 Conclusion

The problem of profound malnutrition in the underdeveloped agriculture countries is increasing day by day, where the majority of the population is occupied in food production and in these countries, demand and supply of protein is broadening further because other protein product sources has not kept step with the population growth. So our next pace is to substantiate the suffering people from malnutrition using protein-enriched food source. In many developing countries especially in India, deficient intake of good quality protein is the major dietary defect, so mushroom is one of the rich sources of protein and high yielded productivity in very less amount of time, where with the help of worthless agricultural waste, fungal mycelium converted into protein source, i.e. mushroom and nutrient enriched organic compost for field crops. India is blessed with a diverse agro-climate, an

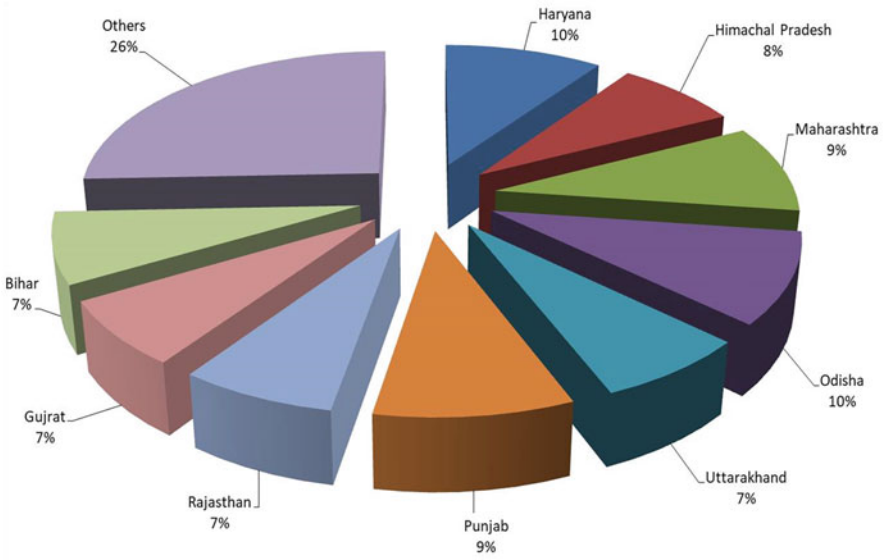

Fig. 28.7 State wise mushroom production in India during 2019–2020

abundance of agricultural waste and manpower that makes it ideal for all types of temperate, tropical and sub-tropical mushrooms to be cultivated. In addition to fruit and vegetable residue, cotton husk, dried leaves, prunnings, coffee husk, rice straw, wheat bran, corn stalks, etc., India is expected to produce 700 million MT of agricultural waste. A large amount of the agricultural waste are burnt and left in the field for composting and incorporation in the soil for fertility. In this process, a large amount of potent source of organic carbon and nutrients are lost, which otherwise could be recycled back to the field as spent mushroom substrate, definitely better compost for field crops.

The issues of unemployment, poverty, and malnutrition in vulnerable parts of society are inextricably connected and are acute. Obviously, these cannot be solved by traditional land based agriculture. The new challenges are the development of opportunities to increase the income of small farmers, landless workers and unemployed young people through the diversification of income generation opportunities through subsidiary jobs. Mushroom production is an indoor activity and land is of little or no importance. It is labor intensive and high profit venture for gainful employment of the poor people. It would also provide good quality protein to bridge the protein gap. Therefore, mushroom cultivation may go a long way in contributing significantly to the solution of the twin problem of poverty and malnutrition in developing countries in general and India in particular.

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Enzymes and Microbes in Agro-Processing 29

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Abstract

Agro-processing is a set of techno-economic activities carried out for preservation and management of agricultural produce to make it usable as food, feed, and other applications. It includes all operations from the stage of harvest till the material reaches the consumers in a desirable form. The major unit operations of agro-processing are cleaning, drying, milling, etc. Agro-processing plays a crucial role in value addition to agricultural produce. The post-harvest shelf life of most of fruits and vegetables are very limited and spoiled before utilization due to their perishable nature. Therefore, these need to be properly processed to improve their quality and safety. Chemical processing affects taste, texture, and nutritional status of an ingredient or food. Nowadays, the enzyme-based processes are preferred to the chemical ones due to their eco-friendly nature, efficient process control, high yield, low refining costs, and process safety. Microorganisms have served the mankind for thousands of years in a variety of ways such as food production and in solving major agricultural and environmental issues. Enzymes play a significant role in different food and fruit processes like peeling of citrus fruits, clarification of juices, reducing processing time, better extraction of fruit components with higher yield, improving the color, flavor, and texture of food, and production of value added products such as fermented foods, beverages, SCP, polysaccharides, etc. Due to their easiness in production and availability, they are mainly used by the food industry to achieve desired organoleptic characteristics of the final product and also help in processing of wastes generated from agro-processing to produce valuable products such as ethanol, etc. Some examples of use of microbial enzymes in agro-processing industries are xylanases and cellulases for the polishing of rice, hemicellulase, cellulase, and protease for

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separation of wheat flour into starch and gluten, pectinases, cellulases, hemicellulases, and amylases in extraction of fruit and vegetable juices, proteases, papain, pepsin, trypsin in seafood processing and phytases, carbohydrases, and proteases in poultry feed processing. In the present chapter, the role of microorganisms and their enzyme in agro-processing will be discussed.

Keywords

Enzymes · Agro-processing · Microbes · Food Industry · Fruit and Vegetable processing

29.1 Introduction

Agro-processing includes all the post-harvest activities involved in the transformation, preservation, and preparation of agricultural produce for intermediate or final consumption. It involves the series of biological, physical, mechanical, and biochemical operations taken to transform agricultural products into a consumer-finish product. It encompasses both traditional and scientific manipulation of agricultural produce at farmers or consumers or industrial level to substantially enhance its shelf life and nutritional value. Agro-processing industries include both agro food processing and agro non-food processing industries. Due to the huge diversity of these industries, various opportunities exist for the application of microbes and enzymes. Microbial enzymes play a major role in these industries because they are more stable than plant and animal enzymes. Microorganisms including bacteria, fungi, and yeasts have served the mankind for thousand years in a variety of ways such as food production and in solving major agricultural and environmental issues. Further, the use of enzymes provides many benefits over traditionally used chemical methods in industries. Traditional chemical methods used are nonspecific and cannot be controlled easily which result in undesirable side effects and generation of large number of toxic wastes. However, enzyme mediated processes are easily controllable as small amount of enzyme brings the desirable change in the raw materials.

Uses of microbial enzymes in agro-processing industries are numerous and increasing rapidly due to greater efficiency, thermal and operational stability, increased product specificity, high turnover, high biodegradability, non-toxicity and eco-friendly characteristics. Enzymes and microbes play a significant role in agro-processing processes like in production of cheese, yogurt, and other milk products, peeling of citrus fruits, clarification of juices, reducing processing time, better extraction of fruit components with higher yield, improving the color, flavor, and texture of food, and production of value added products such as fermented foods, beverages, single cell proteins, polysaccharides, etc. These also help in processing of wastes or agricultural residues generated from agro-processing to produce valuable products such as ethanol, etc. Some examples of use of microbial enzymes in agro-processing industries are xylanases and cellulases for the polishing

of rice, hemicellulase, cellulose, and protease for separation of wheat flour into starch and gluten; and pectinases, cellulases, hemicellulases, and amylases in extraction of fruit and vegetable juices. Cellulases, amylases, and pectinases facilitate maceration, liquefaction, and clarification during fruit juice processing, proteases, papain, pepsin, trypsin in seafood processing and phytases, amylases, and proteases in poultry feed processing. Amylases especially α-amylases are widely used in baking industry as anti-staling agent and flavor enhancer to improve the bread quality.

In agro non-food processing industries, microbial enzymes have found applications in textile and cotton, paper and pulp industries, and leather processing industries. In textile industries, enzymes such as amylases are commercially used for desizing, cellulases and laccases for denim finishing, and proteases are incorporated in detergent formulations. In paper industry, enzymes are used for the reduction of starch viscosity for appropriate coating of the paper. The use of enzymes and microbes in agro-processing industries also reduces energy costs associated with processing.

29.1.1 Agro-Processing

Agro-processing includes set of techno-economic activities which are carried out for conservation and handling of agricultural produce to make it better usable as food, feed, fiber, fuel, or industrial raw material. Hence, the scope of agro-processing involves all operations from the stage of harvest till the material reaches the consumers. It plays a crucial role in value addition to agricultural produce. Since the post-harvest shelf life of many of products is very limited and get rapidly spoiled before utilization, therefore these products need to be properly processed to improve their quality and safety. There are various conventional physiochemical methods used for the processing. Although the chemical treatment processes are effective for huge amount of raw materials, yet they are laborious, expensive, less efficient, and adversely affect the environment/human health.

29.1.2 Physical and Chemical Methods Used in Agro-Processing Industries and their Hazardous Effects

Many of the agro-products (food and non-food) after post-harvest require the processing before their final utilization. Several physical and chemical methods are used in agro-processing industries that date back to the prehistoric ages. Food processing includes many forms of processing from home cooking to complex industrial methods to make edible foods. Food processing can be primary, secondary, and tertiary depending on the type of food required.

In primary food processing, agricultural products such as raw wheat kernels or livestock are converted into edible forms. This category includes various chemicals and physical processes such as drying, threshing, winnowing, milling grain, shelling

Table 29.1 Food processing methods and their effects on human health

Food processing method	Quality change/Health effects
Milling	Rancidity and subsequent off-flavors Loss of vitamins and minerals
Bleaching	Loss of nutrients
Pasteurization	Generation of off-flavors
Canning	Change color, texture, and flavor of foods Loss of vitamins
Addition of excess salt	Increases blood pressure (hypertension), risks of cardiovascular disorders including heart attack, stroke, and kidney failure
Added of excess sugar	Increase the risk for heart disease, obesity, dental cavities, and type 2 diabetes

nuts, butchering animals for meat, deboning, cutting meat, freezing, and smoking of meat. It also includes extraction and filtration of oils, canning and preservation of food through irradiation, homogenization, and pasteurization. Contamination due to chemicals and spoilage in primary food processing can lead to significant public health threats (Table 29.1). Secondary food processing is the everyday process, which involves preparation of foods from ingredients that are ready to use such as cooking methods, baking bread, fermenting fish, making wine, beer, and other alcoholic products. Sausages are a common form of secondary processed meat, formed by grinding of primary processed meat.

Tertiary food processing is the commercial production of ready-to-eat processed food. Processing of food can reduce the nutrient content depending on the food and processing method, e.g., destruction of vitamin C by heating. Therefore, canned fruits possess less vitamin C than their fresh counterparts. Using some food additives (e.g., sweeteners, preservatives, and stabilizers) presents another safety concern. The health risks of any given additive vary greatly from person to person; for example, using sugar as an additive endangers diabetics.

The food processing by chemical and physical methods has some benefits, such as making food last longer and making products more convenient. However, heavily processed foods also have some drawbacks. Highly processed food with more fat, added sugar, and salt can increase the risk of cancer, type 2 diabetes, obesity, high cholesterol, and heart diseases (Table 29.1). Processing of foods often involves nutrient losses, which can make it harder to meet the needs of consumer if these nutrients are not added back through fortification or enrichment. For example, refined grains, which have less fiber, vitamins, and minerals than whole grains increase health risks.

Commercially processed foods sometimes contain the unhealthy trans-fats that increase the risk of consumer for having high cholesterol, heart disease, and stroke. Generally, processed foods are obtained from laboratories with modifications at genetic level. These genetically modified food products may cause gastrointestinal disorders, infertility and can damage organs. Although the preservatives and other food additives used in many processed foods are generally recognized as safe, a few

Table 29.2 Textile processing methods and their adverse effects (Toprak and Anis 2017)

Process	Pollutants	Adverse effects
Desizing	Starch, glucose, polyvinyl alcohol (PVA), resins, fats, waxes	Suppression of hematological system Carcinogenic, mutagenic and affects central nervous system
Scouring	Caustic soda, waxes, soda ash, sodium silicate, and fragments of cloth	Kidney and liver problem
Bleaching	Hypochlorite, chlorine, caustic soda, hydrogen peroxide, acids	Causes lung irritation and also irritation in eyes, prolonged exposure will affect kidney and liver
Mercerizing	Caustic soda	Harmful health hazards
Dyeing	Dye stuff, mordant, and reducing agents like sulfides, acetic acids, and soap	Skin irritation and the respiratory system problems, dermatitis, allergic conjunctivitis, rhinitis, occupational asthma

may cause problems for some individuals, including sulfites, artificial sweeteners, artificial colors and flavors, sodium nitrate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), olestra, caffeine, and monosodium glutamate.

In non-food processing industries like textile and cotton, pulp and paper and leather industries, various chemical methods are used. In textile industry, high amounts of chemicals are used including dye and carrier agents like butyl benzoate, aromatic ester, chlorinated solvents, chlorobenzene derivatives, non-volatile phenolic derivatives, aromatic hydroxyl derivative, phenolic compounds, etc., which are toxic in nature (Shenai 2001; Khandare and Govindwar 2015). The chemical substances used in textiles evaporate into the air, discharged to the environment in the wastewater and also absorbed by human skin by clinging to the fabric. Waste water from the production and processing of textiles contains fat and grease, phosphates, sulfates, sulfides, chromium, copper, acidic and basic compounds, and salts of heavy metals. The increased amounts of these substances in the food chains lead to bioaccumulation and affect human/animal health (Chavan 2001; Toprak and Anis 2017) (Table 29.2).

The processing of leather can be divided into three basic sub-stages such as preparatory, tanning, and crusting (Sivakumar et al. 2010). Preparatory stage includes preservation, soaking, liming, unhairing, fleshing, splitting, reliming, deliming, bating, degreasing, bleaching, pickling, and depickling. Tanning converts the raw hide into a stable material using vegetable and chrome methods (Krishnamoorthy et al. 2012). Crusting involves several steps like wetting back, splitting, shaving, rechroming, neutralization, re-tanning, dyeing, fat liquoring, filling, stuffing, stripping, whitening, fixating, setting, drying, conditioning, milling, staking, and buffing. A wide variety of chemicals are used in order to bring the leather in the usable form during preparation of a variety of products. The negative impact of tannery wastes on environment includes generation of wastewater, hazardous chemicals such as chromium, synthetic tannins, oils, resins, biocides, and

Table 29.3 Toxicity of chemicals used in leather industry (Dixit et al. 2015)

Chemical	Uses	Toxicity/Target organs
Benzyl butyl phthalate	Leather coating	Eyes, lungs, liver, reproductive system, carcinogenic, and teratogenic
N-methyl pyrrolidone	Coalescence, plasticizer, wetting agent	Reproductive toxin
Formaldehyde	Leather finishing, cross linker	Eyes, lungs carcinogen
Chromium	Dyeing	Kidney, central nervous system (CNS)
Organotin compounds (Dibutyl tin)	Catalyst	Gastrointestinal tract, liver, hematopoietic system carcinogen
Azo dyes (Orange II)	Dyeing	Carcinogenic and allergenic blood, liver, testes
Sodium dichromate	Chrome tanning	Carcinogen, blood, kidneys, heart, lungs, eye
Anthracene	Tanning agent	Carcinogen kidney, liver
Methylisothiazolinone	Biocide, microbiological protection	Carcinogen skin, eyes

detergents (Islam et al. 2014). Table 29.3 summarizes the toxicity of chemicals used in leather industry.

In wood and paper industry, the pulp has been processed by mechanical and chemical methods. The production of pulp and paper is an important industrial trade in India. The process mainly involves conversion of fibrous lignocellulosic raw material into pulp and paper. Paper making includes steps like debarking, pulping, bleaching, washing, and sizing (Singh et al. 2019). The working environment of these industries imposes exposure to a variety of hazardous substances depending on the methods used for processing. The main occupational hazards have been asbestos, chlorine compounds, formaldehyde, organic solvents, wood dust, terpenes, latex, clay, carboxymethyl cellulose, silica, and bleachery chemicals in paper and pulp industry. The overexposure of calcium carbonate dust used as filler in paper making process can cause irritation of eyelids, redness of eye, runny nose, sneezing, and coughing. Silica has been known to cause progressive granulomatous and fibrotic disease in the lung. The use of toxic chemicals for pulping and bleaching paper can lead to pollution that causes negative health impacts on paper industry workers. The release of toxic pollutants likes chlorine, lead, mercury, and phosphorous by the industry could cause some genetic damage in workers (Tharshanapriya et al. 2017).

29.1.3 Role of Enzymes and Microbes in Agro-Processing

Microorganisms and enzymes have significantly influenced the agro-processing sector through the production of a variety of products and by improving the conventional agro-processes to produce products with better quality and reduced

cost (Bhalla and Chatanta 2000). Enzymes used in conventional catalytic reactions are either in free or in immobilized forms, which is dependent on the specificity of enzyme. Therefore, specific enzymes are used to perform specialized catalytic reactions in bio-processes.

With the recent advances in biotechnology, enzymes can be designed or engineered according to the requirement of the processes. Various molecular techniques such as protein engineering, biochemical engineering, and metagenomics have been applied to improve the quality and performance of microbial enzymes for their wider applications in many industries. The GRAS (generally recognized as safe) organisms are preferred choice for enzyme production as its application in food sector is considered safe. Thus in agro-processing activities, various microbial enzymes are used for the processing of raw materials into final value added products for consumers. Apart from this, microorganisms also play an important role in management of agriculture residues and wastes generated by agro-processing and convert them either to some value added products or recycle the nutrients in the environment.

29.2 Microorganisms in Management of Agriculture Residues

An enormous amount of agriculture wastes such as paddy and wheat straw, crop stalk, cotton stalk, fruit and vegetable waste, sugarcane waste, etc. are produced every year. Cellulose and lignocellulosic materials are the most abundant agricultural residues in the world. These agriculture wastes, if not managed properly, create major environmental problems. Most of the times these waste products are burned in the field causing the emission of harmful gases that lead to air pollution. Agricultural waste mainly consists of crop residues having high amount of organic carbon content and plant nutrients. Microorganisms play a pivotal role in the management and recycling of agricultural wastes. There are many technologies used for decomposition of these wastes, which mainly include composting, anaerobic decomposition, fermentation, etc. All these processes depend upon the ability of different microorganisms present in the soil.

One of the best and eco-friendly methods is to decompose these agricultural wastes by composting. In this process, microorganisms present in the soil and wastes degrade the solid wastes into compost. Fungi are responsible for decomposing more than 80% of the cellulose. Aerobic bacteria, actinomycetes, and fungal species such as *Aspergillus, Basidiomyces, Penicillium,* and *Trichoderma* play important role in the decomposition of various kinds of agro-waste. The compost generated from agriculture waste offers several benefits such as enhanced soil fertility and soil health, which further improves agricultural productivity and biodiversity of soil.

However, the big challenge is inaccessibility of cellulose to cellulolytic enzymes due to complex association of lignin and cellulose and crystalline nature of native cellulose in lignocellulosic wastes. This may require acidic and thermal pre-treatment before composting. Use of chemicals and high temperature is not favorable due to energy consumption and negative impact of chemicals to

environment. Enzymes such as cellulase could be used to treat the lignocellulose waste, but the cost of industrial enzymes limits their use at large scale. The application of thermophilic cellulolytic microorganisms including fungi and bacteria capable of producing lignocellulolytic enzymes is used that further expedite the process of composting and recycles the lignocellulosic waste with high economic efficiency. Several fungi like *Trichoderma harzianum, Basidiomyces* sp., *Pleurotus ostreatus, Polyporus ostriformis,* and *Phanerochaete chrysosporium* are known to play important role in composting of lignocellulosic materials.

29.3 Microorganisms as Source of Enzymes

Microorganisms are generally preferred as a source of enzymes over plants and animals due to easy availability, consistent production, and cost-effectiveness. Besides, they can be cultured easily in short span of time and their enzyme production can be enhanced by genetic manipulations. Some of the extremophiles are also used in the production of some enzymes that can operate under extreme conditions on commercial scale.

29.4 Applications of Microbial Enzymes in Agro-Processing Industries

From twentieth century onwards, there is a rapid increase in the knowledge and applications of enzymes in various industries including agro-processing sector. Food and beverage, pharmaceuticals, detergent, and biofuel industries are using enzymes in several unit operations while some other such as natural gas conversion, fine chemical production, and many others are on their way to reap the advantages of the applications of enzymes. Use of enzyme particularly in agro-processing sector has the potential to increase productivity, efficiency, and quality output in agro-industrial processes. The broad applications of some of enzymes in various industries are given in Table 29.4.

As the products of agro-industries are both edible and non-edible, the agro-processing can be classified as agro-food processing (or merely food processing industries) and agro non-food processing.

29.5 In Agro-Food Processing

Enzymes have occupied an important place in food processing because of their ability to transform simple raw materials into improved food products. In food processing industries, enzymes are mainly used in baking and brewing processes, fruit juice processing, starch processing, oilseed processing, meat processing, dairy industry, wine industry, and in production of a variety of fermented products

Table 29.4 Applications of some microbial enzymes in various agro-processing industries (Kalia et al. 2001; Abada et al. 2017)

Enzyme	Sources	Industry
Bacterial enzymes		
α-Amylase	*Bacillus licheniformis, B. amyloliquefaciens, B. subtilis*	Starch industry
β-amylase	*Bacillus* sp., *B. coagulans*	Starch industry
Glucose isomerase	*Bacillus* sp.	Fructose syrup
Protease	*B. Amyloliquefaciens, B. licheniformis*	Dairy industry, oilseed processing industry
Pullulanase	*B. acidopulluliticus, Klebsiella* sp.	Starch industry
Hemicellulase	*Aspergillus niger*	Oilseed processing industry
Fungal enzymes		
α-Amylase	*Aspergillus* sp.	Baking industry
Glucoamylase	*Aspergillus* sp.	Starch industry
Catalase	*Aspergillus* sp.	Food industry
Cellulase	*Aspergillus* sp., *Trichoderma* spp.	Fruit juice industry, oilseed processing industry
Glucose oxidase	*Aspergillus* sp.	Food industry
Lipase	*Rhizopus* sp., *Aspergillus* spp., *Penicillium* spp.	Food industry
Pectinase	*Aspergillus* sp.	Fruit juice industry
Yeast enzymes		
Invertase	*Saccharomyces cerevisiae*	Confectionery

(Raveendran et al. 2018, Ozatay 2020). The important applications of enzymes in various agro-food processing industries are discussed below.

29.5.1 Application in Dairy Processing

Dairy industry is an important component of agro-processing sector involving a number of processes that require the use of microorganisms or their enzymes. In dairy industry, microbial enzymes are utilized to produce diverse products like yogurt, curd, cheese, buttermilk, etc. Dairy enzymes are used to enhance the quality of milk products through the development of peculiar aroma, flavor, and color in these products. Proteases and lipases are the main enzymes used in dairy industry. The other enzymes having limited applications in dairy processing include glucose oxidase, catalase, lactase, esterase, aminopeptidase, superoxide dismutase, sulfydryl oxidase, lactoperoxidase, and lysozymes. These enzymes are used for the production of cheese, yogurt, and other milk products. Enzymes are mainly used as coagulant, flavor enhancers, bio-protective agent to improve the shelf life, taste, and safety of the dairy products.

Table 29.5 Applications of some commonly used enzymes in dairy industry

Enzyme	Microorganism	Application
Aminopeptidase	*Lactobacillus* sp.	Faster cheese ripening
Catalase	*Aspergillus niger*	Cheese processing
Esterase	*Lactobacillus* sp.	Flavor generation
Lipase	*Aspergillus niger*	Faster cheese ripening
Lactase	*Aspergillus niger*	Sweetener
Protease	*Aspergilllus oryzae*	Milk coagulation

Proteases have found additional applications in cheese production and in acceleration of cheese ripening. During cheese production, the coagulation of milk into solid curds is mainly done by rennet, which is a mixture of chymosin and pepsin. It is obtained from microorganisms such as *Aspergillus oryzae*, *Rhizomucor miehei*, *R. pusillus*, *Endothia parasitica,* and *Irpex lactis*.

The lipolysis of milk fat, flavor improvement, and faster cheese preparation are mainly done by lipase. Lipases improve the characteristic flavor of cheese by hydrolyzing milk fats to produce free fatty acids. Some examples of lipase-producing microorganisms are *Serratia marcescens, Bacillus subtilis, Pseudomonas aeruginosa,* and *Staphylococcus aureus*. Microbial lactase also called as β-galactosidase catalyzes the hydrolysis of milk sugar lactose into glucose and galactose, and is used to address the problem of lactose intolerance. The sweetness of milk products, solubility as well as digestibility can be improved by lactase. Some examples of microorganisms producing lactase are *Aspergillus niger, Aspergillus oryzae,* and *Kluyveromyces lactis*. List of commonly used enzymes in milk processing is given in Table 29.5.

29.5.2 In Baking Processes

Baking is a process for the production of baked foods, such as bread, cake, pastries, biscuits, cookies, and pies. Wheat flour acts as main substrate for enzymes in baking processes. In bread making, three basic operations are involved, i.e., mixing, fermentation, and baking. Enzymes are usually added during the mixing step of the bread making process. The enzymes most commonly used in bread making are the amylases (mainly starch-converting enzymes belonging to the α-amylase family) extracted from different sources like cereals, fungi, and bacteria. This enzyme is widely used in baking industry as flavor enhancement and anti-staling agent to improve bread quality.

Other enzymes used on a commercial scale in the production of bread, baked goods, crackers, and waffles include proteases, lipases, glucose oxidase, transglutaminase, etc. (Table 29.7). These enzymes can be used to reduce mixing time, decrease dough consistency, assure dough uniformity, regulate gluten strength in bread, control bread texture, and improve flavor. These enzymes directly or indirectly improve the strength of the gluten network and so improve the quality

Table 29.6 Applications of some enzymes in baking processes

Enzyme	Microorganism	Application
Amylase	*Aspergillus* sp., *Bacillus* sp.	Flour adjustment, bread softness
Maltogenic α-amylase	*Bacillus stearothermophilus*	Enhance shelf life of breads
Glucose oxidase	*Aspergillus niger, Penicillium* sp.	Dough strengthening
Lipase	*Aspergillus Niger*	Dough stability and conditioning
Phospholipase	*Fusarium oxysporum*	Dough modification
Transglutaminase	*Streptoverticillium* sp., *Streptomyces* sp.	Laminated dough strength
Protease	*Bacillus* sp.	Reduce gluten allergenicity

Table 29.7 Application of some enzymes in brewing processes (Singh et al. 2016)

Enzyme	Microorganism	Application
α-Amylase	*Bacillus* spp.,, *Aspergillus* spp.	Starch hydrolysis
β-Amylase	*Bacillus* spp., *Streptomyces* spp., *Rhizopus* spp.	Starch hydrolysis
β-Glucanase	*Bacillus subtilis, Aspergillus* spp.	Restrict haze formation
Cellulase	*Aspergillus niger, Trichoderma atroviride*	Fruit liquefaction
Glucose oxidase	*Aspergillus niger*	Oxygen removal from beer
Protease	*Aspergillus niger*	Restrict haze formation
Naringinase	*Aspergillus niger*	Debittering

of the finished bread. Microbial proteases have also been increasingly used to reduce or eliminate the immunogenic effects of gluten by its enzymatic activity, especially in baked goods. Phospholipase from *Fusarium oxysporum* having both phospholipase and lipase activity is marketed by Novozymes A/S (Denmark) for baking application under the name Lipopan F®. Application of some enzymes used in baking processes is given in Table 29.6.

29.5.3 In Brewing Processes

Brewing industry is one of the oldest food processing industries. The raw materials for beer production include cereal (barley malt, rice, or maize), hops, and yeast. Malting process converts the starch in the cereals into fermentable sugar. Hops are basically used as preservative and yeast converts the sugars into alcohol during fermentation. In brewing, enzymes are utilized in four main processes, viz. germination, mashing, fermentation, and clarification. The most commonly used enzymes in brewing are β-glucanase, protease, α-amylase, and β-amylase. Application of some enzymes used in brewing processes is given in Table 29.7. The enzymes used in brewing are needed for saccharification of starch (bacterial and fungal

α-amylases), breakdown of barley β-1,4- and β-1,3- linked glucan (β-glucanase), hydrolysis of protein (neutral protease). Cellulases are also occasionally used, particularly where wheat is used as adjunct and also for liquefaction of fruits if used. In addition, enzymes are used to lower alcohol concentration and calories in beer.

29.5.4 In Wine Making

The most widely used enzymes in wine making include cellulases, pectinases, glucanases, xylanases, and proteases. Cellulases along with other enzymes are used in winemaking to increase the yield and quality of wine. Cellulases are also reported to reduce the viscosity of wort. The main advantages of using these enzymes are improved maceration, better color development, must clarification, wine stability, and overall improvement in wine quality. β-glucosidases and glycosidases contribute in imparting the typical aroma to wine through the modifications of glycosylated precursors. Pectinases help in clarification and processing of wine and hence improve the filterability of wine. In addition, urease and glucose oxidase are used in reduction of ethyl carbamate formation and alcohol levels, respectively.

29.5.5 In Processing of Fruits and Vegetables

A large part of the yearly harvest of fruits and vegetables are processed to produce a variety of daily consumer goods such as fruit juice, wine, sauces, pulps, purees, jams, jellies, canned fruits, and vegetables. Enzymes play an important role in the processing of fruits and vegetables and improving the quality of these products. Most of the fruits and vegetables contain substances such as pectin, cellulose, hemicellulose, starch, tannin, and lignin, which impart cloudiness to the juices. Clarification of juices mainly involves the removal of pectic substances and other polysaccharides as well as carbohydrates.

The enzymes involved in fruit and vegetable processing are pectinases, cellulase, hemicellulase, amylase, tannase, and naringinase. Enzymes such as pectinase, cellulase, hemicellulase, and amylase are employed in processing of fruits and juices to increase juice yield, decrease viscosity, and improve cloud stability in juices. Pectinase breakdown pectin, a polysaccharide found in plant cell walls; cellulase hydrolyzes the cellulose into simple sugars present in fruits cell walls; hemicellulase hydrolyzes the hemicellulose constituents of fruits and vegetables; and amylase involves breakdown of insoluble compounds to make the juice clearer and sweeter. *Aspergillus niger, A. oryzae, Agaricus bisporus, Trichoderma reesei, Cephalosporium sacchari, Bacillus amyloliquefaciens, B. circulans,* and *B. licheniformis* are some of the sources of enzymes used in fruit juice extraction and clarification.

Table 29.8 Different combinations of enzymes used for fruit juice clarification

Fruit Juice	Enzymes	References
Banana juice	0.084% pectinase and 0.02% amylase	Lee et al. (2005)
Mausambi juice	0.0004% pectinases from *Aspergillus niger*	Rai et al. (2004)
Kiwifruit juice	0.025% amylases+0.025% pectinases+0.05% mash enzyme	Vaidya et al. (2009)
Apple juice	Polygalacturonase (45 °C)	Gupta et al. (2003)
White grape juice	0.048% commercial pectinase	Hassan and Krishnaswamy (1992)
Peach juice	Pectinase	Santin et al. (2008)

Some other biomolecules, e.g., tannins impact the organoleptic properties of juices like astringency, brown color, and turbidity of juices. The enzyme tannase also known as tannin acyl hydrolase is an inducible extracellular enzyme produced by microbes. It is basically involved in the hydrolysis of tannins by hydrolyzing the ester bond in tannic acid which results in the release of gallic acid and glucose. Naringin (the main bittering component) present in citrus fruits is hydrolyzed by naringinase and converts it into rhamnose and pruning, thus improves the quality attributes of fruit juices. *Aspergillus niger, A. flavus, Staphylococcus xylosus,* and *Williopsis californica* are the microorganisms reported to produce this enzyme. Enzymatic treatment of fruit juice has many advantages over traditional processing that include improved pulp liquefaction, enhanced clarification by reducing turbidity and viscosity due to hydrolysis of pectic substances, and increased fruit juice yield. Microbial enzymes are also utilized in peeling and segmentation of fruits. Laccases having antioxidant activity help in preservation of flavor and color of fruit juices by removing residual oxygen present in the juice. Some of the enzymes combinations used in fruit juice processing are given below in Table 29.8.

29.5.6 In Sugarcane Processing

Sugarcane is the world's largest crop in terms of quantity of production and contributes largely to energy and fuel production and in chemical synthesis. In sugarcane industry, large amounts of lignocellulosic residues are generated, which includes bagasse, straw, and tops. Ethanol produced from the lignocellulosic biomass of sugarcane provides most suitable alternative for fossil fuels (gasoline) because it is renewable and less carbon intensive. The biological process of conversion of lignocellulosic biomass to ethanol involves pre-treatment for the removal of lignin or hemicellulose to liberate cellulose, depolymerization of carbohydrate polymers to produce free sugars by cellulase action, fermentation of hexose or pentose sugars to produce ethanol, distillation of ethanol. Bioethanol contributes to mitigate climate change by reducing greenhouse gas emissions.

Microorganisms like white rot fungi are effective for biological pre-treatment of lignocellulosic materials. These microorganisms are producing lignin-degrading

enzymes such as peroxidases and laccase. Brown rot fungi mainly attack cellulose, while white and soft rot fungi attack both cellulose and lignin. This pre-treatment is eco-friendly because of its low energy use and mild environmental conditions. The enzymatic hydrolysis of cellulose requires three classes of cellulolytic enzymes: (1) endo-β-1,4-glucanases which attacks regions of low crystallinity in the cellulose fiber and creating free chain ends; (2) cellobiohydrolases or exoglucanase which degrades the molecule further by removing cellobiose units from the free chain ends; and (3) β-glucosidases which hydrolyze cellobiose to produce glucose. Some of the cellulase producing genera of different microorganisms include *Clostridium, Cellulomonas, Bacillus, Thermomonospora, Ruminococcus, Bacteroides, Erwinia, Acetovibrio, Microbispora, Streptomyces, Sclerotium rolfsii, Phanerochaete chrysosporium, Trichoderma, Aspergillus, Schizophyllum, Penicillium,* and *Saccharomyces.*

29.5.7 In Processing of Coarse Cereals

Cereal crops like wheat, rye, barley, oat, rice, millet, sorghum, and corn provide essential nutrients and energy in human diet. In coarse cereal processing, bio-polishing technology helps to protect sensitive nutrients from thermal degradation. The enzymes used for the bio-polishing of cereal grains are cellulases, xylanases, β-glucanases, esterases, etc. It has been observed that the enzymatic modification of bran with xylanases resulted in enhanced soluble dietary fiber content. Cereals grains with β-glucanase combinations result in improved ileal digestibility of the cereal protein as it disrupts the integrity of endospermic cell wall. On the other hand, esterases have the potential for nutrient recovery of coarse cereals apart from its carbohydrate degrading activity.

29.5.8 In Processing of Pulses

In pulses processing, milling is done which involves the removal of the seed coat to produce polished seed (dehulling) and cleavage of the two cotyledons to produce split seeds (splitting). There are various methods used for pre-treatment such as wet method, heating method, use of edible oils, chemicals and enzymes for dehulling of pulses. Use of enzymes for partial hydrolysis of cell walls was explored for the partial hydrolysis of pigeon pea hull. Xylanases, cellulases, and proteases have been used extensively for processing of pulses (Chaudhary et al. 2018). The crude enzyme produced by *Aspergillus fumigates* (NCIM-902) on wheat bran medium was used for pre-treatment. However, the use of enzyme in pre-treatment for easy dehulling of pulses is still limited to laboratory stage because the overall effect of enzyme on the product quality is still to be ascertained.

29.5.9 In Oilseeds Processing

The major oilseeds are produced from groundnut, rapeseed, mustard, castor seed, sesame, linseed, safflower, sunflower, and soybean. There are three most commonly used processes for recovery of oil from oilseeds, i.e., physical grinding, aqueous extraction, and solvent extraction. These processes are combined with enzymatic treatments to increase the productivity, efficiency, and provide quality output.

Lipases and other lipolytic enzymes are used to improve the quality of oils and fats through their modification. These enzymes catalyze three types of reactions, namely, hydrolysis, transesterification of glycerols, and esterification of fatty acids. In hydrolysis, there is cleavage of specific fatty acids, transesterification involves the rearrangement of triglyceride esters of fatty acids, while esterification involves building of di- and tri-acylglycerols from monoglycerols with the addition of certain fatty acids. Phospholipases on the other hand are applicable in the degumming of vegetable oils during processing.

Non-lipolytic enzymes such as proteases, cellulases, and hemicellulases are used which enhance the extraction processes through digestion of cell walls. Proteases are used to hydrolyze proteins in cell membranes as well as inside the cytoplasm for softening of seed coat. Pre-treatment of oil seeds is necessary to break cell walls of seeds to extract lipid reserves stored in it. The enzymes used in pre-treatment of oil seeds are proteases, cellulases, and hemicellulase obtained from *Bacillus licheniformis*, *Trichoderma viride,* and *Aspergillus niger,* respectively.

29.5.10 In Meat Processing

In meat processing industry, microbial proteases and proteases derived from other sources such as papain and bromelain derived from papaya and pineapple plant, respectively, have been used for tenderization of meat. Among protein cross-linking enzymes, transglutaminases (TGase) have been used as texture improvers for several years. Proteases are also used in production of protein hydrolyzates from different by-products of meat such as bones.

29.5.11 In Functional Food Processing

Bioactive peptides are the peptide sequences that provide health functions beyond nutrition when introduced into the human body. Proteases catalyze the hydrolysis of peptide bonds in proteins to give protein hydrolyzates, peptides, or amino acids. Industrial scale production of bioactive peptides is mainly done by microbial proteases from *Bacillus* spp. and lactic acid bacteria. In addition, enzyme invertase isolated from *Saccharomyces cerevisiae* SAA-612 has found application in the synthesis of prebiotic fructo-oligosaccharides, which help in the growth of probiotic microorganisms (Bhalla et al. 2017).

29.6 Agro Non-food Processing

The importance of enzymes in various agro non-food processing industries such as textile and cotton; paper and pulp and leather is described in the following sections.

29.6.1 In Textile and Cotton Processing

In textile industry, there is a vast generation of waste from desizing of fabrics, bleaching chemicals, and dyes that leads to environmental pollution. Thus enzymes are used to develop eco-friendly technologies in fiber processing and improve the final product quality. Enzymes such as amylase, pectinase, peroxidase, cellulase, catalase, ligninase, and laccase have been used in textile industry in different unit of operations such as desizing, scouring, bleaching, dyeing, finishing, denim finishing, and bio-polishing.

The hydrolase group of enzymes including amylase, cellulase, cutinase, protease, pectinase, and lipase/esterase are mainly involved in the processes like cotton softening, antifelting of wool, bio-polishing and bioscouring of fabric, desizing, wool finishing, etc. Catalase, laccase, peroxidase, and ligninase are involved in the processes like bio-bleaching, dye decolorization, bleach termination, wool finishing, etc. Although the enzymes have been used in textile industry since the middle of last century, further advancements in enzyme technology will provide possibilities for the development of new enzyme-based processes for more eco-friendly approach in the textile industry. Some of the enzymes used in textile and cotton processing are given below in Table 29.9.

Table 29.9 Applications of some enzymes in textile and cotton processing (Araujo et al. 2008; Singh et al. 2016)

Enzyme	Microorganism	Application
Amylase	*Bacillus* sp., *B. licheniformis*	Desizing
Catalase	*Aspergillus* sp.	Bleach termination
Cellulase	*Aspergillus niger, Penicillium funiculosum*	Cotton softening, denim finishing
Collagenase	*Clostridium histolyticum*	Wool finishing
Cutinase	*Pseudomonas mendocina, Fusarium solani pisi, Thermomonospora fusca*	Cotton scouring, synthetic fiber modification
Laccase	*Bacillus subtilis*	Non-chlorine bleaching, fabric dyeing
Ligninase	*Trametes versicolor, Phlebia radiata*	Wool finishing
Lipase	*Candida Antarctica*	Denim finishing
Pectate lyase	*Bacillus* sp., *Pseudomonas* sp.	Bioscouring
Protease	*Aspergillus niger, B. subtilis*	Removal of wool fiber scales, degumming of silk

29.6.2 In Processing of Paper and Pulp

In paper and pulp industry, use of microbial enzymes has grown steadily to reduce adverse effects on ecosystem and provides a sustainable solution. The main constituents of paper and pulp (composed of natural polymers) are cellulose, hemicellulose, and lignin that could be potentially modified by natural enzymes (Ghosh et al. 2019). In paper industry, enzymes are mainly used to increase pulp fibrillation, reduce beating time in virgin pulps and in the prebleaching of kraft pulps through the process called as delignification. On commercial scale, hemicellulase and laccase enzymes are used in pulp bleaching, endo-β-xylanase and hemicellulase are used to enhance delignification of kraft pulp. Xylanases are also used along with other lignolytic enzymes which are shown to improve the effectiveness of enzymatic treatment. Amylases, proteases, esterases, lipases, and cellulases are the enzymes used in deinking process and thus help in recycling of the waste paper. Use of enzymes in paper and pulp processing provides advantages like reduction in processing time, energy consumption, and chemicals. Enzymes are also used to enhance deinking and waste treatment by increasing biological oxygen demand (BOD) and chemical oxygen demand (COD). Some of the enzymes used in paper and pulp processing are listed in Table 29.10.

29.6.3 In Processing of Leather

In leather processing industry, enzymes are required during different leather processing steps such as curing, soaking, liming, dehairing, degreasing, and tanning for enhancing leather quality. The enzymes used in leather industry are alkaline proteases, neutral proteases, and lipases. The processing of leather involves the removal of non-fibrillar proteins during soaking and bating which is done to make leather soft, supple, and pliable by the action of alkaline proteases. In dehairing process, mainly neutral and alkaline proteases are used to reduce wastage of water. During degreasing, lipases are used to remove fats. In liming process, enzymes are

Table 29.10 Application of some enzymes used in paper and pulp processing (Kenealy and Jeffries 2003; Singh et al. 2016)

Enzyme	Microorganism	Application
Amylase	*Bacillus licheniformis*	Deinking, drainage improvement
Cellulase	*Bacillus* sp., *Aspergillus niger*	Deinking, drainage improvement
Laccase	*Bacillus subtilis*	Non-chlorine bleaching, delignification
Protease	*Bacillus subtilis*	Biofilm removal
Xylanase, hemicellulase	*Trichoderma reesei, Thermomyces lanuginosus, Aureobasidium pullulans*	Bleach boosting

Table 29.11 Application of some enzymes used in leather processing

Enzyme	Microorganism	Application
Alkaline protease	*Alcaligenes faecalis*	Dehairing, bating
Amylase	*Aspergillus* sp., *Bacillus subtilis*	Fiber splitting
Lipase	*Aspergillus oryzae, A. flavus,*	Degreasing
Neutral protease	*Aspergillus niger, A. flavus, Bacillus subtilis*	Dehairing, soaking

beneficial over chemicals in terms of reduced odor, low BOD and COD in effluents. Some of the enzymes used in leather processing are given in Table 29.11.

29.7 Advantages and Limitations of Enzymes and Microbes over Chemical Methods in Agro-Processing Industries

As mentioned earlier, the chemical processing methods are used in primary, secondary, and tertiary processing stages in various food and non-food industries and the drawbacks accompanied with these methods are toxicity, health hazards, and negative impacts on the environment. The wastewater generated from the production and processing of agro-products contains fat, phosphates, sulfates, sulfides, chromium, copper, organic solvents, wood dust, terpenes, latex, clay, heavy metal, acidic and basic compounds, etc. The rise of these substances in the food chains leads to bioaccumulation and affects humans/animals health.

The other factors include cost of chemicals and instruments which are relatively high and their transportation is potentially hazardous. Some chemicals are explosive when concentrated and can be fatal if swallowed. Due to these reasons, there is a great interest in the improving and implementing new safe, cost effective, and eco-friendly agro-processing methods. The introduction and implementation of stringent standards for food processing have necessitated the need for the development of enzymatic processes. The use of enzymes or microorganisms in food preparations is an age-old process. Microorganisms such as bacteria, yeast, and fungi and their enzymes are widely used in several food preparations for improving the taste and texture. The need of microbial enzymes in agro-processing industries is increasing rapidly due to reduced processing time, low amount of chemicals, low energy input, non-toxic, and eco-friendly characteristics. Microbial enzymes are also capable of degrading toxic chemical compounds of industrial wastes (phenolic compounds, nitriles, amines, etc.) either via degradation or conversion (Singh et al. 2016). Moreover, with the advent of recombinant DNA technology and protein engineering, microbe can be manipulated and cultured in large quantities to meet the increased demand (Liu et al. 2013). Applications of microbial enzymes in agro-processing industries are numerous and rapidly increasing as compared to conventional methods due to less harm to the environment, greater efficiency, and higher quality products. The major advantages associated with enzymes include operation over a wide range of pH, temperature and salinity, absence of shock loading effects, low BOD and COD in effluents, and improved recovery. However, microbial

enzymes have certain drawbacks like they are expensive as it requires high purity and recovery from the reaction mixture.

29.8 National and International Status of Enzymes and Microbes in Agro-Processing

In many parts of the world, agro-processing is seen as critical to the expansion and diversification of the agricultural industry. In developed countries, the use of enzymes and microbes in agro-processing is quite large as compared to developing countries. Microbes and their enzymes have the potential to increase productivity, efficiency, and quality output in *agro-processing* operations in many developing countries. Enzymes have currently found application in several different agro-processing operations and new areas of application are constantly being explored. There is an ascending demand for industrial enzymes especially in emerging economies such as China, India, and Japan.

The global enzyme market is dominated by food and feed applications, which account for 55% to 60% of the market. The USA is the leader in enzyme production and consumption at global level, which is attributed to increased expenditure in better quality products and consumer preference for processed foods. Moreover, the increasing awareness about better quality products made by using microbes and enzymes and the advancements in food processing technologies is further driving the market. Commercially available major enzymes used in non-food agro-processing industries are amylases, proteases, lipases, cellulases, xylanases and catalases, etc. that are used in processing of textile and paper & pulp. Most commonly used food enzymes include amylase, cellulase, xylanase, pectinase, protease and lipase, etc. that are widely used in baking industry, wine making and brewing, treatments of fruit juices (Keerti et al. 2014), dairy industry, and cheese manufacturing and thus have become an essential part of global food additive industries. The use of enzymes in food processing is continuously increasing based on research and development (R&D) efforts focused on discovery of new enzymes, cloning and expression of desired enzymes and enzyme engineering to impart desirable properties to the enzymes.

In India, enzyme market is witnessing robust growth due to the increasing demand for processed foods and wide applications of the enzymes in the processed food industry. The Indian food enzymes market is projected to register a CAGR of 3.8% during 2020–2025. India's food processing sector is expected to be worth over half a trillion dollars by the year 2025 and will potentially attract $33 billion investments. A number of opportunities exist for the use of enzymes in various operations of agro-processing applications in developing countries. Thus, constant innovations in research and development activities are required in enzyme technology to explore novel enzymes and to overcome existing problems related to the use of enzymes like unstable nature of enzyme, cost of production, and non-availability of specific biocatalysts under industrial conditions. Hence, screening of new and better microbial enzymes from extreme environmental sources needs to be explored

which will work efficiently compared to existing enzymes used in agro-processing. These consistent efforts will eventually increase application of enzymes in various activities of agro-processing to produce final product of high value to consumers.

29.9 Challenges

In agro-processing industries, main issues of concern are environmental protection, waste treatment, hygienic practices, higher quality control, and technological applications. Additionally, continuous efforts are needed to increase the productivity of food and fiber crops and to improve quality of their products through microbes and their enzymes. Although enzymes have diverse application in agro-processing, but extraction of enzymes from microorganisms and their purification leads to additional cost in agro-processing. So there is need for development of more rational and cost-effective enzyme-based processes.

29.10 Future Prospects

The agro-processing industries are becoming more promising to provide new biological solutions to traditional chemical processes and promote sustainability in a growing world. New enzymatic processes are being developed with innovative applications, which can replace the use of chemicals in conventional methods of agro-processing. Research and development techniques are focusing on enzyme engineering to impart desirable properties to the enzymes. The use of enzymes as replacement of chemical treatments in various industries has surely reduced waste chemical discharge into the environment. There is a need to increase the application of existing technologies and develop new ones, which maximize the use of raw material inputs in agro-processing. New technologies focusing on enzyme engineering will help in improving the quality of existing enzymes for better usability. Increasing numbers of microbial genome sequences are available, but gene annotation is still a limitation. A large number of putative genes still have an unknown function. Assigning functions to these genes using the appropriate methods like transcriptomics, proteomics, etc. will improve the knowledge about the biology and physiology of the microbes and contribute to our understanding about their enzymes. With increasing accessibility to CRISPR technologies, the development of new and improved microbial strains will enable production of many novel enzymes for novel applications in agro-processing.

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Application of Bioinformatics in Crop Improvement

30

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Abstract

The branch of bioinformatics has evolved tremendously in the twenty-first century after human genome project and other whole genome projects, including crop plants. It has entered into almost all the basic and applied biological research work and crop improvement is no exception to it. There have been many recorded milestones being achieved in the field of crop improvement and have bioinformatics in its central stage. Crop improvement is carried out to have better yield, better trait of stress tolerance and/or disease resistance. This can be accompanied in a very rational way by exploiting the biological data available with us in the form of genomic, proteomic, transcriptomic, interactomic, metabolomic and other—omics data. The proper storage, retrieval and analyses of these data require different statistical and bioinformatics tools and techniques. The present chapter attempts to review the application of bioinformatics tools at genomic, proteomic and metabolomic levels in crop improvement programmes.

Keywords

Plant breeding · Genomics · Proteomics · Metabolomics · Plant database

30.1 Introduction

Bioinformatics is an emerging science of converging technologies and aims to solve biological problems using computational tools. It encompasses interactive applications from different branches of science and technology, including statistics

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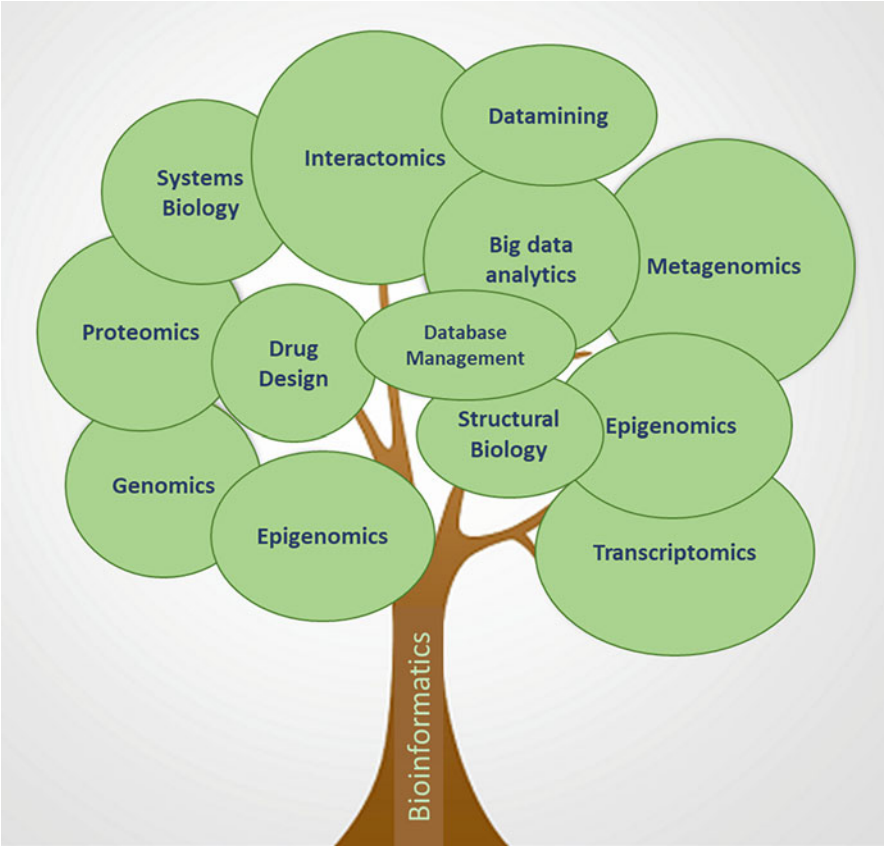

Fig. 30.1 The growing tree of Bioinformatics with major branches

and mathematics with computational and biological science in the core, serving as the root system of the Bioinformatics tree. Several jargons have been added to the vocabulary of bioinformatics in recent past which includes—genomics, proteomics, transcriptomics, interactomics, metagenomics, etc. and they constitute the branches of present-day bioinformatics tree (Fig. 30.1).

30.2 Recent History of Bioinformatics

The biological research witnessed three major milestones during later half of the twentieth century and early twenty-first century, namely: (1) Double helical model proposed by Watson and Crick in 1953 (de Chadarevian 2003); (2) Development of PCR technology by Kary Mulis in 1985 (Pai-Dhungat 2019) and Human Genome Project (Gibbs 2020). Since the development of PCR technology, there was a revolutionary change in the approaches to view and handle biological problems

and led the solution searches lower down to gene and genomics level. With the advancements in genomic tools and techniques like Next-gen sequencing in the last three decades, the biological research witnessed a flood of biological data, worldwide. This in turn gave an invitation to computational science to handle the logarithmic growth of biological data, majorly genomic data. This has given an evolutionary push to the current form of bioinformatics to serve the purpose of proper storage, annotation, retrieval and analysis tools for these biological data. Several biological databases like Genbank at NCBI, European Molecular Biology Lab at EBI, DNA Data Bank of Japan at National Institute of Genetics, Shizuoka, and their consortial approach of International Nucleotide Sequence Database Collaboration (INSDC) served as the repository for submission, maintenance and retrieval of nucleotide data generated worldwide (Karsch-Mizrachi et al. 2018). Also, during the last three decades, the increasing growth rate of other biological data like proteomic data, transcriptomic data, literature data, etc., also paved the path for other types of biological data repositories like—Uniprot KB, Protein Data Bank (PDB), PubMed, PubChem, etc. Table 30.2 provides comprehensive information of different biological databases and reflects the penetration of bioinformatics in all branches of biological research, ranging from plants, animals, humans, microbes to metagenomes, white biotechnology and synthetic biology. Therefore, bioinformatics is also known as "Gold Biotechnology".

30.3 Crop Improvement

"Documents can wait, hunger cannot". With this famous quote of Stalin and repeated by M.S. Swaminathan in favour of evergreen revolution, crop improvement has emerged as the most important assignment for current plant breeders, looking into the shrinking size of cultivable land and increasing world population. In general terms, crop improvement deals in the genetic modifications of plants to meet human requirements. Since the mankind started cultivation and brought a few hundred species after selecting them from several lakhs of available species, the branch of plant breeding and crop improvement has become an integral part of agriculture and agricultural practices, worldwide. The same integrity becomes imperative, looking into the increasing demand of food and other agricultural products and is attained through genetic engineering to provide plants with better quality and quantity of yield (Sedeek et al. 2019).

In simplest form, crop improvement is either for variety/quality enhancement or for production/quantity elevation. Transgenic variety creation is the core of crop improvement with the changing concept of biotechnology, with our transition from the twentieth to twenty-first century. The science of transgenesis relies heavily on genomic data, which in turn gives bioinformatics an important fundamental place in the comprehensive crop improvement programmes, worldwide.

30.3.1 Role of Bioinformatics in Crop Improvement

Plant system has evolved to deal with and adapt to the changing environmental conditions. So, the same cultivar with same genotype has different phenotypic expressions in different climatic conditions. This lab to land paradox is resolved by strict evaluations of cultivars in different lab/greenhouse environment before being released as new variety by plant breeders. Apart from this recognized complex paradox, the genetic basis of crop improvement still occupies the central stage of plant breeding programmes. Bioinformatics play the pivotal role in basic three classified approaches of crop improvement (Table 30.1).

30.3.1.1 Bioinformatics at Genomic Level Approach of Crop Improvement

There has been a focused approach on whole genome projects of plants since the whole genome project of *Arabidopsis thaliana* was completed in the last decade of the twentieth century using shot gun sequencing technology (Cao et al. 2017). Whole genomes of 1000 different plant species were sequenced recently under the umbrella of a mega "One Thousand Plant Genome Project (1KP)" (Matasci et al. 2014; Wong et al. 2016; Cheng et al. 2018) and the final draft including a "capstone" publication was released in 2019 (One Thousand Plant Transcriptomes Initiative 2019). There are about 250 angiosperm species, including crop plants, whose genomes have been sequenced out of ~3,50,000 angiosperm species. A comprehensive list of major angiosperm plant's genome databases/resources is as given in Table 30.2, which reflects the ocean of genomic data available of raw/annotated form, which has been plant genetic analysis and creating transgenic, including crop plants. The genome databases serve as a repository genomic data for further analysis and annotations. The major drawback of these whole genome databases/initiatives include limited data sharing in public domain; non-integrative or individualistic approach of each database; lack of comprehensive toolkit for data analysis and use of cloud storage for big data. Many of these issues have been highlighted recently and are getting addressed well to apply bioinformatics resources and tools in crop improvement (Chen et al. 2018). There is a growth in comprehensive databases in recent years, with different toolkits (Table 30.3).

30.3.1.2 Bioinformatics at Proteomic Level Approach of Crop Improvement

Proteome is defined as the complete set of proteins expressed in a cell in a given set of conditions at a particular time and defines the property and capability of a cell and organism in-toto. Contrary to the genome (static), proteome is dynamic in nature and varies with time and environmental conditions (Smolikova et al. 2020). Hence, the study of proteome or the expressed genome becomes imperative and significant in solving any biological problem, including crop improvement (Varshney et al. 2020). Proteomics is an important component of present-day omics-world and involves large-scale analyses of proteins using different automated/robotic systems and computational biology (Fig. 30.2).

Table 30.1 List of major biological databases/resources used in bioinformatics

S. no.	Biological resource/ database	Nature of data/applications	Weblink
(A) *Genomic resources*			
1	NCBI assembly	Genomic sequence and metadata	https://www.ncbi.nlm.nih.gov/assembly
2	NCBI biocollections	Culture, museums, and herbaria collections	https://www.ncbi.nlm.nih.gov/biocollections
3	NCBI BioProject	Genomic and genetic data	https://www.ncbi.nlm.nih.gov/bioproject
4	NCBI BioSample	Experimental assays information	https://www.ncbi.nlm.nih.gov/biosample
5	NCBI consensus CDS	Human and mouse protein coding regions	https://www.ncbi.nlm.nih.gov/projects/CCDS/CcdsBrowse.cgi
6	NCBI dbSNP	SNPs	https://www.ncbi.nlm.nih.gov/snp/
7	GenBank	Annotated genetic sequences	https://www.ncbi.nlm.nih.gov/genbank/
8	NCBI influenza virus	Annotated influenza virus genetic and protein sequences	https://www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-select.cgi?go=database
9	NCBI pathogen detection project	Bacterial pathogen genomic sequences	https://www.ncbi.nlm.nih.gov/pathogens/
10	NCBI nucleotide database	Genomic sequences	https://www.ncbi.nlm.nih.gov/nuccore
11	NCBI RefSeq gene	Genomic sequences	https://www.ncbi.nlm.nih.gov/refseq/rsg/
12	NCBI reference sequence (RefSeq)	Gene and Protein sequences	https://www.ncbi.nlm.nih.gov/refseq/rsg/
13	NCBI sequence read archive (SRA)	NGS data	https://trace.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?
14	NCBI trace archive	DNA sequence chromatograms	https://www.ncbi.nlm.nih.gov/Traces/trace.cgi
15	NCBI BioSystems	Biomedical literature and sequence data	https://www.ncbi.nlm.nih.gov/biosystems/
16	NCBI gene	Gene	https://www.ncbi.nlm.nih.gov/gene
17	Gene expression omnibus (GEO) database	Genomics data repository	https://www.ncbi.nlm.nih.gov/geo/
18	Gene expression omnibus (GEO) datasets	Curated gene expression	https://www.ncbi.nlm.nih.gov/gds
19	Gene expression omnibus (GEO) profiles	Gene expression and molecular abundance profiles	https://www.ncbi.nlm.nih.gov/geoprofiles/
20	Genes and disease	Genetic disorders summary	https://www.ncbi.nlm.nih.gov/books/NBK22183/

(continued)

Table 30.1 (continued)

S. no.	Biological resource/ database	Nature of data/applications	Weblink
21	Genetic testing registry (GTR)	Registry of genetic tests and laboratories	https://www.ncbi.nlm.nih.gov/gtr/
22	OMIM	Genetic (genes and disorders)	https://www.omim.org/
23	Retrovirus resources	Retrovirus genome and protein analysis	https://www.ncbi.nlm.nih.gov/genome/viruses/retroviruses/
24	Genome	Whole genome sequences	https://www.ncbi.nlm.nih.gov/genome
25	Genome reference consortium (GRC)	Human and mouse reference genomes	https://www.ncbi.nlm.nih.gov/grc
26	HIV-1, human protein interaction database	Protein interaction	https://www.ncbi.nlm.nih.gov/genome/viruses/retroviruses/hiv-1/interactions/
27	NCBI PopSet	DNA sequences	https://www.ncbi.nlm.nih.gov/popset
28	Viral genomes	Viral genomes	https://www.ncbi.nlm.nih.gov/genome/viruses/
29	Database of genomic structural variation (dbVar)	Genomic	https://www.ncbi.nlm.nih.gov/dbvar
30	Database of genotypes and phenotypes (dbGaP)	Genotyping and phenotype interaction	https://www.ncbi.nlm.nih.gov/gap/
31	Burkholderia genome database	Genome annotation	https://www.burkholderia.com/
32	ZFIN	Zebrafish genomic/genetic data	https://zfin.org/
33	ggKbase	Genome annotation	https://ggkbase.berkeley.edu/PLM2016-NC-80p/organisms
34	Nucleome data Bank	3D genome visualization	https://ndb.rice.edu/
35	ENCODE	Genomic/ DNA elements	https://www.encodeproject.org/
36	Gene ontology	Genomic, gene function, gene ontology	http://geneontology.org/
37	PASTAA	Genomic Sequences	http://trap.molgen.mpg.de/cgi-bin/pastaa.cgi
38	Bgee	Gene expression	https://bgee.org/
39	SwissRegulon portal	Tools and data for regulatory genomics	https://swissregulon.unibas.ch/sr/
40	EPD	Eukaryotic promoter database	https://epd.epfl.ch//index.php
41	V-pipe	Viral genomics pipeline	https://github.com/cbg-ethz/V-pipe

(continued)

Table 30.1 (continued)

S. no.	Biological resource/ database	Nature of data/applications	Weblink
42	SwissOrthology	Phylogenomic database	https://swissorthology.ch/service/search
43	Ensembl	Genomic data	Genomic sequence and metadata
44	DNA data Bank of Japan	Genomic data	https://www.ddbj.nig.ac.jp/ddbj/index-e.html
(B) *Proteomic resources*			
45	NCBI computational resources from NCBI's structure group	Protein domains and structures	https://www.ncbi.nlm.nih.gov/Structure/index.shtml
46	NCBI influenza virus	Annotated influenza virus genetic and protein sequences	https://www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-select.cgi?go=database
47	NCBI conserved domain database (CDD)	Sequence alignment and protein domains data	https://www.ncbi.nlm.nih.gov/cdd
48	HIV-1, human protein interaction database	Protein interaction	https://www.ncbi.nlm.nih.gov/genome/viruses/retroviruses/hiv-1/interactions/
49	Retrovirus resources	Retrovirus genome and protein analysis	https://www.ncbi.nlm.nih.gov/genome/viruses/retroviruses/
50	Identical protein groups	Proteins in annotated coding regions in GenBank and RefSeq	https://www.ncbi.nlm.nih.gov/ipg
51	Protein clusters	Related protein sequences	https://www.ncbi.nlm.nih.gov/proteinclusters
52	NCBI BioProject	Genome and genetic data	https://www.ncbi.nlm.nih.gov/bioproject
53	NCBI BioSystems	Biomedical literature and sequence data	https://www.ncbi.nlm.nih.gov/biosystems/
54	Protein database	Protein sequence record	https://www.ncbi.nlm.nih.gov/proteinclusters
55	Protein family models	Protein models	https://www.ncbi.nlm.nih.gov/protfam
56	NCBI reference sequence (RefSeq)	Gene and Proteon sequences	https://www.ncbi.nlm.nih.gov/refseq/rsg/
57	M-CSA	Enzyme reaction	https://www.ebi.ac.uk/thornton-srv/m-csa/
58	Uniprot	Protein sequence	https://www.uniprot.org/
59	Protein data Bank (PDB)	Protein structure	https://www.rcsb.org/pdb/static.do?p=general_information/about_pdb/index.html

(continued)

Table 30.1 (continued)

S. no.	Biological resource/ database	Nature of data/applications	Weblink
60	M-CSA	Enzyme reaction	https://www.ebi.ac.uk/thornton-srv/m-csa/
61	Protein data Bank (PDB)	Protein structure	https://www.rcsb.org/pdb/static.do?p=general_information/about_pdb/index.html
62	PRIDE	Protein and peptide identification or quantification data, along with the spectra	https://www.ebi.ac.uk/pride/
63	PEAKS	Protein MS peaks	https://www.bioinfor.com/peaksdb/
64	antiSMASH	Secondary metabolite	https://antismash-db.secondarymetabolites.org/
65	METLIN	Mass spectral database	https://www.sisweb.com/software/ms/wiley-metlin.htm
66	KEGG	Enzyme pathways	https://www.genome.jp/kegg/
67	UniProtKB/Swiss-Prot	Protein knowledgebase	https://www.uniprot.org/database/
68	STRING	Protein-protein interaction networks and functional enrichment analysis	https://string-db.org/
69	neXtProt	Human protein knowledgebase	https://www.nextprot.org/
70	SWISS-MODEL	Protein structure homology-modelling	https://swissmodel.expasy.org/
		(C) *Metabolomic resources*	
71	KEGG	Enzyme pathways	https://www.genome.jp/kegg/
72	LipidMAPS	Lipid structure database	http://www.lipidmaps.org/data/structure/
73	mzCloud	Mass spectral database	https://www.mzcloud.org/
74	Swiss lipids	Lipid knowledgebase	http://www.swisslipids.org/#/
75	Human metabolome	Metabolites	https://hmdb.ca/
(D) *Structural and Networking resources*			
76	NCBI computational resources from NCBI's structure group	Protein domains and structures	https://www.ncbi.nlm.nih.gov/Structure/index.shtml
77	NCBI BioSystems	Biomedical literature and sequence data	https://www.ncbi.nlm.nih.gov/biosystems/
78	NCBI structure (molecular modelling database)	3D protein structures	https://www.ncbi.nlm.nih.gov/structure
79	STRING	Protein-protein interaction networks and functional enrichment analysis	https://string-db.org/
80	ggKbase	Genome annotation	https://ggkbase.berkeley.edu/PLM2016-NC-80p/organisms

(continued)

Table 30.1 (continued)

S. no.	Biological resource/ database	Nature of data/applications	Weblink
(E) *Chemical/toxicological/pharmaceutical resources*			
81	NCBI BioSystems	Biomedical literature/ small molecules and sequence data	https://www.ncbi.nlm.nih.gov/biosystems/
82	NCBI PubChem BioAssay	Record of bioactivity assays	https://www.ncbi.nlm.nih.gov/pcassay
83	NCBI PubChem compound	Chemical structures	https://www.ncbi.nlm.nih.gov/pccompound
84	NIST	Chemical/molecular data	https://webbook.nist.gov/chemistry/
85	ChEMBL	Bioactive molecules	https://www.ebi.ac.uk/chembl/
86	The Cambridge crystallographic data Centre	Chemical structure	https://www.ccdc.cam.ac.uk/structures/?
87	REAL	Chemical structure/SMILES	https://enamine.net/library-synthesis/real-compounds/real-database
88	ChemSpider	Chemical structure	http://www.chemspider.com/
89	Drug Bank	Pharmaceutical information	https://go.drugbank.com/
90	SwissDrugDesign	Computer-aided drug design	https://www.molecular-modelling.ch/swissdrugdesign.php
(F) *Literature resources*			
91	NCBI TPA	Experimental or inferential results of annotated genes	https://www.ncbi.nlm.nih.gov/genbank/tpa/
92	NCBI trace archive	DNA sequence chromatograms	https://www.ncbi.nlm.nih.gov/Traces/trace.cgi
93	NCBI bookshelf	Biomedical books	https://www.ncbi.nlm.nih.gov/books
94	NCBI ClinicalTrials	Clinical studies	https://clinicaltrials.gov/
95	Database of genotypes and phenotypes (dbGaP)	Genotyping and phenotype interaction	https://www.ncbi.nlm.nih.gov/gap/
96	NCBI gene	Gene	https://www.ncbi.nlm.nih.gov/gene
97	GeneReviews	Peer-reviewed disease descriptions on the NCBI bookshelf	https://www.ncbi.nlm.nih.gov/books/NBK1116/
98	Genes and disease	Genetic disorders summary	https://www.ncbi.nlm.nih.gov/books/NBK22183/
99	Genetic testing registry (GTR)	Registry of genetic tests and laboratories	https://www.ncbi.nlm.nih.gov/gtr/
100	PubMed	Biomedical literature	https://pubmed.ncbi.nlm.nih.gov/

(continued)

Table 30.1 (continued)

S. no.	Biological resource/ database	Nature of data/applications	Weblink
101	PubMed central (PMC)	Biomedical and life sciences journal literature	https://www.ncbi.nlm.nih.gov/pmc/
102	Journals in NCBI databases	Subsets of journals	https://www.ncbi.nlm.nih.gov/nlmcatalog/journals
103	MeSH database	Literature	https://www.ncbi.nlm.nih.gov/mesh
104	MedGen	Medical genetic	https://www.ncbi.nlm.nih.gov/medgen
105	National Library of medicine (NLM) catalog	Bibliographic data for all the journal	https://www.ncbi.nlm.nih.gov/nlmcatalog/journals
106	Cochrane database	Literature	https://www.cochranelibrary.com/cdsr/reviews
107	Data dryad	Literature and data repository	https://doi.org/10.5061/dryad.7m0cfxppz

Table 30.2 List of whole genome database and resources of angiosperm food plants available for public access

S. no.	Crop plant name	Web link
1	*Eleusine coracana*	ncbi.nlm.nih.gov/bioproject/667839
2	*Hordeum vulgare*	phytozome.jgi.doe.gov
3	*Oryza indica*	plants.ensembl.org
4	*Oryza sativa*	rice.plantbiology.msu.edu/
5	*Setaria italica*	phytozome.jgi.doe.gov
6	*Sorghum bicolor*	gramene.org/
7	*Triticum aestivum*	phytozome.jgi.doe.gov
8	*Triticum turgidum*	gigadb.org
9	*Zea mays*	plants.ensembl.org
10	*Secale cereale*	pgsb.helmholtz-muenchen.de
11	*Glycine soja*	soybase.org
12	*Prunus avium*	ftp://ftp.bioinfo.wsu.edu
13	*Punica granatum*	ncbi.nlm.nih.gov/bioproject/694423
14	*Arabidopsis thaliana*	arabidopsis.org/index.jsp
15	*Citrus grandis*	citrus.hzau.edu.cn
16	*Citrus ichangensis*	citrus.hzau.edu.cn
17	*Citrus medica*	citrus.hzau.edu.cn
18	*Chenopodium pallidicaule*	ncbi.nlm.nih.gov/bioproject/326220
19	*Chenopodium quinoa*	phytozome.jgi.doe.gov
20	*Fagopyrum esculentum*	buckwheat.kazusa.or.jp

Table 30.3 List of comprehensive whole genome database of angiosperm food plants available for public access

S. no.	Comprehensive database	No. of genomes	Web link
1	CoGe	22	genomeevolution.org/CoGe
2	EnselmlPlantsV36	37	plants.ensembl.org
3	Plant Genome Duplication Database (PGDD)	43	chibba.agtec.uga.edu/duplication
4	Plant Genome and Systems Biology (PGSB)	12	pgsb.helmholtz-muenchen.de
5	PhytozomeV12	59	phytozome.jgi.doe.gov
6	plantgdb	23	plantgdb.org
7	PLAZA	28	bioinformatic.psb.ugent.be/plaza
8	VISTA	38	genome.lbl.gov/vista

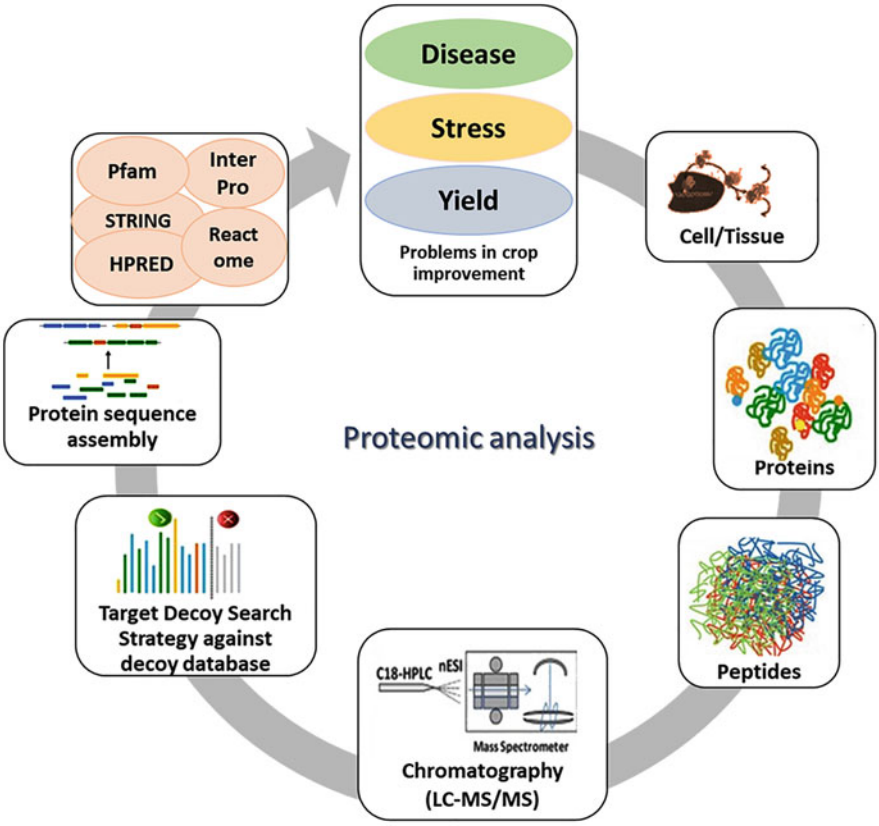

Fig. 30.2 A schematic layout of typical proteomic analysis involving bioinformatics tools for solving biological problems of crop improvement

In context to the plant research and crop improvement, proteomic studies have significantly shown its presence in the last two decades (Smolikova et al. 2020), particularly in the field of identifying and characterizing proteins and their modulations in response to different biotic and abiotic stresses and their effect on crop yield and quality. There are majorly two approaches of proteomic studies: (a) Gel bases proteomics (1D-, 2D- Gel electrophoresis) and (b) Gel free proteomics (Chromatographic analysis) (Jorrín-Novo et al. 2015). The proteomic studies can also be classified into three broad categories on the basis of information collected as;

1. *Expressional proteomics*: A comparative study of expression levels of complete proteome or a sub-set of proteome of different samples under different conditions. These information are very helpful in deciphering the novel plant protein (s) related to stress (Jiang et al. 2020; Chaudhary et al. 2019) or diseases (Razzaq et al. 2019; Wang et al. 2019), which are used to design and develop new plants for crop improvement programmes.
2. *Functional proteomics*: It encompasses a specific and directed proteomic approach towards studying a particular group/type of protein(s) and provides significant information about protein–protein and protein–ligand interactions, which helps in deciphering disease mechanisms. This information is very handy in developing resistant and improved traits in plants including crop plants (Varshney et al. 2020).
3. *Structural proteomics*: This category of proteomic study involves identification and characterization of protein's structure and generating its "cell map" (cellular location). The study deals in identifying all the proteins present in an organelle or protein complexes and characterizing all of their protein–protein interactions (Cruz et al. 2020).

30.3.1.3 Bioinformatics at Metabolomic Level Approach of Crop Improvement

Metabolomics has been a fast evolving branch of omic-science in the last two decades. It is mainly involved in identifying and estimating metabolites and chemical footprints of different metabolic processes occurring in an organism. A metabolome represents the entire pool of metabolites in an organism and is used to identify and estimate environmental variations (Razzaq et al. 2019). Metabolomic approaches have been successfully applied to identify different metabolic networks associated with biotic and abiotic stress and their tolerance strategies, which can be used to develop efficient screening methods for enhanced yield and stress tolerance or disease resistance in crop plants (Piasecka et al. 2019; Ghatak et al. 2018; Tian et al. 2016).

The application of metabolomic-assisted plant breeding (MAPB) permits an organized and rationale approach for transmission of increased product yield and/or stress tolerance like heat tolerance (Raza 2020) and needs to be duly

incorporated in every plant breeding programmes for crop improvement (Razzaq et al. 2021; Thao and Tran 2016). The metabolic profiling results in a huge amount of data that requires different statistical and bioinformatics tools to evaluate and analyse these data. The use of data-mining and machine learning approaches in metabolomics, when integrated with other omic (genomic, proteomic and transcriptomic) level data, lead us to magnified understanding of biochemical, genetic and physiological processes at cellular or organism level, while they have cross-talks with different favourable or unfavourable environmental conditions. This provides a holistic platform for developing successful plant breeding programmes for crop improvement (Piasecka et al. 2019; Tian et al. 2016).

30.4 Conclusion

The role of bioinformatics in crop improvement has now been established and is progressing fast to occupy the central stage of plant breeding programmes in present omics era. The different levels of application of bioinformatic tools and techniques in crop improvement are shown in Fig. 30.3 in a comprehensive way. These approaches, when incorporated, give rise to a better rationalization of crop improvement programmes in terms of reduced manpower, money and time required to achieve a better cultivar in context to yield and quality.

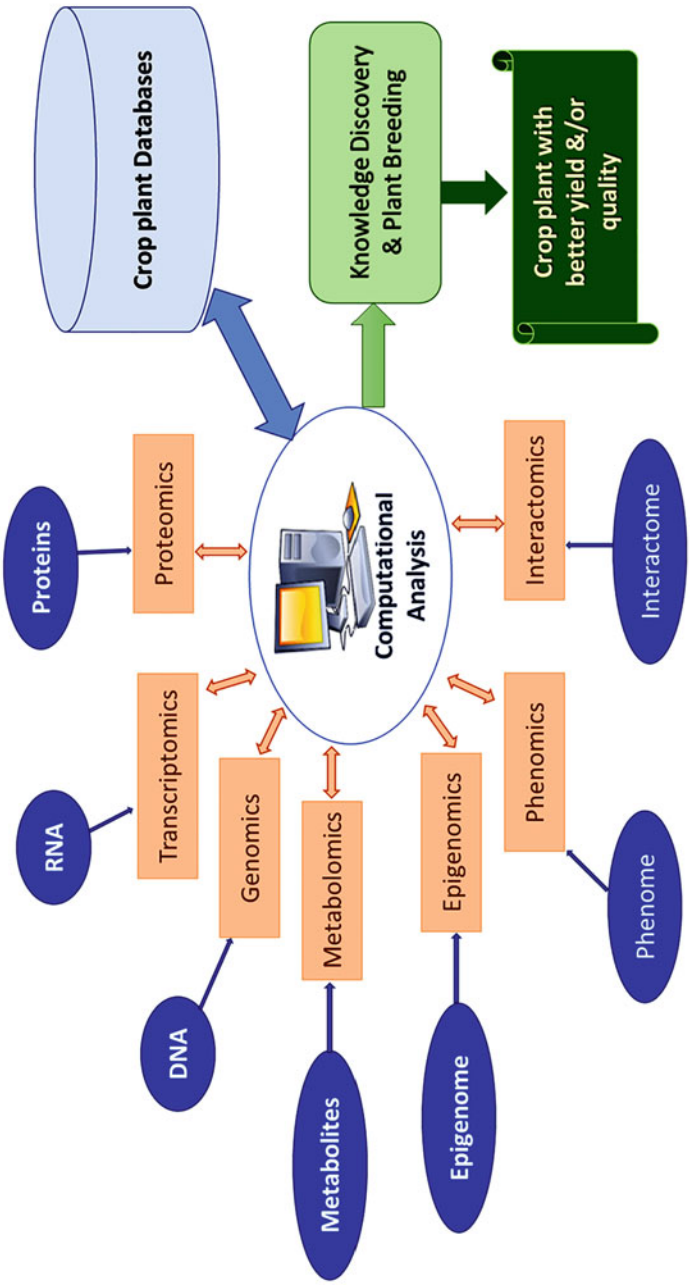

Fig. 30.3 A schematic comprehensive layout of application of different bioinformatics tools in crop improvement

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