P. B. Kavi Kishor
M. V. Rajam
T. Pullaiah *Editors*

Genetically Modified Crops

Current Status, Prospects and Challenges Volume 1

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Editors

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Foreword

To get easy access to food and to improve the productivity of crop plants, humans have used methods of domestication and improvement through selective breeding, based on useful phenotypic traits. It was through the work of Gregor Mendel that we learnt about the genetic basis of plant traits. The first hybrid corn was developed in 1922 by an intelligent breeding strategy. Following the discovery of DNA as the genetic material, the work of a number of groups led to the concept of gene as the unit of DNA that controls a phenotypic character of an organism. And it was in 1973 that Herbert Boyer and Stanley Cohen developed genetic engineering by inserting DNA from one bacterium to another. Around the same time Jeff Schell and Marc Van Montagu discovered that it is due to the transfer of the plasmid DNA of *Agrobacterium tumefaciens* that results in tumor formation in plants. This research was a by-product of curiosity-driven science and based on fundamental scientific discovery. Using this information and developing plant transformation technology, the group of Mary-Dell Chilton and R. Fraley and scientists from Monsanto Company created the first transgenic plant. During the mid-1990s, with the creation of GM tomato, the initial wave of GM plants was set in motion. However, due to certain issues of public acceptability and stringent regulatory laws that were put in place in different countries, the growth of this technology was slowed down. Van Montagu, whom I have had the pleasure of meeting and knowing for a long time, wrote an insightful article in the *Annual Review of Plant Biology* in 2011 titled, "It is a long way to GM agriculture." Even then this technology has been used in many crops, and the global biotech crop area is steadily increasing within many countries which have adopted this technology for crop improvement in their agriculture systems. Unfortunately, due to various social and political issues the adoption of this technology has received resistance. This trend needs to be reversed. In the meanwhile, one has seen the emergence of new technologies like RNAi to silence the expression of genes to understand their role as also to develop novel transgenic plants with useful traits. And since 2015, gene editing technologies have evolved which have become useful and efficient tools to manipulate DNA in plant cells. And now we are moving onwards to precision genome engineering through prime genome editing, which does not involve double-strand breaks and donor DNA templates. Hopefully, these interventions will not be subjected to as much stringent regulatory procedures and will also find better acceptability in the society.

An article was published in *EMBO Reports* by Fagerstrom et al. in 2013, entitled "Stop Worrying Start Growing" with the subtitle, "Risk research on GM crops is a dead parrot: it is time to start reaping the benefits of GM." This is even more true today. The present volumes by Professors Kavi Kishor, Rajam, and Pullaiah have been compiled to convey the same message by presenting achievements and opportunity of employing different technological tools for genetic improvement of plants. I have known the editors of this volume for a long time. They have themselves made significant contributions in the area of plant biotechnology and are well acquainted with GMOs, in all its perspectives. They are also aware of the views of opponents of this technology. Accordingly, taking these into considerations, they have broadly outlined the status, prospects, and challenges of different genetic interventions in various plants of economic importance for improving traits like developing resistance to viral, insect, and other diseases and for conferring tolerance to abiotic stresses. With rapid advancements in genome sequencing methodologies and functional genomics tools, it has now been possible to identify the genes that can be deployed in a very precise manner using efficient transformation techniques.

These volumes cover, among cereals, a chapter on rice that deals with the use of GM technology to address the problem of food and nutrition security and a chapter each on wheat and finger millet. Legumes, which remained recalcitrant for a long time and an efficient transformation system was not available, have now been tamed. This family of plants have received special attention, and a chapter each on pigeonpea, chickpea, cowpea, and peanut has found a place in this volume. Among vegetables there is a detailed account on the present status on brinjal, tomato, and cucurbits and one chapter each on redpepper and capsicum. Other plants of importance which have been included are sugarcane, cassava, banana, papaya, citrus, mulberry, and jatropha. The work on two oil plants, sunflower and safflower, has been presented in two independent chapters. This approach of illustrating the use of the technology for each species separately, rather than group them on specific trait, I find, provides a better perspective to evaluate the importance of GM technology with respect to each plant species.

These volumes, I am very sure, will be useful to all students and practitioners of biotechnology, be in colleges, universities, and private organizations, as well as for policy makers and regulators in the government agencies. I look forward to the deployment of the safe use of new tools and techniques of genetic manipulation for the improvement of important plants on a large scale in our agriculture and horticulture system. This will help, along with other breeding methodologies, including marker-assisted breeding, to sustain productivity with limited inputs. We hope to see a hunger-free world in the years to come.

International Centre for Genetic Engineering and Biotechnology, New Delhi, India
June 06, 2020

Sudhir K. Sopory

Preface

Plants provide us many essential things in life, including food, feed, cloth, wood, paper, medicinal compounds, industrial products, and most importantly the life-sustaining molecule oxygen to breath. Plants are also crucial to clean lifesaving water. There are only six crop plants, *viz*., rice, wheat, corn, potato, sweet potato, and cassava, which provide about 80% calories to humans. There are other important crops like sugarcane, barley, sorghum, bean, soybean, coconut, and banana, which are also being consumed by humans. But crop plants are vulnerable to various biotic factors (pathogens and pests) and extreme environmental conditions or abiotic stresses (e.g., high salinity, drought, heat and cold, heavy metals, and submergence) because of their sessile nature. These stresses cause a colossal loss of crop yields and impair nutritional quality. Otherwise, one can realize the potential and harvest 100% agricultural productivity from all crops. In addition, global warming, shrinking water resources, arable land, and population growth are aggravating the problem of food security. In fact, these are key scientific issues in agriculture besides post-harvest losses and impairment in nutritional quality. Then the critical question that arises in our minds is how to harness the full yield potentials of crops without compromising the quality component. The answer lies evidently in the exploitation of diverse technologies, particularly plant breeding and genetic engineering. Between plant breeding and genetic engineering, the former has contributed significantly for more than seven decades to crop improvement and in fact almost all the new and improved varieties were virtually derived through breeding strategies. However, breeding methods suffer from certain limitations like incompatibility barriers or narrow mobilization of useful genes between closely related species. This leads to the problem of using only limited gene pool and there is no way to transfer a single beneficial gene since we generally transfer a cluster of genes/chromosomes during the crosses, thus subjecting F_1 hybrids for 4–5 backcrosses to chuck away the unacceptable. It takes nearly 10–12 years to develop a new variety with desirable traits and may not be cost-effective. In contrast, genetic transformation by *Agrobacterium* or other gene delivery systems or transgenic technology offers several advantages such as precise gene transfer from any source to crop plants. This means a huge gene pool exists for the transfer of desirable traits across species and takes relatively 7–9 years to develop a transgenic line of interest. Consequently, genetic engineering holds great promise for crop improvement and is essential since huge gap exists between food production and rate of population

growth. Today's world population is about 7.7 billion and is expected to reach 9.7 billion by 2050, and further to an estimated 11 billion by 2100. Human hunger and malnutrition are the major problems, especially in Asian countries due to accelerating birth rates. So, it is a challenge for plant biologists and biotechnologists to resolve the problem of human hunger and malnutrition through crop improvement programs. In reality, about 70% increase in food production is required by 2050 to feed the growing masses; otherwise we may face great famines in the near future. Indeed, this suggests that a second green revolution is the need of the hour to bring food security to the world population, and this can only happen if we couple the conventional breeding strategies with genetic engineering technologies.

Transgenic technology has already proven to be novel and a potential alternative for crop improvement, and a handful of transgenic varieties like cotton, corn, soybean, and canola have been commercialized globally. This has led to a substantial increase in crop yield and quality, reduced use of harmful pesticides, reduction in CO_2 emissions, and decrease in the cost of crop production, besides improving the economy of marginal farmers. The first transgenic variety, *flavr savr*—the slow ripening tomato, was commercialized in 1994 in the USA, and since then there is a steady increase in the adoption of the first generation of genetically modified (GM) crops such as corn, cotton, and soybean for insect resistance, herbicide tolerance, and improvement of oil quality. In 2018, about 475 million acres (191.7 million hectares) of land was under the cultivation of various GM crops in 26 countries (21 developing and 5 developed countries), including 5 top countries—USA, Argentina, Brazil, Canada, and India (with the adoption of only Bt cotton) with the largest area of GM crops grown, and an additional 44 countries imported these GM crops. To date, about 525 different transgenic events in 32 crops have been approved for cultivation in different parts of the world. Currently, the next generation of transgenic plants displayed potential for the production of bio-ethanol, bio-plastics, and many pharmaceutically important recombinant proteins and compounds. Interestingly, the recent genome engineering or editing technology is quickly gaining importance for maneuvering genes in crop plants using the gene editing tool, the CRISPR-Cas system. This technology is aiding us in the improvement of many agronomically important traits such as yield, stress tolerance, and nutritional quality. Soon, the gene-edited crop plants with new traits, but not having an alien gene, will be commercialized. Such an endeavor will assist us in meeting the increasing food demands and global food security. This technology can be safely exploited since it has minimum or no regulatory issues. GM crops have the most rapid adoption rate in the history in spite of public concerns as compared to the traditional hybrids like corn, which took more than seven decades for global penetration. Transgenic varieties were released only after passing the tests against environmental aggressiveness, toxicity, allergenicity, after fulfilling the stringent regulatory guidelines laid down by the respective countries, and after exhibiting their superiority for field performance vis-à-vis the untransformed or wild-type plants.

The present book brought in two volumes has updated information about the current status of GM crops. While the first volume covered genetic modification studies in cereals, pulses, and oil-yielding crops, the second one included information on

important vegetable, fruit-yielding, and commercial crops. These volumes on GM crops will be handy to students of life science stream of both undergraduate and postgraduate studies, research scholars, postdocs and researchers working in plant and agricultural biotechnology organizations, faculty members, biotech companies, and professionals alike.

Lastly, we would like to express our heartfelt gratitude to Springer-Singapore for kindly consenting to bring out this book in two volumes and for extending support through various phases and for the timely completion of publishing. Our heartfelt thanks are also due to Prof. Sudhir K. Sopory, ICGEB, New Delhi, for writing the foreword. We would like to thank all the authors/coauthors who have contributed the review articles and also for their cooperation and erudition.

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Genetic Tinkering of Crops for Sustainable Development: 2020 and Beyond

P. B. Kavi Kishor, M. V. Rajam, and T. Pullaiah

Abstract

The advent of gene isolation from diverse organisms and their transfer into different vectors along with promoters and selectable marker genes are the milestone events in the annals of molecular biology. Further, varied efficient protocols developed for transferring alien genes into the host genomes have unfolded the evolution of transgenic plants for biotic and abiotic stress tolerance, nutritional quality improvement and refinement of many other agronomically important traits. Such transgenic events if occupy the agricultural landscape world over can not only aid to meet the evergrowing food demands alongside the nutritional quality but also help us in sustainable development. Ongoing endeavours all over the world in different laboratories showcased the development of genetically modified (GM) crop plants using candidate genes with different promoters. This has proved beyond doubt that the genetic engineering technologies evolved over time are robust and reproducible. Though a large number of candidate genes including transcription factors have been transferred for conferring diverse agronomic traits, majority of them have not been tested in the open fields and not released for the consumption of general public. Governments across the globe are exercising a caution with the apprehension of spread of engineered genes into the wild species and environmental degradation too. Effective measures and policies therefore must be evolved to clear the uncertainties/anxieties raised by the

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general public and environmentalists alike for the safety of our environment before the release of transgenic crop plants into the open fields.

Keywords

Transgenic plants · Genetic modification · Stress tolerance · Nutritional improvement · Sustainable development · Biosafety

1 Introduction

There is a huge demand for food as the world's human population is expected to reach 9.7 billion by 2050 and further to an estimated 11 billion by 2100 (Raman 2017). Moreover, there are many challenges in agriculture, including the shrinking of resources like water and arable land for crop production, crop yield loss due to pathogens and pests, post-harvest losses, etc. Therefore, the enhancement of food production by both conventional and non-conventional approaches is a matter of the utmost importance to bridge the gap between population growth and food production, and food security, if not taken care, might lead to great famines in the foreseeable future. In this regard, the transgenic technology appears to be a novel and potential alternative to enhance the food production, achieve food security and alleviate the human hunger and malnutrition. In fact, the biotechnological intervention, particularly genetically modified (GM) crops has been proposed to lessen the environmental footprint by improving food quality and enhancing crop productivity (Barros et al. 2019).

Deliberate manipulation of the genes using diverse methods of gene transfer generates transgenic or GM crops (Hundleby and Harwood 2019). Many countries are now able to grow transgenics that help farmers to significantly enhance crop productivity by ~22%, reduce the dependency on agro-chemicals (pesticides) by ~37% for controlling against various biotic stresses and also increase farmer profits by ~68% (Klümper and Qaim 2014; Gruissem 2015). In the USA, transgenic corn acreage is seeded with 92% of the GM crop growing area in 2018 compared with 85% in 2009 and 25% in 2000 (NRC 2002, 2018). There is an overall agreement that our agricultural landscape covering transgenics has improved the yields world over in varied crops showcasing the evidence what genetic engineering technology can do. Despite uncertainties in the field to accept GM crops by the consumers, the potential of the technology is enormous as evident from the experimental material being tested across many countries over a period of time. This introductory chapter focuses on what transgenic lines are being grown or under sale for use with desirable traits alongside their benefits in the wake of climate change.

2 GM Crops Currently Being Grown

The first generation of transgenic crops was raised based on single-gene transfers. Flavr Savr tomato was the first GM crop developed using a single gene and introduced in the USA in the year 1994 (Kramer and Redenbaugh 1994). Flavr Savr tomato has been modified genetically to slow down the process of fruit ripening, cell wall softening and rotting. Though gene transfer technology using *Agrobacterium*-mediated gene transfer into tomato was robust and reproducible, the Flavr Savr tomato produced was not successful as a commercial crop. In 1996, 1.7 MHa of GM crops were planted all over the world, but by 2015, the GM crop-growing area was increased to 179.7 MHa. Over 10% of the world's land (179.7 million hectares) was used to grow GM crops in 28 countries by the year 2015, and the acreage is increasing year after year. While the USA grows nearly 71 million hectares (MHa), smaller countries like Argentina (24.5 MHa) and Brazil (44.2 MHa) also grow GM crops in huge amounts of their agricultural areas. India grows only GM cotton in approximately 11.6 MHa (Dunwell 1998, 1999; Raman 2017). Besides, controlled trials are still being tested in several countries including the UK, Africa and Canada. Major crops being grown commercially include aubergine or brinjal (Bangladesh), cotton (nearly 15 countries), maize (17 countries), oilseed rape (Canola) (4 countries including Canada), papaya (the USA, China), potato (the USA), soybean (11 countries), squash (the USA) and sugar beet (North America). While GM crops such as soybean accounts for 83% of the world production (92.1 MHa), cotton represents 75% in the year 2015 (Raman 2017). Several of the European countries like Spain, Portugal, Romania and Slovakia grow mostly maize, but not other crops. Many GM crops produced in the mid-1990s protected the crops against pathogens, insects and herbicides. Though crop plants with abiotic stress tolerance were developed, they were not tested at the field level barring corn (Dunwell 1999; Checker et al. 2012; Chang et al. 2014). Transgenic drought tolerant corn was developed but not yet released to the farmers. Some of the transgenic crops like soybean which is glyphosate resistant, cotton and corn resistant to insects due to *Bt* genes attained commercial success (Dunwell 1996, 1999; James 1998, 2011). Thus, the first-generation transgenics included several crops that were resistant mostly to biotic stresses (Raman 2017; Askari-Khorasgani and Pessarakli 2018).

In 2015, while the USA grew ten GM crops, Canada produced only four varieties. GM varieties like alfalfa, apple, eggplant, poplar, potato and squash were grown in one country each. In 2015, Brazil had approved GM crops like *Phaseolus vulgaris* and eucalyptus for commercialization. Likewise, transgenic rice, wheat, sorghum, cassava, banana, camelina, citrus, chickpea, cowpea, groundnut, mustard, pigeon pea, chestnut (*Castanea dentata*) and safflower (*Carthamus tinctorius*) were in various stages of progress (James 2014, 2015). Data for commercially grown GM varieties are available for nine food crops, three non-food crop plants and also two types of flowering plants for the year 2015 (James 2015). Among them, maize and soybean crops were the widely grown across the globe. In 2018, a total of 70 countries adopted GM crops through cultivation and importation (NRC 2018). About 191.7 million hectares of GM crops were planted in 26 countries (21 developed and

5 industrialized). The USA, Brazil, Argentina, Canada and India are the top five countries with the largest area of GM crops planted, collectively occupying 91% of the global GM crops area. The cultivation of new-generation herbicide tolerant cotton and soybean, low gossypol cotton, roundup ready (RR) and low lignin alfalfa, omega-3 canola and insect resistant (IR) cowpea has been approved for plantation in 2019 (NRC 2018).

3 Commercial GM Crops and Vandalism

Regrettably, general public is still not acceding and endorsing the GM food. Destruction of public and governmental experiments of GM crops were reported in many countries during trials in the open fields. Kuntz (2012) reported destruction of a trial of a wide variety of GM crops in France, Germany, the UK and Switzerland. The loss from such damage has been estimated at 1.2 million Euros. Sadly, there is a widespread rejection of GM foods all over the world. This is something worth pondering in the right spirit, understood and debated from different sections in the scientific circles, politicians, policymakers, nongovernmental organizations and general public. GM crops draw the public attention and hence needs discussions. Needless perhaps to mention that genetic engineering is not discovered by humans, but a naturally happening phenomenon. It is a continuous process. The fact is that every organism is genetically modified, but naturally. Utilization of GM crops must be discussed and debated in this context for the larger benefits of the society. GM crops have been rejected by European Union (EU), yet large number of European countries import GM agricultural products like soybean meal and soybeans as a feed for livestock. European countries import GM soybean meal and soybean from Argentina, Brazil and the USA to the tune of $9 and $6.5 billion per year, respectively (Dunwell 2014). How can countries that do not grow GM crops in their own farm lands are importing from other countries? (Masip et al. 2013). That is seemingly absurd and certainly paradoxical.

4 The Second-Generation Transgenics with Industrial Applications

While the first-generation transgenics were concentrated on transfer of single genes that influenced distinct agronomic characters, researchers then focussed to develop transgenics with a wide spectrum of genes that influenced industrially important products. Such a product generation would depend upon genome-wide screening, identification and validation of candidate genes. The appearance of next-generation transgenics greatly impacted the environment and industry. Transgenic yellow poplar (*Liriodendron tulipifera*) with bacterial mercuric reductase gene can now be used for phytoremediation of industrial wastes such as ionic mercury (Rugh et al. 1998). Transgenics release elemental mercury at significantly higher levels compared to wild-type plants. Transgenic mustard with increased tolerance to cadmium

(Zhu et al. 1999) and tobacco engineered for degrading hydrocarbon pollutants have also been generated (Dunwell 1999).

4.1 Bioenergy and Ethanol Production

It is known that poplar and eucalyptus are being used as feedstock for the production of ethanol. By improving cellulose content using biosynthetic pathway gene manipulations, Arioli et al. (1998) and Hu et al. (2013) generated transgenics with improved biomass. Similarly, overexpression of cellulase enzyme resulted in improved ethanol production to be used in automobile industry (Lebel et al. 1998). Further, reduction in lignin by downregulation of lignin biosynthetic pathway genes improved cellulosic biomass and alcohol production (Bauscher et al. 1998; Lapierre et al. 1999; Prashant et al. 2011; Hu et al. 2013).

4.2 Bioplastic Industry

Since plastics have been banned, bioplastics need to be developed. Poirier (1999) has reported that Monsanto developed a transgenic oilseed rape which expresses polyhydroxybutyrates (PHB) in the leucoplasts nearly 8% of its dry biomass. As a novel concept, the genes have been expressed in rubber trees. This helps us to harvest the bioplastics continuously by tapping the latex and without demolishing the plants (Arokiaraj et al. 1998).

4.3 Coloured Cotton and Textile Industry

Cotton is best known for its insect resistance with the incorporation of *Bt* gene. It is being grown widely in several countries including India (John 1997; Dunwell 1999). But introduction of pigment compounds such as melanin for black colouration and also other colours would be of interest since it can preclude the dyeing of cotton fabric. Permission to grow such transgenic coloured cotton would help the textile industry. Use of fibre-specific promoters can help create such fabrics which is certainly the need of the hour. Natural brown and green-coloured fibres exist but poor fibre quality limits the utility of such coloured cotton (Liu et al. 2018). Therefore, transgenic coloured fibres were developed which is of immense help to the mankind. But like natural coloured fibres, transgenic coloured fibres are not only weaker but also shorter than wild-type controls (Liu et al. 2018). Thus, it is clear that potential exists for the genetic manipulation of flavonoid biosynthetic pathway genes to alter the colour of cotton fibre as well as quality. Further, it is of interest to note the synthesis of polyhydroxybutyrates in the fibre cells has helped thermal properties of the cotton fibre (Chowdhury and John 1998; Hankermeyer and Tjeerdema 1999; Poirier 1999). Thus, the textile industry would be benefitted if the GM plants are permitted to grow.

4.4 Paper and Pulp Industry

Reduction in lignin content has a bearing in pulping process and paper industry. Field trials of several transgenics are still on and pulping tests are being conducted for use of GM plants in paper industry (Bauscher et al. 1998). If such transgenics are brought to use, the paper industry would be massively benefitted.

4.5 Production of Terpenoids and Mint Oil

Several mono- and sesquiterpenes are used in flavour, perfume and pharmaceutical industries. One such molecule is mint oil with nearly $6 billion industry including its processed products (Lange and Croteau 1999). Genetic manipulation for the production of mint oil, especially *p*-menthane monoterpene metabolism in peppermint industry has resulted in (−)-menthone to (−)-menthol (Lange and Croteau 1999). Likewise, attempts to increase the density of glandular trichomes of *Mentha* species are being made. If they succeed, GM crops with better yields of terpenoid compounds would be available for use in flavour and fragrance industries.

4.6 Transgenic Plants in Pharmaceutical Industry and Veterinary Applications

Transgenic plants have been developed for the production of many pharmaceutically important compounds, valuable chemicals, vaccines, antigens, antibodies, enzymes and growth factors (Lee et al. 1997; Arakawa et al. 1998; Gruber et al. 1998a; Somerville and Bonetta 2001; Daniell et al. 2001; Fischer et al. 2004; Ortiz and Swennen 2014; Ankita et al. 2016). Among plant-derived compounds under the category, plant protein toxin called ricin produced by the *Ricinus communis* has considerable use in pharmaceutical industry as a therapeutic agent (in cancer and apoptosis). Sehnke and Ferl (1999) produced safe recombinant ricin, but not yet commercialized. More importantly, human haemoglobin (Dieryck et al. 1997) and collagen (Gruber et al. 1998b) have been produced in plants. These products have the potential for commercialization but have not been launched. Environmental effects of transgenic plants have been thoroughly discussed at diverse fora by scientific experts (Domingo 2016; Kumar et al. 2018; Giraldo et al. 2019), but the scope and adequacy of regulation is always under hammer in majority of the countries (National Research Council 2002). Domingo (2016) reported that the assessed GM soybeans, rice, maize and wheat are shown to be safe like that of parental species. Where controversies exist, there he noticed lack of proper reports for many GM crops. The report of WHO as well as the assessment of published literature by Domingo (2007) reveals that the GM products (canola, corn, cucumber, peas, pepper, potatoes, rice, sweet pepper, soybean, and tomatoes) being used currently on the international market have passed risk assessments conducted by respective national authorities. Not surprisingly, different assessments have not recorded any

potential toxicity of GM or risk to human health (Domingo 2007). Gene flow has occurred from transgenics to wild/related species, but no one cited any example that demonstrated an adverse environmental effect of such a gene flow from GM crops. However, long-term studies are crucial on the safety and health effects of GM crops with reliable scientific data. The National Research Council recommends "public-sector investment in GM crop risk analysis, better methodologies and protocols for development of GM plants". Committee on GM crops assessed the rigour of all available evidences that support or negate the claims about the potential human health risks/benefits of several GM foods (NASEM 2016). Further, FDA in the USA have not allowed any GM food until such food is proven safe for human consumption (NASEM 2016). The outcry by the researchers for the legitimate release of GM crops is valid, but it is perhaps vital to improve the transgenic methods that will reduce the risks to the environmental safety. It is also recommended that GM crops must be subjected to safety testing if they have intended or unintended qualities if any with potential hazards to animals and humans. A comparison of the molecular profiles of the GM crops with those of their counterparts already in use is perhaps recommended. Also, the governance of all GM crops should be transparent and participatory before they are released to the public. This would instil confidence and also widespread acceptance among the consumers. It is therefore essential to monitor GM crops for the effects on the environment, the spread of transgene to the wild relatives, on animal and human health and also intense research on social, economic and value-based issues that damage and devastate our precious environment. Research on such aspects is urgently warranted since we need to bring the fruits of GM crops on to the table by minimizing the environmental and human health risks if any.

5 Policy Issues

- Policy issues may change as the type of transgenics changes. But, research funding for hazard identification and risk assessment studies is meagre world over.
- We need to develop scientifically sound protocols to find out if the transgenes are causing any damage to the environment (Devos et al. 2016) and also to the non-target organisms. Protocols available at our disposal today are effective in finding out the toxic chemicals being spread if any and the sequence of their broad ecological consequences.
- The effect of horizontal transgene transfer to pollinators, soil microbiota and conservation of species must be evaluated for several seasons across the countries (Giacomo et al. 2016).
- Further, the movement of transgene if any needs to be traced in the wild relatives.
- It is also vital for us to comprehend if the genetic modifications are affecting the invasiveness of the species.
- Regulatory systems that are in place across the globe must be effective and efficient to assess the GM crops and the ecological damage, animal and human health risks if they are causing. Existing regulatory issues need to be strength-

ened, improved and modified. Such regulatory policies must be based on our vast experience, sound scientific principles and methodologies being used.

- Possible environmental hazards or ecological effects of the transgenes must be carefully and critically assessed independently and monitored by several scientific groups rigorously for a long time. Such a mammoth effort certainly reduces the risk of transgenes and their potential environmental hazards if any.
- GM crops or their products that are substantially equivalent with their counterparts can only be given approval for commercialization, and such GM crops must be evaluated both spatially and temporally in a cost-effective manner.
- More importantly, the methods of gene transfer or modifications should reduce the risks and improve benefits to ecosystems. The methods of tissue culture can cause genetic variation (somaclonal variation), hence must be avoided for gene transfer. Instead, the technology of gene editing like CRISPR-Cas9 may be a superior way of gene editing for getting required benefits.
- The change in nutritional characteristics in GM crops as compared to their counter parts should be evaluated carefully over a period of time (Pauwels et al. 2015).
- A detailed study on the toxicity and allerginicity of GM foods should be performed (Domingo 2007; De Santis et al. 2018).
- Also, transgenic events with single-gene insertions are preferred to avoid gene silencing in subsequent generations and for subsequent safety assessment (Tiwari and Singh 2018). In several labs, such a procedure is being followed which can ensure us stable integration and expression of the transgenes.
- An intensive research must also be carried out in different countries if gene stacking or trait stacking is leading to the sale of GM seeds that are exorbitant than what resource-poor farmers can afford.
- The labelling of GM foods should be mandatory (Huffman and McCluskey 2017; Kamle et al. 2017; Moghissi et al. 2018).
- GM traceability which enables tracking of GM food or feed products at all stages of the supply chain should be considered (Giraldo et al. 2019).
- If better technologies such as CRISPR-Cas9 are adapted, then it must be debated publicly, and consensus opinion must be arrived. The consequences of such genetic tinkering on ethical, legal and social issues must be resolved and addressed properly before the release of GM foods.
- Besides product safety, policymakers should also carefully address all issues related to technology governance, be it a private or public sector, and also competing interests of stakeholders and associated trade-offs.

6 Conclusions

The ability to isolate and insert genes of interest into crop plants at will with tissue-specific promoters is a milestone. We now have the potential tools to introduce multiple genes into the host plant of interest that can affect polygenic traits. Newer protocols with improved efficiency and single gene insertions have been developed for a majority of crop plants and irrespective of the genotype. Despite the generation

of diverse GM crops with remarkable improvement in tolerance to biotic and abiotic stresses, herbicide tolerance and nutritional quality improvement, we do not grow many GM crops in the field conditions. Further, governments do not have the resolve to strictly follow the regulatory systems so as to take care of the ecosystems. General public has been opposing the introduction of GM crops tooth and nail, but without much debate on safety issues. WHO has been assessing the human health risks due to consumption of GM food, but could not find any potential toxicological risks. The results obtained through several independent projects, and nearly four decades of transgenic research data generated in both public and private sectors around the world revealed that GM foods *per se* are not risky in comparison with plant breeding technologies (European Commission 2010). These facts infer that genetic engineering technologies, and the GM crops are not risky to animal and human health and do not cause any harm to the environment. It is time for us to review the current and future commercial status of GM crops and their benefits/risks to the society at large. The situation in Europe is totally different since we have dichotomy of experience. Paradoxically, they do not grow GM crops but import the same from other countries. Our attitude towards GM crops must change in future since opportunities and benefits abound with GM crops, but with a note of caution about environmental safety, ecological security and animal and human health risks.

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Genetic Improvement of Rice for Food and Nutritional Security

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Abstract

Rice is a staple food consumed by almost half of the world's population. However, in a natural environment, like any other plant, rice is exposed to various abiotic stresses such as salinity, drought, and high temperature, which in turn affect its yield. Therefore, to meet the demand of the world's growing population, it is imperative for scientists to come up with novel strategies of combating these abiotic stresses. Over the years, transgenic rice showing improved performance under stresses such as salinity, drought, and cold have been developed using genetic engineering approaches. Additionally, scientists have also developed rice that has higher nutrient content such as, golden rice, folate-biofortified rice, iron-fortified rice, and zinc-fortified rice. In this chapter, we discuss how plants respond to heat, cold, salinity, drought, and flooding stress with an emphasis on the physiological, biochemical, and molecular mechanisms of stress tolerance. Further, we also present a few representative success stories where attempts have been made towards improving the nutritional value or for enhancing stress tolerance in rice. This information may help in promoting the interdisciplinary studies designed to assess the stress-responsive genes and their role under various abiotic stresses along with a target of improving the nutritional value in rice.

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Abiotic stress · Tolerance · Genetically modified rice · Golden rice · Nutrition

1 Introduction

Practices of crop selection and breeding to enhance yields have been adopted since the beginning of the agricultural era which dates back to about 10,000 years (Voss-Fels et al. 2019). The mode of choosing for higher and better crops is followed mostly to compensate the increase in demands with the rise in population. It is estimated that by the year 2050, the world population would reach nine billion (https://www.un.org/development/desa/en/news/population/world-population-prospects-2017.html, accessed on 5 March 2020). To feed this growing population, it is estimated that the global food production should increase by 44 million tons each year (Qaim 2009). However, with the changing climate causing severe environmental degradation leading to drastic reduction in soil fertility and the severity of drought, salinity, high temperature, cold, etc. becoming more prevailing (Pareek et al. 2020), a challenge is laid before us to produce such a high volume of crops.

In spite of high carbohydrates and sugar content, the nutritional value for most of the rice types are found to be low (Gregory et al. 2017). At the same time, nearly half of the world's population is dependent on rice as a staple food. In Asia alone, it is estimated that approximately 1.3 billion people consume rice every day (Maclean et al. 2013). Therefore, if rice with high nutritional contents can be developed, more than half of the world's population will be free from malnutrition. This is one of the reasons why rice draws attention of crop scientists who are working towards food and nutritional security missions of the countries.

Genetic engineering for crop development was introduced in the early 1980s (Wieczorek and Wright 2012), and by mid-1990s, the first genetically modified (GM) plant, tobacco with resistant to herbicide was released in France and USA. However, the first commercially available GM food, tomato with delayed ripening was released in 1994 from the University of California, Agriculture and Natural Resources by the name *Flavr Savr* (Bruening and Lyons 2000). Subsequently, other crops such as maize, canola, and soybean with various traits were also genetically modified and released commercially in several countries including Argentina, Canada, and the USA (Anderson et al. 2004). Crops developed through genetic engineering do not vary much from those pursued by conventional breeding. However, the benefit of using genetic engineering over traditional breeding is that it is a targeted approach and takes shorter time to develop the desired traits. Through this technique, several traits that were impossible through conventional breeding have also been developed (Qaim 2009; Zafar et al. 2019).

Keeping in mind the importance of rice as a staple food crop, there is an urgent need to have food security along with nutritional security with a clear focus on this crop. In this chapter, we present a few representative success stories targeting the development of genetically modified rice for nutrient enrichment and enhanced

tolerance to various abiotic stresses. Additionally, we also briefly touch on the economic benefits of GM rice.

2 Traditional Methods to Develop the New Rice Types

The conventional approach to develop improved rice varieties primarily relies on the tools of plant breeding. Several novel genotypes carrying a desired character have been generated by crossing different parental lines (Schaart et al. 2016; Hickey et al. 2017). One of the best example in this category is the development of dwarf wheat genotypes during green revolution (1960s). This dwarf variety of wheat was high yielding and resistant to lodging (Swaminathan 2000). After this successful breakthrough, several plant breeders and scientists continued further work on developing rice varieties through conventional breeding approaches that resulted in improved grain quality (nutritional) and disease resistance (Breseghello and Coelho 2013).

Broadly, conventional breeding can be categorized as follows: (1) *Pedigree breeding*, in this approach, two contrasting parental lines are crossed to generate segregating populations, and cultivar with desirable characteristics is selected. This method can be applied only to self-pollinating species for developing suitable quantitative traits like disease resistance and plant architecture-related traits like shape or color of plant parts (Crossa et al. 2017). (2) *Ideotype breeding*, this approach is based on the hypothesis that complex traits can be improved by modifying the individual traits that govern specified phenotype. Ideotype breeding addresses the strategy to improve the pedigree method, so that yield can be promoted (Rasmusson 1987; Peng et al. 2008). (3) *Population breeding*, this approach focuses on the methods designed for the intermating population so that their phenotypic performance can be improved. To achieve this goal, frequency of favorable alleles is increased that is controlling the desirable traits (Breseghello and Coelho 2013). (4) *Hybrid breeding,* in this breeding technique, two homozygotic but genetically different parental lines are crossed resulting in the development of heterozygotic offspring (Cui et al. 2020). Using these breeding techniques, considerable efforts have been made to develop rice varieties that have high nutrient content and are tolerant to stress. One of the recent examples is the development of "the new plant type" (NPT), a rice variety, by a group of scientists from the International Rice Research Institute (IRRI), Philippines, which produces more than 200 grains per panicle. This variety has dark green leaves that are erect and thick, whereas the panicles are larger and stronger than the parental lines and thus can withstand the weight of the grains (Uphoff et al. 2015).

3 Raising Genetically Modified Rice with Nutrient Enrichment

Over the years, several successful attempts have been made to improve the nutritional content of crops through genetic engineering (Ye et al. 2000; Akhtar et al. 2013). For brevity sake, some of these success stories that have left an impact at a global scale against the fight for malnutrition and hunger are summarily presented in Table 1.

Table 1 Representative success stories for the genetic modification in rice for the selected traits

GM rice	Target gene(s)	Method of transformation	Trait improvement	Reference(s)
Genetically modified rice for nutritional enrichment				
Golden rice	*Psy* and *CrtI*	*Agrobacterium*-mediated transformation	Provitamin A enrichment	Ye et al. (2000)
Folate-fortified rice	*GTPCHI* and *ADCS*	*Agrobacterium*-mediated transformation	Folate biosynthesis and enrichment	Storozhenko et al. (2007)
Iron-fortified rice	*AtNAS1* and *Pvferritin*	Biolistic-mediated transformation	Iron enrichment	Vasconcelos et al. (2003)
Zinc-fortified rice	*OsNAS1*, *OsNAS2*, and *OsNAS3*	Biolistic-mediated transformation; *Agrobacterium*-mediated transformation	Zinc enrichment	Vasconcelos et al. (2003), Johnson et al. (2011)
Genetically modified rice for enhanced stress tolerance				
Glyphosate-tolerant rice	*CP4-EPSPS*	*Agrobacterium*-mediated transformation	Glyphosate tolerance	Chhapekar et al. (2015)
BT rice	*CryI*	Electroporation transformation	Resistance to Lepidopteran pests	Fujimoto et al. (1993)
Salinity-tolerant rice	*SOS1, SERF1, SOS2, SOS3, STRK, CNAtr, MYB2, MnSOD, GS, katE, ADC, codA, SAMDC, NHX1,* Os*KAT1*, Os*Cyp2* Os*HBP1b*, Os*GATA8*, Os*PGK2-P*, Bj*GLY I*, and Os*GLY II*	*Agrobacterium*-mediated transformation	Salinity tolerance	Tanaka et al. (1999), Hoshida et al. (2000), Roy and Wu (2001), Mohanty et al. (2002), Ma et al. (2005), Nagamiya et al. (2007), Verma et al. (2007), Singh et al. (2008), Kumari et al. (2009), Joshi et al. (2016), Lakra et al. (2015), Gupta et al. (2018), Nutan et al. (2020)
Drought-tolerant rice	*P5CS2, ICE1, HOS1, OsNAC14, COX1, PKDP, bZIP1, AP2-EREBP, Hsp20, DREB* family, *ABF3, SNAC1, COC1*, and *OsLG3*	*Agrobacterium*-mediated transformation	Drought tolerance	Singh et al. (2008), Saakre et al. (2017), Shim et al. (2018), Xiong et al. (2018)

(continued)

Table 1 (continued)

GM rice	Target gene(s)	Method of transformation	Trait improvement	Reference(s)
Cold-tolerant rice	*P5CS2, ICE1*, and *HOS1*	*Agrobacterium*-mediated transformation	Cold tolerance	Hur et al. (2004), Jung et al. (2013)
Heat-tolerant rice	*HIL1, OsHsfB2c*, *PM19*, and *Hsp90*	*Agrobacterium*-mediated transformation	Heat tolerance	Rerksiri et al. (2013), Higashi et al. (2018)
Submergence-tolerant rice	*Sub1A, Sub1B*, and *Sub1C*	*Agrobacterium*-mediated transformation	Submergence tolerance	Xu et al. (2006)

3.1 Golden Rice for Curing Night Blindness

In every rice plant, the activity to synthesis β-carotene, a precursor of vitamin A, is active in the leaves; however, in the seeds/grains, this machinery gets switched-off. Two groups of scientists, Peter Beyer and Ingo Potrykus, developed golden rice by introducing plant phytoene synthase (*psy*) and a bacterial carotene desaturase (*crt I*) in the endosperm of rice grain which enables the synthesis of β-carotene (Ye et al. 2000). With this introduction, the seeds could synthesize roughly 1.6 μg/g of β-carotenoids. Paine et al. (2005) further improved the golden rice by introducing *psy* gene obtained from maize. This further led to the increased synthesis of β-carotene by almost 23-fold (37 μg/g) than the normal rice. This was later commercialized as "Golden rice 2" (Paine et al. 2005).

Golden rice is developed to fight against vitamin A deficiency (VAD) that is prevailing in most of the underdeveloped countries. Deficiency of vitamin A leads to several health problems such as night blindness, diarrhea, respiratory diseases, and measles (Akhtar et al. 2013). According to the present report recorded in the World Health Organization (WHO) database, about 250 million pre-school children are affected by VAD. In the region severely affected by VAD, majority of them are pregnant women which further complicates the child they carry. It is also recorded that about 250,000–500,000 children become blind due to VAD every year and almost half of them are dying by the age of 12 months after losing sight (https://www.who.int/nutrition/topics/vad/en/ accessed on 5 March 2020).

3.2 Folate-Fortified Rice

Folate or folic acid is a type of vitamin B (B12) that helps in DNA biogenesis, DNA repair, and production of red blood cells (RBC) (Moll and Davis 2017). This water-soluble vitamin is present richly in fruits, green vegetables, and liver. In the USA, folate is added in the grain as a dietary supplement (Khan and Jialal 2019). Folate deficiency leads to various disorders, including neural tube defects, a defect of

brain, spine, or spinal cord of the child by birth (Safi et al. 2012). However, in most of the developing countries, deficiency of folate is still very common. Therefore, several programs for folic acid fortification to fight against folate deficiency are common in countries such as India, South Africa, and some parts of Asia (Hoddinott 2018).

Folate is made up of three molecules, which include one or more glutamate residues, a pterin moiety, and *para*-aminobenzoic acid (PABA). Folate biofortification of rice has been carried out successfully through molecular engineering by overexpression of *Arabidopsis thaliana* GTP cyclohydrolase I (GTPCHI) and aminodeoxychorismate synthase (ADCS) genes in rice endosperm by a group of scientists from the Unit Plant Hormone Signaling and Bio-imaging Laboratory, Department of Molecular Genetics, Ghent University, Belgium. By overexpressing these genes, they have obtained a total of 89% increase in folate accumulation in the form of 5-methyltetrahydrofolate in rice (Storozhenko et al. 2007).

3.3 Iron-Fortified Rice

Iron deficiency is a prevalent nutritional disorder across the globe (Bailey et al. 2015). Although current global statistics are not available, according to the WHO/United Nations International Children's Emergency Fund/United Nations University (UNICEF/UNU), iron deficiency cases have been reported in most preschool children and pregnant women in developing countries. Fortification of iron is not easy as most soluble compounds of iron, that the body can absorb, such as $FeSO_4$ are not palatable (Nagpal and Choudhury 2004; Wirth et al. 2009). A major breakthrough on iron-fortified rice has been achieved by Wilhelm Gruissem and team from the Department of Biology, Plant Biotechnology, ETH Zurich, Universitätstrasse, Switzerland. They expressed *Arabidopsis thaliana* nicotianamine synthase 1 (AtNAS1), and *Phaseolus vulgaris* ferritin (Pv*ferritin*) genes in rice and observed more than six fold increase in iron concentration in the endosperm of the transgenic rice (Wirth et al. 2009). Similarly, Johnson and team at the University of Melbourne, Australia, 2011, constructed three transgenic rice populations overexpressing *OsNAS1*, *OsNAS2*, and *OsNAS3*. The overexpression of *OsNAS* genes increases not only the iron but also zinc concentration in rice (Johnson et al. 2011). Swapan K. Datta and his team in India transformed the *Indica* rice with the soybean *ferritin* gene. The ferritin-transgenic lines of *Indica* rice accumulated higher iron and zinc content even after seed polishing (Vasconcelos et al. 2003).

4 Raising Genetically Modified Rice with Enhanced Tolerance to Abiotic Stresses

Abiotic stresses such as salinity, drought, extreme temperature, mineral toxicity, and nutrient deficiencies reduce the growth of plants and thus decrease crop yield (Roy et al. 2011; Tripathi et al. 2012). Stress episodes can be transient or chronic,

and the impact of stress on crop viability and yield is determined by the timing of stress as well as on the developmental stage of plants. Stresses during vegetative stage causes slow growth in terms of cell expansion and division. However, stresses during the reproductive stage can considerably reduce their productivity (Basu et al. 2016; Sehgal et al. 2018). Under natural conditions, stresses occur in combination or succession, thus necessitating the plant to develop diverse mechanisms for adaptation (Mickelbart et al. 2015; Bahuguna et al. 2018).

Plants being a sessile organism and stresses a complex phenomenon, plants have developed multiple sensors to perceive stress signals (Vranová et al. 2002). After the stress signal is recognized, a signaling cascade is initiated, which involves the relay of secondary messengers, ultimately resulting in generation of stress response by activating stress-related genes (for review, see Nongpiur et al. 2019). Stress-responsive genes help the plant to withstand stress conditions either through a short-term or long-term response (Joseph et al. 2010). Some of the stress-responsive genes comprise genes that encode enzymes for reactive oxygen species (ROS) scavenging, compatible solute biosynthesis, and modifying lipid saturation in the membrane, heat shock proteins (HSPs), transcription factors, late embryogenesis-abundant proteins (LEA), and proteins required for maintaining ion homeostasis (Zhang et al. 2000). The products of the stress-induced genes mainly contribute to tolerance via two major mechanisms: stress tolerance and signal transduction (Mittler 2002). These include biosynthesis of chaperones, osmolytes, and detoxification enzymes (Joseph et al. 2010). Some of the abiotic stresses that cause decline in crop yield are discussed below.

4.1 Heat Stress

Heat stress affects plants at all phases of its life cycle, i.e., seed germination, vegetative, and reproductive stages (Zinn et al. 2010; for review see Sehgal et al. 2018). The lipid component of the cell membrane is responsible for its fluidity. Heat stress affects the fluidity of the membrane by decreasing the polyunsaturated acyl groups, mainly hexadecatrienoate (roughanic acid) (16:3) and α-linolenate (18:3) of the chloroplast. These polyunsaturated acyl groups are mostly bound covalently to the monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) (Higashi et al. 2018). Mechanism for tolerance against heat stress is well-defined in plants, one of which involves a signaling cascade in which heat shock factors (HSF) get activated in response to heat shock and results in further activation of several HSPs as shown in Fig. 1. The adverse effect of heat shock includes accumulation of unfolded or misfolded proteins in the endoplasmic reticulum and cytosol that hamper the interaction between transcriptional factors and molecular chaperons. These events trigger the activation of unfolded protein response (UPR), mediated by several transcription factors belonging to Arabidopsis transcription activation factor 1 (ATAF), basic leucine zipper (bZIP) or no apical meristem (NAM), and cup-shaped cotyledon (CUC) (NAC) family (Zhang et al. 2017). The quality control mechanism

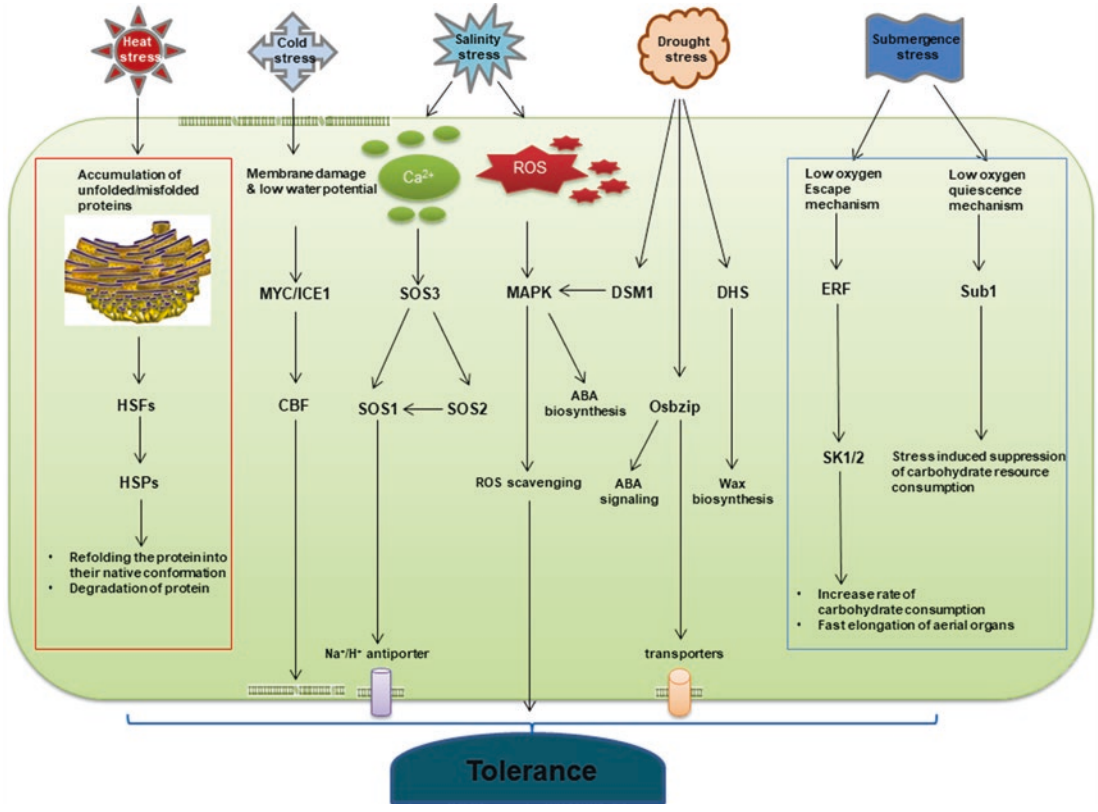

Fig. 1 The complexity in abiotic stress response machinery in rice. Upon heat stress, several unfolded or misfolded proteins start accumulating in endoplasmic reticulum and cytosol that leads to the activation of HSFs which in turn activates HSPs. The HSPs deal with misfolded proteins by either refolding them into their native states or promoting their degradation. Cold stress results in membrane damage and low water potential that activate many transcription factors like

MYC and ICEI which in turn activates CBF. Upon salt stress, cytosolic calcium production increases, leading to the activation of the *SOS3* gene, which in turn activates the *SOS2* gene. Upon activation, both SOS2 and SOS3 regulate the expression of *SOS1* that in turn enhances the expression level of Na^+/H^+ antiporter-related genes. Likewise, ROS level also increases in the cell which activate MAPK pathway through which ROS scavenging takes place thus resulting in tolerance. Upon drought stress, MAPK pathway gets activated by DSM1 and results in ROS scavenging and ABA biosynthesis, whereas transcription factor OsbZIP, via ABA signaling, enhance the tolerance. On the other hand, DHS works ABA-independently and takes part in wax biosynthesis, which in turn increases the tolerance. Submergence stress in plants is tackled by two prime mechanisms, one is 'low oxygen escape mechanism' and other one is 'low oxygen quiescence mechanism'. Low-oxygen escape mechanism involves traits such as increased rate of carbohydrate consumption that results in the fast elongation of aerial organs (by the action of SK1/2, which in turn are regulated by the ERF genes). The low oxygen quiescence mechanism involves stress-induced suppression of carbohydrate resource consumption regulated by the *Sub1* genes. *HSFs* heat shock factors, *HSPs* heat shock proteins, *CBF* c-repeat/dehydration-responsive element binding factors, *ICEI* inducer of CBF expression1, *SOS1/2/3* Salt Overly Sensitive 1/2/3, *ROS* reactive oxygen species, *MAPK* mitogen-activated protein kinases, *DSM1* drought-hypersensitive mutant1, *ABA* abscisic acid, *Osbzip* basic leucine zipper, *DHS* drought hypersensitive, *SK1/2* SNORKEL1/2, *ERF* ethylene-responsive factor, *SUB1* submergence1

deals with misfolded proteins by either refolding them into their native states or promoting their degradation via distinctive ubiquitin-proteasome pathways. The degradation of misfolded proteins is initiated by molecular chaperones like HSP70 and HSP90 (Gil et al. 2017).

4.2 Low Temperature Stress

Low temperature or chilling stress affects different aspects of plant growth and development. Several cold-responsive genes such as MYC transcription factor, INDUCER OF CBF EXPRESSION1 (ICE1), get activated during cold stress and thus induce many transcription factors such as C-repeat/dehydration-responsive element binding factors (CBFs) (Fig. 1), which in turn regulate the downstream genes which ultimately result in cold responses (Oh et al. 2005; Jung et al. 2013). Like heat stress, cold stress also influences membrane fluidity by changing the composition of lipids and proteins. Leaf ultrastructure is also found to be changed during cold stress. Organic molecules such as proline accumulate during low and high temperature, drought, salinity, and heavy metal stress (Hur et al. 2004).

4.3 Salinity Stress

Salinity stress affects plant growth drastically and disturbs cellular homeostasis. However, some plants belonging to halophytes can tolerate high salinity (Wungrampha et al. 2019a, b) without compromising their yield (for review, see Sharma et al. 2016). High salinity leads to physiological drought causing cellular dehydration. This further stimulates the biosynthesis of a phytohormone abscisic acid (ABA). Under high salinity, accumulation of excess ROS, such as superoxide anion and hydrogen peroxide (H_2O_2) through nicotinamide adenine dinucleotide hydrogen phosphate (NADPH) oxidase-dependent H_2O_2 production, is also observed which further causes osmotic stress to the plants. ROS, under a minimum threshold concentration, has been reported to be involved in various biological processes (Schmidt et al. 2013; for review, see Wungrampha et al. 2018). Principal mechanisms of salinity stress tolerance in plants include (1) ion homeostasis and compartmentalization, (2) biosynthesis of compatible solutes and osmoprotectants, (3) ion transport and uptake, (4) synthesis of polyamines, (5) hormone modulation, (6) activation and synthesis of antioxidant enzymes and compounds, respectively, and (7) generation of nitric oxide (NO). Plants cannot withstand high salinity in their cytoplasm, so most plants have developed a mechanism in which they transport the excess salt into the vacuole or in older tissues, which would be sacrificed eventually (Gupta and Huang 2014). Salinity responses in plants are multigenic in nature. Salt Overly Sensitive (SOS) pathway is one of the well-understood mechanisms adopted by plants to tolerate high-salinity conditions. SOS1 is a Na^+/H^+ antiporter which acts as the primary regulator for ion homeostasis under salinity stress (Ji et al. 2013). SOS pathway is a calcium-dependent protein kinase pathway that involves several genes, including *SOS3*, *SOS2*, and *SOS1*. *SOS3* senses the

salinity-induced calcium signal and activates a serine/threonine-protein kinase, i.e., SOS2. As shown in Fig. 1, SOS2 and SOS3 upon activation regulate the expression of *SOS1* that in turn enhances the expression level of Na^+/H^+ antiporter-related genes (Zhu 2002; Wu 2018).

Two-component system (TCS) is one of the crucial signal sensing machinery that regulates the cellular responses of the plant toward the environmental stimuli. Chang et al. (1993) were the first to discover this system in plants. Since then, two-component machinery has been reported in several plants, including rice (Pareek et al. 2006). It is also known as His-to-Asp phosphorelay, as histidine kinase (HK) that gets autophosphorylated in the presence of a signal, by using adenosine triphosphate (ATP) as a substrate, transfer a phosphate group to the histidine phosphotransferase (HPT). Further, this phosphate group is transferred to the response regulator (RR) by HPT (Sharan et al. 2017) (Fig. 2).

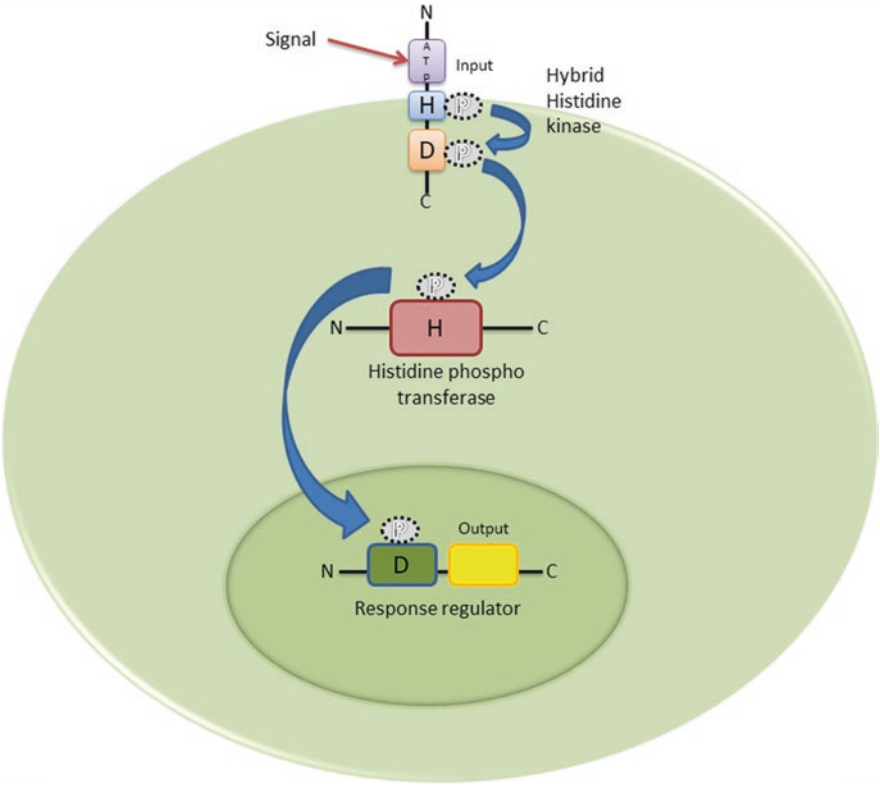

Fig. 2 Schematic representation of two component system (TCS) in rice. A hybrid-type TCS in which the conserved histidine and aspartate are found in the same protein, which serves as the sensory histidine kinase and is usually membrane bound. The phosphotransfer protein is a shuttle protein which acts as a mediator for the transfer of the phosphoryl group between the histidine kinase and the response regulator which is residing in the nucleus. *H* His, *D* Asp, *P* phosphoryl group, *N* amino-terminal of protein, *C* carboxy-terminal of protein

TCS in rice consists of a total of 51 genes that encodes for 73 proteins, from which 22 HKs, 7 HPTs, and 44 RRs are encoded by 14 histidine kinase genes, 5 phosphotransfer genes, and 32 response regulator genes. HKs consist of three families: the typical OsHK family which is a cytokinin receptor homologs, an ethylene receptor (ETR) homolog family (further divided into subfamilies—OsETRs and OsERSs), and the phytochromes (OsPHYs). These HK families have a predicted range of 359–1186 amino acids in size showing considerable variation in structure and function (Pareek et al. 2006). *AHK1*, *AHK2*, and *ETR2* genes show downregulation under cold stress in roots of *Arabidopsis*, whereas *AHK3* shows downregulation in shoots. *PHYC* gene shows downregulation in both shoots and roots, whereas the *PHYE* gene shows upregulation in shoots only. Under drought stress, *AHK1*, *ETR2*, and *PHYB* genes show upregulation in the *Arabidopsis* plant. In the case of heat and osmotic stress, the *AHK1* gene shows downregulation, and the *ETR2* gene shows upregulation in roots. Under salt stress, all the HK genes show upregulation except *AHK1* and *PHYA* in roots. In rice, *OsHK3* and *OsETR3* show upregulation, whereas *OsHK2* shows downregulation of expression under drought stress. HPT genes show the opposite behavior as compared to HKs in *Arabidopsis* under all these stresses. Under osmotic stress, only *AHP4* and *AHP6* genes show upregulation, whereas other HPTs show downregulation in shoots. Under cold stress, only the *AHP6* gene exhibits downregulation in shoots. Furthermore, only the *AHP4* gene displays upregulation under heat stress. In rice, *OsHPT4* and *OsHPT5* show upregulation in expression under drought and salinity stresses. As for RRs, under cold stress conditions, the *APRR9* gene shows upregulation in both shoots and roots. *APRR4*, *APRR9*, and *ARR15* genes show upregulation in roots, whereas *APRR4*, *APRR9*, *ARR11*, and *ARR21* genes show upregulation in shoots. Under osmotic stress, only the *APRR9* gene shows upregulation in expression in the shoots. Likewise, in rice, the *OsRR1* gene exhibits downregulation under salinity and drought stresses (Singh et al. 2015).

Another mechanism for salinity tolerance involves the activation of antioxidant enzymes, which is mediated by ROS signaling. ROS including the hydroxyl radical ($OH^{\bullet}$), superoxide ($O_2^{\bullet}$), and H_2O_2 are the essential signaling molecules not only for salinity stress but also in other abiotic stresses. High salt concentration in cells results in a high ROS level that further results in the activation of mitogen-activated protein kinases (MAPK) signaling cascade and salinity stress-related genes (Kumar et al. 2012; Che-Othman et al. 2017) (Fig. 1).

Several attempts have been made to generate salinity tolerant rice by either identifying the novel genes responsible for tolerance or manipulating the known genes involved in salinity tolerance pathway. Kumari et al. (2009) identified a rice cyclophilin gene (*OsCYP2*) that confers tolerance against multiple abiotic stresses including salinity, osmotic, oxidative, and high temperature. Likewise, a nuclear-localized histone-gene-binding protein (OsHBP1b) identified in rice known to enhance the salinity tolerance. Scientists have generated *OsHBP1b* overexpression lines and observed that under salinity condition, transgenic plants accumulated low ROS level compared to wild-type plants (Lakra et al. 2015; Das et al. 2019).

Recently, scientists have discovered a *Saltol* QTL-localized transcription factor OsGATA8 and overexpressed it using transgenic approach. Overexpression of the gene results in high biomass accumulation, high photosynthetic efficiency, and approximately 46% higher yield under salinity stress compared to wild-type plants (Nutan et al. 2020).

4.4 Drought Stress

Crop production is severely affected under drought stress. There are diverse strategies adopted by plants to withstand drought stress, one of which is the deposition of cuticular wax on the leaves which acts as a barrier to reduce the water loss. Cuticular wax is made up of long fatty acids. Numerous genes from the family of ethylene-responsive factor/APETALA2 (EFR/AP2) and regulatory proteins such as wax synthesis regulatory gene 1 and 4 (WR1/4) have been identified for the biosynthesis of cuticular wax from wax crystal-sparse leaf mutants (Wang et al. 2012). Drought-sensitive phenotype is observed if these genes get mutated as cuticular wax content gets reduced (Fig. 1). Another protein, known as drought hypersensitive (DHS), which acts as a RING-type E3 ligase, is also found to stimulate wax biosynthesis in rice (Wang et al. 2018). It was recently reported that the expression of drought-inducible genes is controlled in an ABA-independent manner (Joshi et al. 2016). A transcription factor Osbzip-mediated via ABA signaling targets drought-tolerance-related genes (Zong et al. 2016) (Fig. 1). Additionally, drought-hypersensitive mutant 1 (DSM1) is a drought-responsive gene that confers tolerance against drought via the MAPK pathway (Fig. 1). Overexpression of MAPK5 in rice also showed salinity and osmotic stress tolerance by several folds (Ning et al. 2010; Schmidt et al. 2013).

4.5 Submergence Stress

Submergence of plants mostly affect photosynthesis and aerobic respiration. Under these conditions, several survival strategies are employed by the plant, including shifting to anaerobic respiration from aerobic respiration (Xu et al. 2006). Sudden flooding results in deprivation of nutrients due to the reduced level of oxygen and soil pH in rice fields (Singh and Sinha, 2016). Rice plant grows above the water level and restores the exchange of gases during submergence stress. It has also been observed that rice plants can get acclimatized to the submergence situation up to 14 days, but the duration is influenced by light levels, water turbidity, and temperature (Xu et al. 2006). Rice has evolved two mechanisms to deal with submergence stress: (1) low oxygen escape mechanism and (2) low oxygen quiescence mechanism. Low oxygen escape mechanism happens through a range of traits such as an increased rate of carbohydrate consumption that results in the fast elongation of aerial organs, so that leaves remain above the water level. This feature is

conferred by SNORKEL1/2 (SK1 and SK2), which are regulated by the ethylene-responsive factor (ERF) genes (Fig. 1) (Hattori et al. 2009). On the other hand, the low oxygen quiescence mechanism involves stress-induced suppression of carbohydrate resource consumption controlled by the submergence 1 (*Sub1*) region (Singh and Sinha 2016) (Fig. 1). *Sub1* gene family is present in the region on chromosome 9 consisting of three genes, i.e., *Sub1A*, *Sub1B*, and *Sub1C* which gets induced during submergence stress in rice (Xu et al. 2006), of which, tolerance is conferred only by *Sub1A* (Fukao et al. 2011).

5 Adoption of Genetically Modified Crops That Are Stress Tolerant

The USA, Canada, and Argentina have adopted GM varieties of maize, soybean, and canola during the late 1990s (Anderson et al. 2004). Some of the commercially adopted GM food crops are potato, squash, alfalfa, sugar beet in the USA and Canada, brinjal in Bangladesh, papaya in the USA and China. Oilseed rape is grown commercially in 4 countries, soybeans in 11 countries, cotton in 15 countries, and maize in 17 countries (royalsociety.org). Some varieties of GM rice with improved agronomic traits like resistance to pest and herbicide have the potential to increase farm productivity. According to Brookes and Barfoot (2003), within a decade, GM rice is expected to increase the yield per hectare nearly 10% higher for 40% of global production. GM rice such as BT rice, glyphosate-tolerant rice, and Sub1 rice that are some of the most important forthcoming products of genetic engineering are briefly discussed below.

5.1 BT Rice for Insect Resistance

Many tropical countries face severe damage to rice production due to a variety of insect pests. The lepidopteran pest alone annually damages approximately $681 million worth of rice production worldwide (Fujimoto et al. 1993). On the other hand, *Bacillus thuringiensis* (Bt), a Gram-positive bacterium that, upon sporulation, produces a crystal protein named δ-endotoxin encoded by crystal protein gene (*cry*), which is harmful to coleopteran, dipteran, and lepidopteran insects (Palma et al. 2014). With this knowledge, Plantech Research Institute, Japan, in 1993, developed the insect-resistant Bt rice. They used a truncated *cry1A(b)* gene isolated from *Bacillus thuringiensis* var. *kurstaki* into *Oryza japonica* using electroporation method to generate insect-resistant transgenic plant. The altered gene in transgenic rice offers resistance toward lepidopteran pests (Fujimoto et al. 1993). Additionally, a group of Chinese scientists investigated the Bt rice safety in aquatic ecosystem using zooplankton, and their results showed that Bt rice are safe to use even for aquatic ecosystem (Li et al. 2014).

5.2 Glyphosate-Tolerant Rice

Glyphosate (*N*-[phosphonomethyl]-glycine) is a non-selective broad-spectrum herbicide that has a significant effect on broad-leaf weeds and grasses (perennial and annual) (Kurtz and Street 2003). It inhibits 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) and interferes with the shikimate metabolic pathway (Schönbrunn et al. 2001). Therefore, the use of this herbicide gives counter-productive results as the crops are also affected along with weeds. *Agrobacterium* strain, CP4, has a *CP4-EPSPS* gene that enables them to be naturally tolerant against glyphosate (Funke et al. 2006). The same technology has been exploited to make transgenic CP4-EPSPS rice, which confers tolerance against glyphosate. Kumar and his team manipulated a codon-optimized m*CP4-EPSPS* gene and transferred it into IR64 Indica rice to confer glyphosate tolerance (Chhapekar et al. 2015).

5.3 Sub1 Rice

A team of scientists in the USA and Philippines introgressed a genomic region containing *Sub1A* into a high-yielding varieties of Swarna, IR64, Samba Mahsuri, BR11, CR1009, and Thadokkam1 using marker-assisted back-crossing. They observed increased number of panicles, increased number of grains per panicle, and higher grain yield with the addition of submergence tolerance in *Sub1* rice as compared to non-*Sub1* rice (Bailey-Serres et al. 2010). Furthermore, Indian scientists have performed field trials on a submergence tolerant variety named Swarna-Sub1 and observed that it can withstand flood up to 14 days. The study was performed in 120 villages of Orissa, India, and found that this variety increases yield up to 45% (Dar et al. 2013).

6 Conclusion

Although many concerns have been raised for the safety of GM food, one cannot shunt the possibility that GM crops are going to be one of the solutions for future food and nutritional security. Numerous countries have developed and accepted GM crops, but a few have still not allowed their open field cultivation. For example, the Clearfield® herbicide-resistant rice was released and commercialized in the USA but not accepted in any other country. Social awareness and political debate to answer the doubts and concerns the consumer has should go hand in hand along with all the scientific research programs. Nonetheless, improving the stress tolerance and enhancing the nutritional contents in rice are the need of the hour if we need to be geared up to feed the nine billion.

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Improvement of Wheat (*Triticum* spp.) Through Genetic Manipulation

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Abstract

Wheat (*Triticum* spp.) is one of the global leading cereals used as food and forage. It is the major source of carbohydrates and proteins for the human diet. Ongoing global warming and climatic changes significantly limit wheat production and grain quality. To cope up these adverse effects, genetic modifications and gene editing (CRISPR-CAS9) have become the time-effective state-of-the-art tools in the post-genomic era, in comparison with the available classical breeding and genetic approaches. Due to the advent of many gene-editing platforms and useful genes from diverse plant sources, it becomes easier to introduce genes from its source to other plants with desirable traits. However, validation of genes and trait stability are the key points for developing transgenics with better characters. In this regard, transgenic wheat production is useful to study the trait introgression in different genetic backgrounds of crop species. In this chapter, we showcase the available technologies for genetic manipulations in wheat and how candidate gene resources are useful to make the crop more resilient to abiotic stresses, resistant to biotic stresses, tolerant to herbicides, enhanced yield and nutritional improvement.

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1 Introduction

Wheat (*Triticum* spp.) is one of the major grain cereals used as the staple food for 40% of the world's inhabitants. It has a very old cultivation history since the biblical time, and presently, it is grown on more than 200 million hectares worldwide and having a diverse accessions (Frankin et al. 2019). Wheat grain provides majorly carbohydrates (40% of calories) and 13% of proteins (Giraldo et al. 2019). Throughout the world, hexaploid wheat (*Triticum aestivum* L., 2*n* = 42, BBAADD genomes) is produced and used for making bread. Over and above, tetraploid durum or pasta wheat (*Triticum turgidum* L. var. *durum* Desf., 2*n* = 28, BBAA genomes) has good production for making pasta, noodles, and medium-dense bread (Peña 2019). Both biotic and abiotic stresses trigger oxidative stress at the cellular level resulting in the generation of reactive oxygen species (ROS). Subsequently, oxidative homeostasis is disturbed leading to cell death. Agricultural practices include spraying herbicides which also disturbs the crop production. All these factors adversely affect the wheat yield and nutritive traits as a whole (Roberts and Mattoo 2018). Keeping in view of the population explosion, and the global demand for an increase in the grain quantity and quality, we need to adopt newer technologies besides plant breeding and marker-assisted selection (MAS). With the advent of biotechnological approaches and the state-of-art technologies, it becomes possible to make the bridge between the plant breeding and trait improvement through genetic modifications, which can be either transgene overexpression or silencing (knockout) of the targeted gene (Borisjuk et al. 2019). Availability of transformation technologies provides an opportunity to manipulate wheat genome for improved agronomic performance, resistance to biotic and abiotic stresses, higher yields, and grain quality (Alvarez et al. 2000). Due to the large size of the hexaploid wheat genome (about 40 times the size of rice and five times the size of humans), gene manipulation is challenging. Genes for many desired metabolic traits cannot be found in crossable wild relatives of the crop and, therefore, currently can only be introduced by genetic engineering. Metabolic pathway engineering, therefore, has quickly become one of the most intensely pursued practical applications of transgenic technologies in plants (Farré et al. 2014; Misawa 2011).

2 Genetic Manipulation of Wheat for Abiotic Stress Tolerance

Like other crops, wheat is also exposed to drought, salinity, heat and cold temperatures (Roychowdhury et al. 2013; Hasanuzzaman et al. 2013, 2015; Chakraborty et al. 2014). Since abiotic stress factors limit final yields and nutritive values of the

cereals, there is a need to improve the genetic architecture of wheat with genetic engineering technologies. The exploitation of different candidate genes and the traits improved are represented in Table 1.

2.1 Osmotic Stress: A Combination of Drought and Salinity

Drought is the major abiotic stressors that limit the wheat yield and grain quality worldwide. Drought resilience is a multigenic trait and relies on the interaction of different factors like phytohormones, genes, transcription factors (TFs), miRNAs, proteins, and metabolites (Mwadzingeni et al. 2016). TFs, for example, DREB1/CBF, DREB2, AREB/ABF, and NAC, are known to respond to stress signaling flags and direct the expression of many drought-induced downstream genes to confer stress tolerance in wheat (Rabara et al. 2014; Anumalla et al. 2016; Joshi et al. 2016). These TFs additionally function in crosstalk with other abiotic stressors like heat, cold (low temperature), and salinity might help in improving stress response. Basic leucine zipper or bZIP-like ABA-responsive element binding factors (AREBF), AP2/EREBP-like DRE binding protein (DREB) or CRT binding factor (CBF), NAM_ATAF-CUC2 [NAC, like stress-responsive NAC (SNAC)], and zinc finger (like C2H2 zinc finger protein ZFP) TFs have been portrayed in detail and found involved in regulating drought tolerance in wheat (Hu and Xiong 2014). Drought stress-related proteins and enzymes, including dehydrins, vacuolar acid invertase, glutathione *S*-transferase (GST), and late embryogenic abundant (LEA) proteins, have been genetically engineered to validate their impact on improving drought tolerance in wheat (Yang et al. 2010). Dehydration responsive-element binding (DREB) proteins, which belong to the subfamily of AP2/EREBP TFs, have been exploited to engineer tolerance to abiotic stresses in crop plants (Gao et al. 2009). Genes encoding DREB TFs comprise one of the major groups involved in drought response regulation through DREB/CBF signaling pathway. Two significant classes of DREB (DREB1 and DREB2) were found to function in different ways. While DREB1 expression is emphatically upregulated by cold (low temperature), DREB2 is more responsive to drought, heat, and salinity (Liu et al. 1998). Gao et al. (2009) demonstrated that the transgenic wheat expressing a cotton DREB TF (GhDREB) is either influenced by *ubi1* or *rd29A* promoters, displayed enhanced resistance to drought, salinity, and cold stresses. Transgenic wheat accumulated significant levels of soluble sugars than the wild-type under stress (Chen et al. 2007). Expression of a stress-inducible DREB from soybean (GmDREB) under the control of either *ubi1* or *rd29A* enhanced the tolerance to drought and salt in transgenic wheat. Likewise, Jiang et al. (2014) reported improved salt tolerance in wheat through the stress-inducible expression of GmDREB. Transgenic wheat plants displayed elevated levels of proline and betaine and lower levels of malondialdehyde and relative electrolyte leakage in comparison with the wild-types. Proteomic investigation of transgenic plants demonstrated that *GmDREB1* regulates the expression of osmotic and oxidative-stress-related proteins that reduced the occurrence of cell

Table 1 List of various transgenes, their biological sources, and stress tolerance

Sl no.	Target gene(s)	Source(s)	Transgenics	Stress tolerance	References
1.	*AtDREB1*	*Arabidopsis*	Wheat	Low temperature	Liu et al. (1998)
2.	*AtDREB2*	*Arabidopsis*	Wheat	Drought, high temperature, salinity	Liu et al. (1998)
3.	*GhDREB*	Cotton	Wheat	Drought, salinity, low temperature	Gao et al. (2009)
4.	*GmDREB1*	Soybean	Wheat	Drought, salinity	Chen et al. (2007), Jiang et al. (2014)
5.	*AtDREB1A*	*Arabidopsis*	Wheat	Osmotic stress	Pellegrineschi et al. (2004)
6.	*DREB2, DREB3*	Wheat: Maize promoter	Wheat	Dehydration	Morran et al. 2011
7.	*SNAC1*	Rice	Wheat	Drought, salinity	Joshi et al. (2016)
8.	*NAC69*	Wheat: Barley promoter	Wheat	Dehydration	Xue et al. (2011)
9.	*HvDhn4s:TaNAC69*	Barley	Wheat	Drought, salinity	Xue et al. (2011)
10.	*TaERF3*	Wheat	Wheat (overexpression)	Salinity, drought	Rong et al. (2014)
11.	*SeCspA, SeCspB*	*Escherichia coli*	Wheat	Drought, cold	Yu et al. (2017)
12.	*HVA1*	Barley	Wheat	Osmotic stress	Hu and Xiong (2014)
13.	*TaNIP*	Bread wheat	*Arabidopsis*	Salinity	Gao et al. (2010)
14.	*TaAQP8*	Wheat	Tobacco	Salinity	Hu et al. (2012a)
15.	*TdPIP1;1*, *TdPIP2;1*	Durum wheat	Tobacco	Low temperature	Ayadi et al. (2011)
16.	*TaAQP7*	Wheat	Tobacco	Low temperature	Huang et al. (2014)
17.	*DREB1A*	*Arabidopsis*	Wheat	Low temperature	Pellegrineschi et al. (2004)
18.	*WCS19*	Wheat	*Arabidopsis*	Low temperature	Gong et al. (2002)
19.	*WCOR410*	Wheat	Strawberry	Low temperature	Houde et al. (2004)
20.	*TaHSFA6f*	Wheat	Wheat (overexpression)	High temperature	Xue et al. (2014)

(continued)

Table 1 (continued)

Sl no.	Target gene(s)	Source(s)	Transgenics	Stress tolerance	References
21.	*TaMBF1c*, *TaFER-5B*, *TaOEP16-2-5B*, *TaB2*, and *TaGASR1*	Wheat	*Arabidopsis*	High temperature	Zang et al. (2017)
22.	*TaHsfC2a*	Wheat	Wheat (overexpression)	High temperature	Hu et al. (2018a)
23.	*TaWRKY33*	Wheat	*Arabidopsis*	High temperature	He et al. (2016)
24.	*TaHsfA2d*	Wheat	Wheat (overexpression)	High temperature	Chauhan et al. (2013)

damage and injury brought about by high saltiness. Transgenic wheat plants expressing *Arabidopsis thaliana* DREB1A (*AtDREB1A*) with the *rd29A* promoter showed sustained resistance to water stress in comparison with control plants under greenhouse conditions (Pellegrineschi et al. 2004). Transgenic wheat and barley expressing DREB2 and DREB3 TFs isolated from wheat under a constitutive (double 35S) and drought-inducible promoter (maize *rab17*) exhibited resilience to dehydration (Morran et al. 2011). Overexpression of DREB1A has been demonstrated to improve the low-temperature stress tolerance in wheat. *Arabidopsis* DREB1A gene under the control of *RD29A* promoter of cold-regulated (*cor*) genes was transferred into bread wheat using the biolistic method. This promoter also limited the negative effects on plant growth and development (Pellegrineschi et al. 2004).

High salinity triggers two distinct stresses: (1) High salt levels in the soil cause an abatement in soil water potential which makes it difficult for plant roots to uptake water, thus prompting a physiological drought condition; and (2) sodium (Na^+) accumulation in tissues can reach up to toxic levels and cause ionic stress. As Na^+ is taken up through ion transporters or anatomical leaks in the root endodermis, it disrupts typical cellular metabolism (Tester and Davenport 2003). NACs are TFs that play a significant role in plant development and abiotic stress resilience. A subfamily of stress-responsive NAC (SNAC-A) TFs actuated by abiotic stresses and ABA hormone have been suggested to play a key role in drought tolerance of crop plants (Budak et al. 2015). The *SNAC1* gene has been found associated with drought and salinity tolerance in crop plants (Joshi et al. 2016). Transformation of an elite Chinese wheat variety Yangmai12 with *SNAC1* under the *ubi1* promoter resulted in significantly enhancing tolerance to drought and salinity over multiple generations (Saad et al. 2013). Expression studies of *SNAC1* by qPCR revealed that several genes associated with abiotic stress/ABA signaling, such as wheat *1-phosphatidyli nositol3-phosphate-5-kinase*, *sucrose phosphate synthase*, *type 2C protein phosphatases*, and regulatory components of ABA receptor, were successfully regulated. Overexpression of *TaNAC69* under a barley drought-inducible *HvDhn4s* promoter led to enhanced transcript levels of stress upregulated genes and dehydration tolerance in bread wheat (Xue et al. 2011). The *HvDhn4s*:*TaNAC69* transgenic lines

produced more shoot biomass under combined mild salt stress and water limitation conditions. Wheat ethylene response factor *TaERF3* was functionally characterized by Rong et al. (2014), and its overexpression revealed increased salt and drought tolerance in wheat. Seedlings of *TaERF3*-overexpressing transgenic lines exhibited significantly improved tolerance to both salinity and drought stresses. Improved drought resilience in wheat has been additionally conceivable by overexpressing a synthetic bacterial cold shock protein gene (*SeCspA*) (Yu et al. 2017). Cold shock proteins (CSPs) are essential for bacterial growth and development under the adverse environmental conditions such as low temperatures (Blattner et al. 1997). The *Escherichia coli CSP* genes *SeCspA* and *SeCspB* with modified plant preferred codon sequences were utilized for genetic transformation of wheat. Overexpression of *SeCspA* and *SeCspB* resulted in stable and significant improvement in drought tolerance under stress conditions in the field.

Late embryogenesis abundant (LEA) proteins by and large accumulate in the incipient embryo during seed desiccation. LEAs are also incited in vegetative tissues by dehydration and drought stresses. LEAs are associated with the adaptive responses to hyperosmotic conditions through the maintenance of membrane structure, sequestration of ions, and binding of water, also act as chaperons, and help prevent damage to plant cellular and macromolecular structures and to increased ion concentrations (Bray 1997; Yamaguchi-Shinozaki and Shinozaki 2009). Since LEA proteins are increased in crop plants under water deficiency condition, genes encoding LEA proteins have attracted the attention for engineering drought and salinity stress tolerance. The barley *HVA1* gene encodes a group of three LEA proteins and has been found to improve stress tolerance in crop plants including wheat (Hu and Xiong 2014). Because the degree of abiotic stressors varies, the impact of a transgene may differ depending upon environmental conditions. Extensive field experiments are necessary to assess transgene effects across a range of diverse genotypes and environments.

Short non-coding miRNAs are important regulators in plant abiotic stress signaling as their corresponding target genes have key roles in metabolism and signal transduction. Various drought-related cellular pathways are regulated by miRNAs, including auxin signaling, ABA response, antioxidant defence, osmoprotectant, cellular growth, respiration, and photosynthesis. miR169 shows differential expression under drought in bread wheat (Ding et al. 2013). ABA plays a crucial role in plant stress tolerance by accumulating in the guard cells of stomata under osmotic stress condition, triggers its closure to reduce exorbitant water loss, and controls transpiration. Several genes in the ABA metabolism pathway have been found to improve drought resistance in crop plants.

2.2 High-Temperature Stress (Heat)

Genetic engineering and transgenic approaches alleviate the adverse effects of heat stress by improving heat tolerance. It involves the incorporation of genes of interest into the desired genotypes. In any case, the complex nature of the genomic pattern

makes it very hard to explore for genetic modifications in wheat (Akter and Islam 2017). Heat stress quickly alters the expression patterns of heat-related genes in wheat. Heat shock proteins (HSPs) function as molecular chaperones in keeping up the homeostasis of protein folding and are related to the acquisition of thermotolerance (Hasanuzzaman et al. 2013). The small heat shock protein (sHSP26) from wheat chloroplasts was shown to play a positive role in the heat stress tolerance mechanism (Chauhan et al. 2013). The functions of some wheat genes involved in sensing and responding to heat stress were characterized by overexpression in *Arabidopsis* and wheat (Wang et al. 2012). Improved thermotolerance was noticed in wheat plants by overexpressing *TaHSFA6f* gene (Xue et al. 2014). Ectopic expression of wheat *TaMBF1c*, *TaFER-5B*, *TaOEP16-2-5B*, *TaB2*, and *TaGASR1* in *Arabidopsis* could enhance thermotolerance in that species (Zang et al. 2017). *TaHsfC2a*-overexpressing wheat demonstrated improved thermotolerance (Hu et al. 2018a). Also, *TaWRKY33* transgenic lines of *Arabidopsis* displayed enhanced tolerance to heat stress (He et al. 2016). Further, transgenic *Arabidopsis* overexpressing *TaHsfA2d* exhibited improved thermotolerance (Chauhan et al. 2013). *TaLTP3*-overexpressing plants indicated higher thermotolerance than the wild-type plants at the seedling stage (Wang et al. 2014a). Similarly, overexpression of *TaNAC2L* brings about enhanced heat tolerance/resistance by activating the expression of heat-related genes (Guo et al. 2015). MicroRNA-mediated heat stress tolerance was found in rice with the overexpression of wheat *TamiR159*, which is more sensitive to the heat stress relative to the wild-type plants (Wang et al. 2012). Heat stress for a longer period increases protein synthesis elongation factor (EF-Tu) in chloroplasts which are associated with heat tolerance in wheat. Constitutive expression of EF-Tu in transgenic wheat protected leaf proteins against thermal aggregation, reduced thylakoid membrane disruption, enhanced photosynthetic capability, and imparted resistance to microbial infection (Fu et al. 2012). It appears that improvements in wheat transformation technology and the availability of bread and durum wheat mutant libraries will accelerate the progress in functional analysis of heat-responsive genes (Gardiner et al. 2014).

2.3 Low-Temperature Stress (Cold)

Low temperature or cold hinders the root intercede water uptake. Aquaporin proteins (AQPs) are membrane layer-bound protein channels in roots and known to transport water and other small molecules (Roychowdhury et al. 2018, 2019). Till date, more than 35 *AQP* genes have been distinguished in wheat. Some bread and durum wheat *AQP* genes such as *TaNIP*, *TdPIP1;1*, *TdPIP2;1*, and *TaAQP8* have been found to be involved in drought or salt stress tolerance (Hu et al. 2012a). *TaAQP7* encodes a novel plasma membrane intrinsic protein 2 with 286 amino acids and has portrayed the function of the protein in transgenic tobacco. It confers cold stress tolerance by protecting the membrane integrity in transgenic tobacco (Huang et al. 2014). Freezing tolerance of *Arabidopsis* and strawberry (*Fragaria* × *ananassa*) was increased by the ectopic expression of wheat gene *WCS19* in the former

and wheat *dehydrin* gene *WCOR410* in strawberry leaves (Gong et al. 2002; Houde et al. 2004).

2.4 Herbicide Tolerance in Wheat

The most common weeds in wheat are *Phalaris minor* and wild oat (*Avena fatua*) although many other annual and perennial weeds can be found. Due to their similar growth habit with the wheat, these are very difficult to manage by hand weeding which is laborious. One major tool for weed management in modern agriculture is the use of herbicides. Herbicides are chemically inert to the resistant crops but inhibit photosynthesis or cause injury and death to the susceptible weeds or crops depending on the mode of action of herbicides (Nakka et al. 2019). The mode of action depends on the inhibition of photosystem II (PS-II), acetolactate synthase, and long-chain fatty acid synthesis. The breakdown of electron transport process occurs in case of atrazine, which binds to plastoquinone-binding protein in plants. Resistance to herbicides in crop plants, including wheat, has been developed through the introduction of genes from allied sources. Detoxification of herbicides occurs through the activities of glutathione *S*-transferases (GSTs) and cytochrome P450 monooxygenases (CYP450s). Transgenic wheat to the broad-spectrum herbicide phosphinothricin (PPT) tolerance was achieved by microprojectile bombardment with the introduction of *bar* gene (Vasil et al. 1992; Becker et al. 1994; Nehra et al. 1994). Wheat transgenics developed so far for tolerance to herbicides is shown in Table 2.

Table 2 Genetic transformation of wheat for herbicide tolerance/resistance

Sl no.	Target gene(s)	Source(s)	Resistant phenotype/ trait	References
1.	*bar*	*Streptomyces*	Phosphinothricin herbicide resistant	Vasil et al. (1992)
2.	*bar*	*Streptomyces*	Glufosinate ammonium resistant	Becker et al. (1994)
3.	*bar*	*Streptomyces*	Phosphinothricin resistant	Nehra et al. (1994)
4.	*GST-27*	Maize (*Zea mays*)	Alachlor herbicide resistant	Milligan et al. (2001)
5.	*aroA:CP4*	*Agrobacterium tumefaciens* strain CP4	Glyphosate herbicide resistant	Hu et al. (2003)
6.	*aroA:CP4*	*Agrobacterium tumefaciens* strain CP4	Roundup ready	Zhou et al. (2003)
7.	*GS1, GS2* (*Glutathione synthetase*)	*Pisum sativum*	Phosphinothricin herbicide resistant	Huang et al. (2005)
8.	*TaDEP1, TaGW2, TaEPSPS*	*Triticum aestivum*	Herbicide resistance	Li et al. (2018)

3 Genetic Manipulation of Wheat for Disease Resistance (Biotic Stress Tolerance)

Wheat yields are globally affected by pathogens such as virus, fungus, bacteria, and insects. To overcome these difficulties, chemical pesticide application is followed for insect attacks and fungal diseases. But, pesticide usage has limitation in controlling viral diseases (not transmitted by insect vectors). It is neither economic nor ecofriendly due to their cost and serious environmental and health hazards to animals and humans (Satya et al. 2016; Cozma et al. 2017). Efforts are being made to reduce the losses in the yields of wheat through various crop improvement methods. Plant breeding methods supplemented with advanced genome modification technologies have a profound impact on wheat yield improvement. Genotypes with resistant genes/alleles from diverse sources need to be explored for their utilization in the widely used wheat cultivars. This will rejuvenate the genetic makeup of wheat cultivars, which can significantly improve the yield potential. Since the development of transgenic wheat plants by Vasil et al. (1992), significant achievements have been made globally, against the major diseases and pests. Among different diseases in wheat, the incidence of fungal diseases are higher and most common than the viral and bacterial diseases. Efforts have been made to develop wheat plants resistant to many of these biotic stresses using genetic engineering either by particle gun-mediated or by *Agrobacterium*-mediated gene transfer approaches.

3.1 Fungal Resistance

Wheat plants are prone to attack by many fungal pathogens. Among the common wheat diseases, black rust, yellow/stripe rust, brown rust, loose smut, flag smut, powdery mildew (*Erysiphe graminis*), Alternaria leaf blight, *Fusarium* head blight/head scab, ergot, and bunt are predominant, which severely affect the grain yields and quality. The grain quality could be reduced due to microbial or insect attack and even due to the poisonous toxins produced by them. Genetic improvement of wheat to fungal pathogen resistance would also have a significant impact through transgenic approaches with the introgression of antifungal genes from prokaryotes. Expression of microbial-derived genes such as pathogenesis-related (PR) proteins which are induced upon fungal infection in plants such as chitinases, β-1,3-glucanases, and peroxidases is the ideal target for induction of fungal resistance through genetic engineering (Sharma et al. 2011; Shrawat and Armstrong 2018). The first report of fungal resistance in wheat using genetic engineering approach dates back to 1999 when Bliffeld et al. (1999) introduced three PR protein-coding genes such as *glucanase* (*Glu*), *chitinase* (*Chi11*), and ribosome inactivating protein (RIP) from *Hordeum vulgare* against powdery mildew causing fungus. Since then, many genetic transformation events have been made around the globe by various researchers to impart fungal resistance to wheat (Table 2). A variant of RNAi approach is host-induced gene silencing (HIGS) technology. This has also been

used for resistance to fusarium head blight of wheat by introducing *F. graminearum* virulence gene *chitin synthase 3b* (*Chs3b*) (Cheng et al. 2015b).

3.2 Bacterial Resistance

Unlike fungal and viral diseases, bacterial infection in wheat is uncommon. Few bacterial diseases such as bacterial leaf streak (BLS) caused by *Xanthomonas campestris* pv. *translucens*, basal glume rot caused by *Pseudomonas syringae* pv. *atrofaciens*, and bacterial leaf blight (BLB) caused by *Pseudomonas syringae* pv. *oryzae* could harm wheat production (Maraite et al. 2007; Ramakrishnan et al. 2019), although have not shown severe epidemics (Maraite et al. 2007). This is the reason, bacterial disease resistance in wheat was not given serious attention. Recently, Ramakrishanan et al. (2019) identified genomic region associated with resistance to BLS from wheat germplasms through genome wide association study (GWAS) that would be helpful for genomics assisted breeding. Another study demonstrated the mechanism of non-host resistance in wheat plants by expressing *Arabidopsis* EF-TU receptor (*AtEFR*), a pattern recognition receptor (PRR). PRRs contribute first line of defence by recognizing the pathogenic molecules during the infection process. The transgenic plant responded to elf18 peptide when infected with *Pseudomonas syringae* pv. *oryzae* through *AtEFR* expression and conferred immunity in wheat (Schoonbeek et al. 2015).

3.3 Viral Resistance

Along with pathogen-derived resistance (PDR) approach, there are other techniques like antisense RNA expression, double-stranded RNA, siRNA/miRNA-mediated, ribozyme-mediated, genome modifications using different genome-editing technologies. Transgenic wheat was developed using different PDR approaches and crop-infecting viruses, such as WSMV, BYMV, and WYMV besides specific genes from yeast and bacteria (Table 3). Different virus-derived gene sequences such as coat protein (*CP*) (Karunaratne et al. 1996; Sharp et al. 2002; Sivamani et al. 2002; Shoup Rupp et al. 2016), viral replicases (*Nib*) (Sivamani et al. 2000; Sharp et al. 2002; Chen et al. 2014), and nuclear inclusion protein "a" (*Nia*) (Fahim et al. 2010) have been targeted. The *dsRNA-specific RNaseIII* gene from *Escherichia coli* (Zhang et al. 2001) and *Saccharomyces pombe* (Yan et al. 2006) has also been incorporated into wheat for viral resistance especially against WSMV.

3.4 Insect Resistance

The cry protein-encoding gene (*cry*) of *Bacillus thuringiensis* (*Bt*) led to the development of many crop plants to insect resistance including wheat (Shrawat and Armstrong 2018). Despite several insect attacks to wheat plants such as termite,

Table 3 Transgenics wheat for biotic stress resistance/tolerance

Sl no.	Target gene(s)	Source(s)	Resistant phenotype against (virus/ fungus/insect/ nematodes, etc.)	References
Fungal resistance				
1.	*Glu—glucanase ChiII—chitinase RIP—ribosome-inactivating protein*	*Hordeum vulgare*	Powdery mildew (*Erysiphe graminis*)	Bliffeld et al. (1999)
2.	*Sts—chimeric stilbene synthase*	*Vitis vinifera*	Fungal resistance	Fettig and Hess (1999)
3.	*Tlp—thaumatin-like protein ChiII*	*Oryza sativa*	*Fusarium graminearum*	Chen et al. (1999)
4.	*Germin—TaGlp21, HVGlp1*	*Triticum aestivum, Hordeum vulgare*	Wheat powdery mildew (*Blumeria graminis*)	Schweizer et al. (1999)
5.	*Vst1—stilbene synthase*	*Vitis vinifera*	Powdery mildew	Liang et al. (2000)
6.	*Kp4—antifungal protein*	*Ustilago maydis*	*Tilletia tritici*	Clausen et al. (2000)
7.	*PhyA*	*Aspergillus niger*	Fungal resistance	Brinch-Pedersen et al. (2000)
8.	*RIP—ribosome-inactivating protein*	*Hordeum vulgare*	Powdery mildew (*Erysiphe graminis*)	Bieri et al. (2000)
9.	*afp—antifungal protein coding gene ChiII—chitinase RIP1*	afp from *Aspergillus giganteus, ChiII* and *RIP1 from Hordeum vulgare*	Powdery mildew (*Erysiphe graminis*)	Oldach et al. (2001)
10.	*RCH-8 chitinase*	*Oryza sativa*	Wheat scab causing fungus	Wu et al. (2001)
11.	*FSTRI 101*	*Fusarium sporotrichioides*	Fusarium head blight (*Fusarium graminearum*)	Okubara et al. (2002)
12.	*Glu-1,3—glucanase, Chi—chitinase, RIP—ribosome-inactivating protein*	*Hordeum vulgare*	Wheat powdery mildew (*Blumeria graminis*)	Bieri et al. (2003)
13.	*tlp—thaumatin-like protein Chitinase β-1,3-Glucanase, PR protein*	*Triticum aestivum*	Wheat scab causing fungus	Anand et al. (2003)
14.	*Peroxidase* (*PERO*)	*Triticum aestivum*	Wheat powdery mildew	Altpeter et al. (2005)

(continued)

Table 3 (continued)

Sl no.	Target gene(s)	Source(s)	Resistant phenotype against (virus/ fungus/insect/ nematodes, etc.)	References
15.	*vst1, vst2, pss—pinosylvin synthase*	*vst1, vst2—Vitis vinifera, Pinus sylvestris*	*Puccinia recondita, Septoria nodium*	Serazetdinova et al. (2005)
16.	*Ace-amp1* (*antimicrobial protein*) *pina-D1a*	*Allium cepa Triticum aestivum*	Fungal resistance	Roy-Barman et al. (2006)
17.	*kp4—antifungal protein*	*Zea mays*	Stinking smut (*Tilletia caries*)	Schlaich et al. (2006)
18.	*AtNPR1*	*Arabidopsis thaliana*	Fusarium head blight (*Fusarium graminearum*)	Makandar et al. (2006)
19.	*β-1,3-Glucanase*	*Arabidopsis thaliana*	Wheat powdery mildew	Zhao et al. (2006)
20.	*A-1 purothionin, tlp-1 β-1,3Glucanase*	*Triticum aestivum Hordeum vulgare*	Fusarium head blight (*Fusarium graminearum*)	Mackintosh et al. (2007)
21.	*PGIP—polygalacturonase-inhibiting protein*	*Phaseolus vulgaris*	*Bipolaris sorokiniana*	Janni et al. (2008)
22.	*AGScG*	*Fusarium*-specific recombinant antibody from chicken and antifungal peptide from *Aspergillus giganteus*	*Fusarium asiaticum*	Li et al. (2008)
23.	*Chit—barley class-II chitinase*	*Hordeum vulgare*	Fusarium head blight (*Fusarium graminearum*)	Shin et al. (2008)
24.	*TaPIEP1—pathogen-induced ERF*	*Triticum aestivum*	Fungal resistance	Dong et al. (2010)
25.	*Pm3b—powdery mildew resistance*	*Triticum aestivum*	Wheat powdery mildew	Zeller et al. (2010)
26.	*N-terminal fragment of wheat*	Yeast		Di et al. (2010)
27.	*Pm3b—powdery mildew resistance*	*Triticum aestivum*	Powdery mildew fungus	Brunner et al. (2011)
28.	*Pm3b Chitinase β-1,3-Glucanase*	*Pm3b—Triticum aestivum β-1,3-Glucanase—Hordeum vulgare*	Powdery mildew fungus	Kalinina et al. (2011)

(continued)

Table 3 (continued)

Sl no.	Target gene(s)	Source(s)	Resistant phenotype against (virus/ fungus/insect/ nematodes, etc.)	References
29.	*RSAFP2—antifungal protein*	*Raphanus sativus*	*Fusarium graminearum* and *Rhizoctonia cerealis*	Li et al. (2011)
30.	*Acpmei—pectin methyl esterase inhibitor*	*Actinidia chinensis*	*Fusarium graminearum* and *Bipolaris mayidis*	Volpi et al. (2011)
31.	*Pm3b*	*Triticum aestivum, Hordeum vulgare*	Powdery mildew resistance	Álvarez-Alfageme et al. (2011)
32.	*Lr34-encoding an ATP binding cassette transporter*	*Triticum aestivum*	Leaf rust resistance	Risk et al. (2012)
33.	*Pm3 alleles—race-specific powdery mildew resistance pm3a, pm3b, pm3c, pm3d, pm3g*	*Triticum aestivum*	Powdery mildew fungus	Brunner et al. (2012)
34.	*TaGCN2—protein kinase*	*Triticum aestivum*	Fungal resistance	Byrne et al. (2012)
35.	*Harchit, Harcho, antifungal gene, chitinase, chitosan*	*Trichoderma harzianum*	Fungal resistance	Rana et al. (2012)
36.	*BLF—antimicrobial bovine lactoferrin gene*	Bovine	*Fusarium graminearum*	Han et al. (2012)
37.	*TaPIMP1—transcription factor*	*Triticum aestivum*	*Bipolaris sorokiniana*	Zhang et al. (2012)
38.	*PvPGIP2*	*Phaseolus vulgaris*	*Erysiphe graminis*	Ferrari et al. (2012)
39.	*NPR1—non-expressor of PR*	*Arabidopsis thaliana*	Fusarium blight	Gao et al. (2013)
40.	*RC24—rice chitinase class I*	*Oryza sativa*	Stripe rust fungus	Huang et al. (2013)
41.	*SN1—snakin 1*	*Solanum tuberosum*	Fungal resistance	Rong et al. (2013)
42.	*GmPGIP3*	*Glycine max*	Common root rot disease	Wang et al. (2015)
43.	*Ech42, CWp2—endochitinase gene*	Ech42—*Trichoderma atoviride*, CWp2—*Fusarium*-specific recombinant antibody	Fungal resistance	Cheng et al. (2015a)
44.	*Chs3b—chitin synthase 3b*	*Fusarium graminearum*	Fusarium head blight	Cheng et al. (2015b)

(continued)

Table 3 (continued)

Sl no.	Target gene(s)	Source(s)	Resistant phenotype against (virus/ fungus/insect/ nematodes, etc.)	References
45.	*Chitinase* (*chi26*)	*Barley, Hordeum vulgare*	Wheat rust (*Puccinia triticina*), yellow rust (*Puccinia striiformis* f. sp. *tritici*) and powdery mildew (*Blumeria graminis*)	Eissa et al. (2017)
46.	Antifungal defensin *MtDEF4.2*	*Medicago truncatula*	*Puccinia triticina* and *Fusarium graminearum*	Kaur et al. (2017)
47.	Hairpin RNAi constructs of *PtMAP-kinase* (*PtMAPK1*) or a *cyclophilin* (*PtCYC1*)	Sequence homology to *Puccinia triticina*	Wheat leaf rust (*Puccinia triticina*)	Panwar et al. (2018)
48.	Host-induced gene silencing of *PsCPK1*	*Puccinia striiformis* f. sp. *tritici*	Wheat stripe rust (*Puccinia striiformis* f. sp. *tritici*)	Qi et al. (2018)
Bacterial resistance				
1.	*AtEFR—EF-TU receptor*	*Arabidopsis thaliana*	*Pseudomonas syringae* pv. *oryzae*	Schoonbeek et al. (2015)
Viral resistance				
1.	*Coat protein (CP)*	BYMV	Barley yellow mosaic virus (BYMV)	Karunaratne et al. (1996)
2.	*Nib—viral replicase*	WSMV	Wheat stripe mosaic virus (WSMV)	Sivamani et al. (2000)
3.	*rnc70*—mutant *bacterial RNaseIII Coat protein (CP)*	Bacterial RNaseIII (*Escherichia coli*) WSMV	Barley stripe mosaic virus (BSMV)	Zhang et al. (2001)
4.	*Coat protein (CP) Nib*	WSMV	WSMV	Sharp et al. (2002)
5.	*Coat protein (CP)*	WSMV	WSMV	Sivamani et al. (2002)
6.	*Pac1—dsRNA-specific RNaseIII*	Yeast, *Saccharomyces pombe*	Barley yellow dwarf virus (BYDV)	Yan et al. (2006)
7.	*Nia—nuclear inclusion protein "a"*	WSMV	WSMV	Fahim et al. (2010)
8.	*FGmiR395—artificial polycistronic miRNA*	*Oryza sativa* derived miR395	WSMV	Fahim et al. (2012)

(continued)

Table 3 (continued)

Sl no.	Target gene(s)	Source(s)	Resistant phenotype against (virus/ fungus/insect/ nematodes, etc.)	References
9.	*Nib8 viral replicase*	WYMV, *Triticum aestivum*	Wheat yellow mosaic virus (WYMV)	Chen et al. (2014)
10.	RNAi-mediated TriMV *CP—coat protein gene*	TriMV-CP	Triticum mosaic virus (TriMV)	Shoup Rupp et al. (2016)
Insect resistance				
1.	*Vst1 stilbene synthase*	*Vitis vinifera*		Leckband and Lörz (1998)
2.	*Gna—lectin, agglutinin*	*Galanthus nivalis*	Wheat aphid (*Sitobion avenae*)	Stoger et al. (1999)
3.	*Itr1—trypsin inhibitor*	*Hordeum vulgare*	*S. cerealella*	Altpeter et al. (1999)
4.	*Synthetic avidin*	Chicken	Wheat weevil (*Sitophilus granarius*)	Abouseadaa et al. (2015)
5.	*(ppa)-Lectin*	*Pinellia pedatisecta agglutinin*	Wheat aphid (*Sitobion avenae*)	Duan et al. (2018)
Nematode resistance				
1.	*pin2—serine protease inhibitor*	*Solanum tuberosum*	Nematode, *H. avenae*	Vishnudasan et al. (2005)
2.	Esophageal gland gene	*Heterodera avenae*	Nematode, *H. avenae*	Umarao et al. (2009)
3.	*Cre2*, *Cre5*, *Cre7*	*Aegilops* sp.	Nematode, *H. avenae*	Simonetti et al. (2010)

wheat aphid, armyworm/cutworm, American pod borer, brown mite, pink stem borer, and shoot fly, only a few transgenic plants have been developed so far including resistance for aphid and wheat weevil (Altpeter et al. 1999; Stoger et al. 1999; Abouseadaa et al. 2015; Hou et al. 2019; Duan et al. 2018). The major transgenic approaches for insect resistance are based on the introduction of transgenes for the expression of constituent proteins or enzymes against the insect, which act as toxins such as *Bt* crystal protein, α-amylase inhibitor, proteinase inhibitor, vegetative insecticidal protein (*vip*), and others (Sharma et al. 2000; Shrawat and Armstrong 2018). Transgenic wheat plants expressing plant lectin expressing genes and *Guanine nucleotide-binding protein subunit α gene (Gqα)* have been tried against aphid infestation. Plant lectins are carbohydrate-binding proteins known to have insecticidal activity due to their binding with cell surface glycoproteins and glycolipids causing cell agglutination (Bharathi et al. 2010; Chandrasekhar et al. 2014a). Chandrasekhar et al. (2014b) demonstrated the transgenic rice resistance to sap-sucking insects by phloem-specific expression *Allium sativum* agglutinin (ASAL) with the combination of mutated herbicide gene (EPSPS). A gene encoding

mannose-binding lectin from *Pinellia pedatisecta agglutinin* (*Ppa*) was used for creating transgenic wheat that showed low aphid infestation in comparison with wild-type plants (Duan et al. 2018). *Gqα* is required during G-protein-coupled receptor (GPCR)-mediated signal transduction processes in insect olfaction. RNAi approach has been used to produce transgenic wheat for incorporating *Gqα* gene. The transgenic wheat lines displayed less aphid infestation with reduced reproduction and molting and higher grain yield (Hou et al. 2019). In another attempt, *Galanthus nivalis* lectin (*GNA*) gene has been used for generating wheat that can withstand aphid. Further, synthetic *avidin* gene from chicken has been introduced into wheat genome for generating resistance to weevil insects (*Sitophilus granarius*) (Abouseadaa et al. 2015). The caterpillars of angoumois grain moth (*Sitotroga cerealella*), a stored grain pest of wheat, and other related cereals bore into the seeds and later feed on the wheat grains. To manage this pest, transgenic wheat plants expressing barley trypsin inhibitor CMe (*BTI-CMe*) has been developed (Altpeter et al. 1999). A list of insect-resistant transgenic wheat plants is given in Table 3.

3.5 Nematode Resistance

Cereal cyst nematode (*Heterodera avenae*) resistance genes have been identified from wheat and its wild relatives. Genes such as *Cre1* and *Cre8* from *Triticum aestivum*, *Cre2*, *Cre3, Cre4, Cre5, Cre6, Cre7, CreX*, and *CreY* from *Aegilops* species, and *CreR* from *Secale cereale* were utilized for the marker-assisted wheat breeding program (Ogbonnaya et al. 2009). Cytosolic ascorbate peroxidase (*cTaAPx*) genes were induced in roots of transgenic wheat carrying *Cre2*, *Cre5*, or *Cre7* nematode resistance genes. This suggests the possible role of *Cre*-mediated resistance in wheat in response to *H. avenae* (Simonetti et al. 2010). Silencing of dorsal esophageal gland gene of *H. avenae* using RNAi approach resulted in transgenic wheat (Umarao et al. 2009). Managing insect pests through nutrient deprivation could be an ecofriendly approach. Pests utilizing proteinaceous nutrients need serine protease enzyme for cleaving proteins to peptides. Serine protease inhibitors are defence proteins, synthesized de novo in plants, and reduce nutrient utilization in insects (Jamal et al. 2013). Vishnudasan et al. (2005) developed transgenic wheat by expressing *serine protease inhibitor* gene (*pin2*) from potato to tackle *H. avenae* nematode.

4 Nutritional Improvement of Wheat

It is estimated that almost one third of the world's population is currently suffering from malnutrition due to the lack of sufficient proteins, vitamins, and a variety of micronutrients in their daily diet especially iron (Fe) and zinc (Zn). The wheat grain consists of 83% endosperm, 14.5% bran, and 2.5% germ tissues (Balyan et al. 2013).

4.1 Macronutrients

4.1.1 Carbohydrates

Digestible carbohydrates of wheat comprise mainly 70–75% of starch and the starch comprises 75% of amylopectin and 25% amylose (Lafiandra et al. 2014). Higher amylose content in wheat starch is considered to improve human health and reduce the risk of serious non-infectious diseases (Regina et al. 2006). Regina et al. (2006) showed downregulation of starch-branching enzyme (SBEIIa) using RNA interference (RNAi) in the wheat endosperm, which improved amylose content to 70%. RNAi-mediated silencing of *SBEIIa* gene in two cultivars of durum wheat caused alterations in granule morphology and starch composition, leading to high amylose content in grain (Sestili et al. 2010).

4.1.2 Proteins

Proteins play a major role in human nutrition (Balyan et al. 2013). Downregulation of *Gpc-B1* (NAC transcription factor) in transgenic wheat delays the senescence (more than 3 weeks) and reduces the concentration of protein and micronutrients such as Zn and Fe in the grain (>30%). This suggests an association between senescence and nutrient remobilization (Cantu et al. 2011). In order to improve nutritional quality, Tamás et al. (2009) transformed bread wheat with an amaranth (*Amaranthus hypochondriacus*) albumin gene, *ama1*, driven by an endosperm-specific promoter of wheat. Transgenics exhibited an increase in essential amino acid content that lead to an increased lysine (Lys) content from 5 to 6.4% and tyrosine (Tyr) from 3.5 to 3.8%. The sum of the AmA1 protein obtained showed a strong correlation with the increase in wheat flour's essential amino acid content. It indicates that the change in amino acid composition was due to the AmA1 protein expression. Transgenic wheat lines expressing winged bean Lys-rich protein (*wblrp*) gene showed 2–3 times higher levels of free Lys and 10% more bound Lys in leaves than the wild-type plants. This indicates that deployment of *wblrp* genes could effectively improve the quality of wheat nutrition (Meng et al. 2004). Cereal grains typically have low lysine (Lys) levels (Shotwell and Larkins 1989). Efforts to synthesize Lys-rich proteins in plants have been possible through genetic engineering to improve the protein levels in cereal grain. The approaches used were basically the modifications of endogenous protein or synthetic protein sequences. From the cereal embryo, extracted protein translation elongation factor 1α (EF-1α) has been found to be rich in Lys. Lys synthesis can be improved by reducing dihydrodipicolinate synthase (DHPS) responsiveness to Lys inhibition feedback. In both dicot and monocot species, the genes encoding bacterial feedback-insensitive DHPS enzymes were overexpressed (Galili 1995; Galili et al. 2002).

4.1.3 Lipids (Oils)

Wheat contains linoleic acid (LA) representing 50% of the fatty acids (FAs) and lack of gamma linolenic (GLA) and other essential fatty acids (Dunford and Zhang 2003). GLA is the key dietary essential FA required to metabolize different substances (Horrobin 1993). The human body cannot synthesize linoleic and linolenic

acids by itself, and these essential poly-unsaturated fatty acids (PUFAs) must be obtained through the food. Mihálik et al. (2015) overexpressed the codon-optimized *δ-6-desaturase* (*D6D*) gene isolated from the fungus *Thamnidium elegans* in the wheat under high-molecular-weight glutenin subunit (HMWG) promoter. Transgenic wheat showed increased linolenic acid [0.04–0.32% (v/v)] of the total amount of FAs in the grains. Cao et al. (2020) carried out RNAi cassette expression in wheat grains to suppress the expression of the lipoxygenase (*LOX*) gene. GLRW-1, -3, and -5 showed decreased expression of *LOX* gene, lower activity of LOX enzyme, and less lipid peroxidation in the grains. In the grain and flour samples, GLRW lines have substantially increased the content of linoleic and linolenic acids in comparison with the wild-type controls.

4.1.4 Dietary Fiber (DF)

A variety of health benefits have been shown in cereal dietary fiber (DF). It lowers blood pressure and serum cholesterol, increases insulin sensitivity, and reduces the prevalence of certain types of cancer, including intestinal and breast cancers (Cade et al. 2007; Reynolds et al. 2019; Lovegrove et al. 2019). The major components of wheat flour, DF fractions include cell wall polysaccharides, primarily arabinoxylan (AX) around 70 and 20% of (1→3,1→4)-β-D-glucan (β-glucan) (Mares and Stone 1973; Lovegrove et al. 2013). Wheat is a poor source of β-glucan, and it is present in 0.6% of the flour (Šramková et al. 2009). Glycosyltransferases (GT) are involved in arabinoxylan biosynthesis (Anders et al. 2012). RNAi suppression of glycosyltransferases in the family 61 (GT61-1) resulted in decreased total AX (TOT-AX) and reduced water-extractable AX (WE-AX) proportions in two lines (Anders et al. 2012). This demonstrates that arabinosylation pattern and altered AX solubility are associated with DF content. RNAi inhibition of *GT43-2* and *GT47-2* also reduced total AX and decreased aqueous extract viscosity (Lovegrove et al. 2013; Lafiandra et al. 2014). GTs are overexpressed to improve arabinoxylan (Lovegrove et al. 2013). Biosynthesis of β-glucan is regulated by the cellulose synthase-like (*CSLF6*) gene in barley (Burton et al. 2006) and wheat (Nemeth et al. 2010). In barley, overexpression of *CSLF6* gene under an endosperm-specific promoter in transgenic barley resulted in 80% of increment of β-glucan in the grain (Burton et al. 2011).

4.2 Micronutrients

4.2.1 Vitamins

Wheat grain is an excellent source of vitamins B and E (Brinch-Pedersen et al. 2007). Over and above, provitamins A (β-carotene), D (calciferol), and K (phylloquinone) are present in minute quantities in the wheat embryo (Brinch-Pedersen et al. 2007; Shewry and Hey 2015). Transformed wheat with the bacterial phytoene synthase (*CrtB*) and carotene desaturase (*Crt1*) genes displayed an increased amount of provitamin A. Compared to the expression of either *CrtB* or *Crtl* alone, a combination of genes has significantly increased the carotenoid content in the transformed wheat grains. Co-expression of these genes resulted in an approximately

8-fold increase in carotenoid (4.76 mg g^{-1} seed dry weight), a 65-fold increase in β-carotene (3.21 mg g^{-1} seed dry weight), and a 76-fold increase in provitamin A (3.82 mg g^{-1} seed dry weight) (Wang et al. 2014b). *CrtB* and *Crt1* combination has also resulted in an increase in the total carotenoids in transgenic elite wheat lines (Cong et al. 2009). Zeng et al. (2015) engineered wheat by simultaneously overexpressing the carotenoid hydroxylase gene (*TaHYD*) with *CrtB* and endosperm-specific silencing. This endosperm-specific silencing of the carotenoid hydroxylase gene (*TaHYD*) increased the β-carotene content in wheat endosperm by 10.5-folds (1.76 mg g^{-1}) and overexpression of *CrtB* increased it by 14.6-folds (2.45 mg g^{-1}). It is interesting to note that β-carotene content increased to 31-fold (5.06 mg g^{-1}) by using a combination of "move strategy" (*CrtB* overexpression) and "block strategy" (*TaHYD* silencing). Because of the nature of the wheat genome, carotenoid metabolic engineering studies are limited to further increase the carotenoid content in wheat (Cong et al. 2009; Wang et al. 2014b).

4.2.2 Minerals

The most essential microelements for humans are Fe and Zn (Borg et al. 2012). To improve the content of microelements, the main approaches for food crops adopted include agronomic biofortification, plant breeding, and genetic engineering (White and Broadley 2009). Genetic engineering techniques offer a valid alternative to conventional breeding strategies when there is insufficient genotypical variation within the species for the required trait. Since minerals are not synthesized in the plant, plants receive Fe and Zn from the rhizosphere and surrounding environment (Morrissey and Guerinot 2009). Transgenic approaches for improving the content of Fe and Zn in crop plants have focused primarily on increasing plant uptake and utilization efficiency by modulating the expression of transporters (Kerkeb et al. 2008) and minimizing the anti-nutritional factors such as phytic acid (Kumar et al. 2019). Borg et al. (2012) cloned and analyzed the genes of wheat ferritin (TaFer1-A) and demonstrated the ability of ferritin to increase the content of Fe in wheat endosperm. Endosperm-targeted overexpression of the *TaFer1-A* gene controlled by HMW glutenin 1DX5 promoter resulted in an increase of 50–85% ferritin in wheat grains. Overexpression of genes associated with Zn translocation and mobilization with enhanced bioavailability of Zn without yield penalty is an effective way to enhance grain Zn content (Borrill et al. 2014). Among many known cation transporter families, ZIP (ZRT, IRT-related protein) and CDF (Cation diffusion facilitator) families predominate and play a major role in Zn transportation. Biofortification of cereals with NAS alone or in conjunction with ferritin has great potential to counter global human mineral shortages (Lee et al. 2009; Zheng et al. 2010).

4.3 Antinutrients

Substances, such as phytic acid (PA) and oxalates, that reduce the nutrients uptake are known as antinutrients. Phytate or PA (myo-inositol 1,2,3,4,5,6-

hexakisphosphate; PA; IP6) is the major component of wheat seeds and chelates metal ions, thereby reducing their bioavailability and thus reducing grain nutritional value (Bhati et al. 2016; Jiang et al. 2019). It has been possible to reduce the phytate content in wheat grains using RNAi technique by either overexpression of *phytase* or silencing the phytate synthesizing genes. In *phytase* overexpressed lines, enhanced degradation of PA stored in seeds and increased in vitro bioavailability of the mineral nutrients was noticed (Abid et al. 2017). Brinch-Pedersen et al. (2000) overexpressed the *Aspergillus niger* phytase-encoding gene (*phyA*) in wheat with α-amylase signal peptide sequence and without singular peptide under the ubiquitin promoter. The signal peptide-containing transgenic seeds showed up to a fourfold increase in phytase activity while an increase of up to 56% was found in plants. Transgenics expressing *Aspergillus japonicus* phytase gene (*phyA*) have been developed in wheat endosperm, and the plants were tested up to T_3 generation. Transgenics showed 18–99% increase in phytase production and 12–76% decrease in seed PA content (Abid et al. 2017). Bhati et al. (2016) studied *TaABCC13*:RNAi wheat lines targeting *ABCC* transporter gene (ABC-type transporters), and the plants showed a reduction of 34 to 22% in PA levels with a concomitant increase in calcium (Ca^{2+}). In *TaABCC13*:RNAi lines, some of these negative impacts such as reduced seed weight, slightly delayed germination, and slow coleoptile development were noticed (Bhati et al. 2016).

5 Yield Improvement by Genetically Modified Methods

The wheat yield consists of grain size and grain number per unit area. Techniques of genetics and genomics were used to classify significant yield-related genes in wheat (Nadolska-Orczyk et al. 2017; Borisjuk et al. 2019).

5.1 Genes Involved in Photosynthetic Efficiency

In all cereals, photosynthesis is the major metabolic process and contributes 85% of the dry matter accumulation (Ruan et al. 2012). Therefore, yield improvement in wheat includes improvement of ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO) performance and regulation, C_4 traits such as CO_2 concentrating mechanisms, improvement in light-harvesting ability, and better photosynthesis at canopy and spike level. Agronomic traits of C_3 plants like rice and wheat were improved by overexpression of multiple genes like phosphoenolpyruvate carboxylase (PEPC) and malate dehydrogenase (MDH) from C_4 plants. Improving the photosynthetic carbon assimilation of C_3 plants by overexpressing photosynthetic genes for phosphoenolpyruvate carboxylase (PEPC), pyruvate orthophosphate dikinase (PPDK), phosphoenolpyruvate carboxykinase (PCK), nicotinamide adenine dinucleotide phosphate malic enzyme (NADP-ME), and NADP-malate dehydrogenase (NADP-MDH) in single or multiple genes (Ruan et al. 2012). The transfer of C_4-specific enzyme-encoding genes, PEPC, and PPDK have led to promising results in rice and

wheat (Hu et al. 2012b; Zhang et al. 2014). The C_4-specific PEPC-overexpressed wheat transgenics showed 25–50% yield increment compared to wild-type plants (Kershanskaya and Teixeira da Silva 2010). Increased yields were recorded in transgenics due to increasing in the number of grains per spike and their weight, and flag leaf stay green phenotype (Edwards et al. 2001; Kershanskaya and Teixeira da Silva 2010). Transgenic winter wheat expressing the intact C_4-PEPC gene of maize exhibited increased photosynthetic efficiency and transpiration rates (Chen et al. 2004; Dunwell 2014). Overexpression of maize C_4-PEPC in wheat also conferred increased grain yield (Qin et al. 2016), which was found to be associated with improved harvest index (HI), biomass, large spikes, increased grain number, and thousand-grain weight (TGW). Qi et al. (2017) characterized two transgenic wheat lines containing maize PEPC gene, PC27, and PC51 under high-temperature stress. All transgenic wheat lines displayed a higher photosynthetic rate under both non-stress and high-temperature (heat)-stress conditions compared to wild-type plants. Peña et al. (2017) showed ZmDof1 under the influence of light-inducible promoter RuBisCO, which is responsible for increased biomass and yield components in transgenic wheat, while constitutive expression resulted in the downregulation of photosynthetic genes and a related negative impact on crop productivity. In wheat, overexpression of *Brachypodium distachyon sedoheptulose-1,7-biphosphatase* (*SBPase*) gene showed an increase in SBPase activity with an increase in leaf CO_2 assimilation levels and an increase in total seed weight (30–40% higher than wild-type) in total biomass (Driever et al. 2017).

5.2 Transcription Factors for Manipulating Spike Development and Grain Set

5.2.1 Nuclear Factor-Y (NF-Y)

NF-Ys are composed of protein subunits from three distinct transcription factor families A, B, and C (NF-YA, NF-YB, and NF-YC, respectively) (Zanetti et al. 2017). NF-Y transcription factors, known as CCAAT-binding factors, are heterotrimeric DNA-binding proteins, and structurally maintained in all eukaryotic organisms (Mantovani 1999; Romier et al. 2003). Nuclear factor-Ys (NF-Y) are recognized as regulators of plant growth, development, and many physiological processes (Myers and Holt 2018). *TaNFYA-B1*, a low-nitrogen and low-phosphorus-inducible NF-YA transcription factor, significantly increased both nitrogen (N) and phosphorus (P) intake efficiency and grain yield in transgenic wheat under different N and P regimes in field conditions (Qu et al. 2015). Interestingly, overexpression of *TaNFYA-B1* in transgenic wheat stimulated lateral root formation, growth, upregulated the expression of both N and P transporters, and increased N and P uptake resulting in increased grain yields under control, low-N, and low-P conditions. *TaNF-YB4* gene when overexpressed under the constitutive maize zinc ubiquitin (Zn Ubi1) promoter showed a significant increase in 20–30% of grain yields in wheat cultivar Gladius (Yadav et al. 2015).

5.2.2 TaNAC Transcription Factors

TaNAC2-5A transcription factor played a vital role in nitrogen signaling, and its overexpression in wheat enhanced the root growth and nitrate influx rate and improved the root's stable ability to get nitrate from the soil. Such transgenic wheat lines exhibited higher nitrogen accumulation and grain yields (He et al. 2016). Overexpression of an isoform of wheat plastidial *Glutamine synthetase* (GS2) *TaGS2-2Ab* with its own promoter TaGS2-2Ab in winter wheat *cv.* Ji5265 resulted in higher grain yield, increased spike number, grain number per spike, and thousand-grain weight in comparison with wild-type under both low N and high N soil conditions (Hu et al. 2018b).

5.3 Genes Determining Cell Division and Proliferation Effect on Grain Size

Genomic studies of wheat identified *TaGW2* as the regulator of the grain size (Su et al. 2011) and *TaGW2-A* as a functional E3 RING ubiquitin ligase (Bednarek et al. 2012). *TaGW2* homologs A, B, and D-specific RNAi was expressed in bread wheat *cv.* Recital where the grain size and weight decreased sharply (Bednarek et al. 2012). In similar experiments, in Chinese bread wheat *cv.* Shi4185, an increment in grain width and weight was observed (Hong et al. 2014).

5.4 Genes Involved in Carbohydrate Metabolism

5.4.1 ADP-Glucose Pyrophosphorylase (AGP)

Improvement in the synthesis of seed starch is one of the factors which can increase the yields of wheat. ADP-glucose pyrophosphorylase (AGP) catalyzes a rate-limiting step in the biosynthesis of seed starch (Meyer et al. 2007; Smidansky et al. 2007). AGP, the key enzyme in starch synthesis, consists of two small and two large subunits with cytosolic and plastidial isoforms. *TaAGPS1b* was overexpressed in a Chinese bread wheat cultivar and the transgenic wheat lines showed a significant increase in AGPase endosperm activities, starch content, and grain weight. These indicated that its truncated transit peptide targeted the TaAGPS1b subunit into plastids and could play an important role in the starch synthesis in bread wheat grains (Yang et al. 2017). ADP-glucose pyrophosphorylase (AGP), which catalyzes a rate-limiting step in seed starch biosynthesis, was also used in several other genetic engineering studies for yield improvement (Meyer et al. 2007; Smidansky et al. 2007). Smidansky et al. (2002) transformed wheat with a modified form of the *Shrunken 2* maize gene (*Sh2r6hs*) that encodes a large subunit of altered AGP. In growth chamber experiments, transgenic wheat expressing *Sh2r6hs* developed an average of 38% higher seed weight and 31% increased biomass (Meyer et al. 2004) but showed no yield gain under restricted conditions (Meyer et al. 2007). Since transgenic plants have shown no yield benefits under conditions limiting production, higher photosynthetic levels in early seed development stages appeared to be

the key to enhance wheat yield components (Smidansky et al. 2007; Zhang et al. 2014).

5.4.2 Starch Synthase

Tian et al. (2018) showed expression of a rice gene encoding a soluble starch synthase (*OsSS-I*) with increased heat stability. Such an overexpression resulted in 21–34% increase in the yield of T_2 and T_3 generations of transgenic wheat under high-temperature conditions. *OsSS-I*-transgenics also extended the length of the time of photosynthetic growth in bread wheat. Similarly, overexpression of an endogenous gene coding for chloroplastic *TaGS2* in wheat resulted in prolonged photosynthesis in the leaf, and an increased rate of nitrogen remobilization in developing grains at grain-filling stage that translated into higher spike numbers, grain numbers per spike, and total yield in the form of grain weight manipulated by grain size (Hu et al. 2018b).

6 CRISPR-CAS9-Based Genetic Manipulation in Wheat

Using genome-editing technologies, various crop genomes have been modified for better resistance to biotic and abiotic stresses (Roychowdhury et al. 2020; Singh et al. 2020). Due to the large genome size (17 Gb), high ploidy, and many repetitive sequences, hexaploid wheat is difficult to manipulate genetically. However, genome-editing technologies are free from these limitations and ideal tools for gene function analysis and genome modification. In an attempt to apply CRISPR-Cas9 system for precise gene modification in wheat, *inositol oxygenase* (*inox*) and *phytoene desaturase* (*pds*) genes were introduced using wheat cell suspension cultures, which resulted in *indel* mutations (Upadhyay et al. 2013). This study demonstrated successful application of CRISPR-Cas9 system in wheat. Zhang et al. (2017) generated *enhanced disease resistance1* (*edr1*) mutant wheat plants using CRISPR/Cas9 technology by simultaneous modification of the three homoeologs of *TaEDR1*. The *TaEDR1* mutant wheat plant generated through this approach could be an important breeding material for creating powdery mildew resistance wheat crop as it exhibited resistance with no off-target effects. Imidazolinone herbicide resistance wheat has been developed through zinc finger nuclease (ZFN)-mediated, nonhomologous end joining (NHEJ)-directed editing of *acetohydroxy acid synthase* (*AHAS*) gene, which resulted into a loss-of-function gene knockout by a precise single amino acid change into the coding sequence (Ran et al. 2018). A list of the developed wheat plants (*mutants*) is shown in Table 4.

7 Conclusion and Future Perspectives

Theoretically, genetic manipulation can be effectively justifiable, yet its implication and foreseeing the future are quite dim. Albeit it needs to be legitimately acknowledged and accepted by the nation's policies, environmental groups, and society at

Table 4 Genome modification in wheat using different genome editing tools

Sl No.	Target gene(s)	Source(s)	Resistant phenotype/ trait	References
CRISPR/Cas9-mediated				
1.	*Inositol oxygenase* (*inox*) *Phytoene desaturase* (*pds*)	*Triticum aestivum*	Demonstration of CRISPR/Cas system in wheat and tobacco	Upadhyay et al. (2013)
2.	*TaMLO-A1* (*mildew resistance locus*)	*Triticum aestivum*	Powdery mildew resistance	Wang et al. (2014c)
3.	*TaEDR1*	*Triticum aestivum*	Powdery mildew resistance	Zhang et al. (2017)
4.	*TaDEP1*, *TaGW2*, *TaEPSPS*	*Triticum aestivum*	Herbicide resistance	Li et al. (2018)
TALLENs-mediated				
1.	*TaMLO-A1*, *A2*, *A3* (*mildew resistance locus*)	*Triticum aestivum*	Powdery mildew resistance	Wang et al. (2014c)
ZnFNs-mediated				
1.	*Acetohydroxyacid synthase* (*AHAS*)		Herbicide resistance	Ran et al. (2018)

large. Advancements have been made in wheat by manipulating its genetic cassette for increased resilience to different abiotic stresses (drought, salinity, high and low temperature, herbicide), diseases caused by fungus, bacteria, virus, insects, and nematodes. Additionally, research has been focused to improve the nutritional values in wheat flour by enhancing carbohydrates, proteins, lipids, dietary fibers, vitamins, and minerals and reduction of antinutrients in the grains. In wheat, improved grain yield (grain number, grain weight, and size) has also been achieved by genetic manipulation of different nuclear and transcription factor regulated pathways. Importantly, photosynthetic efficiency and carbon assimilation, spike development and grain setting, and grain starch contents have been addressed. Further, CRISPR-Cas9 has become the most promising gene-editing tool to manipulate any undesired trait in wheat. Utilizing different gene-editing technologies, it is possible to dissect the wheat genome and modify the functions of the genes for improving grain quantity and quality and tolerance to a wide range of biotic and abiotic stresses. But effective utilization of technologies, as well as transgenic wheat lines developed, is inescapable in future to meet the ever-growing food demands throughout the world.

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Transgenic Finger Millet [*Eleusine coracana* (L.) Gaertn.] for Crop Improvement

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Abstract

Finger millet [*Eleusine coracana* (L.) Gaertn.] is a nutritionally enriched member of the Poaceae family and sixth most important cereal crop. The crop is mostly cultivated throughout Asia, Africa, and South America. Finger millet is a rich source of minerals, vitamins, dietary fibers, and proteins as compared to other millets. The crop has a diverse gene pool with distinct features and genetic variations. It has several advantages of nutritional quality and quantity, health benefits, and abiotic and biotic stress adaptation over other cereals. Sustainable crop improvement is one of the critical tasks in the present-day plant breeding programs. Conventional methods of plant breeding have improved the crop yields, but have their limitations. In such a scenario, targeted and less time-consuming molecular marker-assisted breeding and genetic engineering-based tools and techniques are encouraged. In this chapter, we have discussed some of the transgenics developed in finger millet for crop improvement.

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Biotic and abiotic stresses · Crop improvement · Finger millet · Genetic engineering

1 Introduction

Millets are the major source of staple food that supplies a major portion of nutrition in Africa and Asia to the larger segment of populations (O'Kennedy et al. 2006; Vinoth and Ravindhran 2017). It serves as a critical plant genetic resource to cover food security for the farmers in Asia and Africa, where lands are arid, infertile, marginal, and poor (Gupta et al. 2017). Generally, seven crops are referred to as millets including barnyard millet (*Echinochloa* spp.), finger millet [*Eleusine coracana* (L.) Gaertn], foxtail millet [*Setaria italica* (L.) Beauv], Kodo millet (*Paspalum scrobiculatum* L.), little millet (*Panicum sumatrense* Roth. ex Roem. & Schult.), pearl millet [*Pennisetum glaucum* (L.) R.Br.] and proso millet (*Panicum miliaceum* L.). Of these seven millet crops, pearl millet conquers the first position with about 95% of the total millet production followed by Foxtail millet (Yadav and Rai 2013; Nedumaran et al. 2014; Vinoth and Ravindhran 2017).

Finger millet [*Eleusine coracana* (L.) Gaertn.] belongs to the family Poaceae, commonly known as ragi, nachani, or nagali (Mundada et al. 2019). It is an allotetraploid crop plant species ($2n = 4X = 36$). The generic name "*Eleusine*" is derived from an ancient city sacred to Demeter called "Eleusis," the Greek goddess of agriculture. The specific epithet "*coracana*" is derived from Sinhalese language of Sri Lanka namely "kurukkan." The common name finger millet is acquired from the finger-like branching of the panicle (Mirza and Marla 2019). It is mainly cultivated in the arid and semiarid regions of the world (Fakrudin et al. 2004). It is grown as a cereal crop in more than 25 countries of Africa, Asia, and South America in warm temperate regions (Phillips 1972). It is the sixth most important cereal crop with tremendous potential but is under-utilized than other consumed cereals. In India, finger millet stands at the sixth position after important cereal crops, viz. wheat, rice, maize, sorghum, bajra, and vital staple food for economically weaker sections. Globally, India is the largest producer of finger millet (Ramakrishnan et al. 2015). More than 34,160 finger millet genotypes are available throughout the world, and about 22,583 genotypes are found in India alone (Ramakrishnan et al. 2016). Worldwide around 12% of the area is under the cultivation of millet accounting for the production of around 4.5 million metric tons per year (Kumar et al. 2016). Annual production of finger millet in Africa is 2.5 million metric tons and ranking first followed by India producing 1.2 million metric tons. Among all the millets cultivated in India, finger millet alone accounts for 85% of the yield and is cultivated over the area of 1.19 million hectares (Sakamma et al. 2018).

Nutritionally, the grains are the ionic source of minerals especially calcium, vitamins, dietary fibers, proteins, and energy as other cereals (Devi et al. 2014). It also contains a useful amount of certain minerals such as copper, iron, manganese, and

phosphorus (Tripathi and Platel 2010). It is an important crop in the diets as "Nachani Satva" for children, pregnant women, and lactating mothers (Gupta et al. 2017). It showed health beneficial pharmacological activities like antidiabetic, antimicrobial, antioxidant, atherosclerogenic, and anti-tumorigenic (Devi et al. 2014). It is also used in folk medicine as well as a source for beer production and valuable as fodder for cattle (Kumar et al. 2016).

In developing countries, malnutrition is a severe problem for pregnant women and fetuses (Datta et al. 2006; Renuka et al. 2016). Micronutrient deficiencies are associated with alteration in compromised commencement, length of development, and fetal growth and development, which can lead to pregnancy loss, preterm delivery, small birth size, birth defects, and long-term metabolic disturbances (Gernand et al. 2016). Finger millet consists of all the quantitative and qualitative traits to serve as a model plant for nutritional supplements. It has better nutritional qualities than that of rice, wheat, and other prominent cereal crops (Latha et al. 2005; Chandrasekara and Shahidi 2010). To combat malnutrition, conventional plant breeding, and genetic engineering are the best ways to improve nutritional components (Stein 2010). Understanding of inheritance and heritability is the most important factor for designing of any breeding program (Grant et al. 2008). In addition to this, the identification of genetic resources with rich nutrients and compositions is an essential step to improve nutritional quality through conventional plant breeding (Ortiz-Monasterio et al. 2007).

2 Crop Improvement by Genetic Engineering

Agriculture practices were started by man almost thousands of years ago. Agriculture is the domestication of wild plants for personal use in humans. Food, cloth, and shelter are considered as the basic need for the sustainability of human life on this planet. To fulfill the demand for food, several crop plants have been harvested from years ago. With the ever-increasing population the demand for food, sustainable crop improvement is the need of the hour. The Major limiting factor for improving the yield of crop plants are biotic and abiotic stresses. From ancient times, man has been adopting different ideas and techniques for the protection of crops against these factors. Despite these efforts, it is not possible to cope up with the increasing demand for food with conventional cultivation practices and rapidly decreasing natural resources. Thus, there is an urgent need to develop new strategies and approaches for the improvement of quality as well as quantity of yield.

In the present era, the transfer of genes between unrelated species for improvement in nutritional quality and development of biotic and abiotic stress tolerance are vital tools in the improvement of crops. Transgenic technology is most widely used for the improvement of maize, rice, and wheat. Many of the transgenic crop plants have been cultivated on large scale in certain countries (Shrawat and Lörz 2006). This has resulted in improved production capacity, high nutritive value, stress tolerance, etc. Genetic engineering tools allow transferring the genes among distinct genera or

species (Ali et al. 2011). Genetic engineering is different than the conventional breeding technique and involves the intended transfer of genetic material from one source to another. It is more effective and requires a short duration for the development of new crop variety than traditional breeding.

Most popular crops developed by genetic engineering include longer post-harvest storage tomatoes (flavor saver), insect-resistant cotton and maize (BT), virus-resistant potato, herbicide-resistant soybean, and canola (Dunwell 2000; Akhtar et al. 2014; Amin et al. 2014; Dar et al. 2014; Tariq et al. 2014; Khan et al. 2015; Puspito et al. 2015). For the efficient transfer of genetic material, a suitable gene transfer technique is required. These techniques include physical, chemical, and biological methods. The techniques used for the development of transgenes and traits in finger millet are discussed further.

2.1 Development of Protocol for Genetic Transformation in Finger Millet

As compared to other cereal crops, very few attempts have been made for genetic improvement despite having several nutritional properties in finger millet (Table 1). The first report on transformation in finger millet used the biolistic method of gene transfer (Gupta et al. 2001). This attempt was made to test the efficiency of different promoters, namely CaMV35S, Act I (rice), UqI (maize), RbcS, and FtuidA using β-glucuronidase (GUS) assay (Gupta et al. 2001). This study concluded that UqI gene promoter was the most efficient for the stable transfer of transgene in finger millet.

Agrobacterium-mediated genetic transformation in finger millet was reported by Sharma et al. (2011). In this protocol, they used green nodular regenerative calli with meristematic nodules of seeds origin as the target tissue. *Agrobacterium* strain EHA-105 bearing plasmid p-CNL 56 with *npt*II as the selectable marker was used for the study. The highest transformation frequency of 44.4% was reported using this protocol. The use of $CuSO_4$ in the regeneration medium enhanced the transformation efficiency during recovery of the transformed plants. The transgenes were confirmed by PCR using *npt*II-specific primers.

Another attempt on the optimization of *Agrobacterium*-mediated gene transfer using shoot apex was reported by Ceasar and Ignacimuthu (2011). Wherein, *Agrobacterium* strain LBA4404 bearing plasmid binary vector CAMBIA1301 with hygromycin phosphotransferase (*hpt*II) as a selectable marker gene and GUS reporter gene was used. In genotype GPU 45, 19% transient expression with 3.8% stable transformation efficiency was achieved using optimal conditions. The transformation was confirmed by southern blotting.

Jagga-Chugh et al. (2012) used seed-derived callus as an explant for the optimization of biolistic mediated transformation protocol under CaMV35S promoter for efficient and accurate transfer of a gene. Optimum conditions for biolistic mediated genetic transformation in finger millet were reported as 1100 psi pressure to rupture disk with 3 cm distance for microcarrier and 12 cm microprojectile travel distance.

Table 1 List of developed protocols for genetic transformation in finger millet

Method of transformation	Promoter/ reporter used	Selection marker	Target tissue/ explant	Outcome	References
Biolistic method	CaMV35S, act I, UqI, RbcS, and FtuidA GUS	*uid*A	Callus	Development of protocol for gene transfer and screening of efficient promoter	Gupta et al. (2001)
Agrobacterium-mediated gene transfer	CaMV35S GUS	*npt*II	Embryonic seeds	Standardization of protocol for *Agrobacterium*-mediated transformation in *Eleusine coracana*	Sharma et al. (2011)
Agrobacterium-mediated gene transfer	CaMV35S GUS	*hpt*II	Shoot apex	Establishment of protocol for *Agrobacterium*-mediated gene transfer by optimizing different physical and chemical parameters	Ceasar and Ignacimuthu (2011)
Microprojectile bombardment method	CaMV35S GUS	*hpt*II	Seed derived callus	Optimization of protocol for biolistic mediated gene transfer	Jagga-Chugh et al. (2012)
Agrobacterium-mediated gene transfer	CaMV35S GUS	*hpt*II	Shoot apical meristem	Improved protocol for *Agrobacterium*-mediated gene transfer in finger millet	Satish et al. (2017)

Treatment of callus with 0.4 M sorbitol enhanced the efficiency of the biolistic method of genetic transformation. This method achieved 45.3% of transformation efficiency in finger millet. Transformation of the transgene was confirmed using DIG-labeled *hpt*II as a probe for Southern hybridization and PCR with *hpt*II primers.

Satish et al. (2017) reported the genetic transformation in finger millet using the *Agrobacterium* strain EHA105 bearing binary vector pCAMBIA1301. An improved protocol was developed using shoot apical meristem as explant. Several parameters like age of explants, concentration of antibiotic (hygromycin), the density of culture, duration of infection, co-cultivation period, and concentration of acetosyringone were optimized. This study confers the maximum transformation efficiency (85.1%) in finger millet using *Agrobacterium* without undergoing the callus phase in 45 days duration. The transformation was confirmed by PCR and Southern blotting.

2.2 Genetic Transformation in Finger Millet for Biotic Stress Tolerance

The yield of finger millet is highly affected due to leaf blast disease caused by *Pyricularia grisea* (Harinarayana 1986) mostly in the rainy season. Most commonly, the disease is controlled by using synthetic chemical fungicides. However, these fungicides affect the environment adversely by destructing biodiversity. Thus, tolerance to such biotic stress can be efficiently conferred by the transfer of exotic genes. However, to date, limited attempts are reported in finger millet (Table 2).

The very first report on the transfer of gene in finger millet came from Latha et al. (2005). They used biolistic method of gene transfer to transform antifungal protein (PIN) from a prawn to confer the resistance to the leaf blast disease caused by *Pyricularia grisea* in finger millet. The PIN gene was synthesized artificially and placed under the CaMV35 promoter in plasmid pPin35S. Stable integration and expression of transgene was confirmed by using Southern and northern blot analysis and fungal bioassay in primary transformants (T_1). However, the stability of transgene among successive generations (T_2–T_3) was not discussed.

For combating the same issue of leaf blast disease, another transgene from rice coding chitinase 11 (*Chi11*) was transformed into finger millet using *Agrobacterium*-mediated gene transfer technique (Ignacimuthu and Ceasar 2012). In this study, the transgene Chill was driven by maize ubiquitin promoter. GUS histochemical staining was performed to check the transient expression of transgene for the confirmation of transformation. Furthermore, the presence and expression of transgene was confirmed using PCR, Southern blotting, and western blotting. The efficacy testing for leaf blast disease tolerance in the transformed plant was carried out by challenging the transgenic plants with spores of *Pyricularia grisea*.

Table 2 Genetic transformation in finger millet for biotic stress tolerance

Method of transformation	Promoter/ reporter used	Selection marker	Transgene	Target tissue/ explant	Outcome	References
Biolistic method	CaMV35S *uid*A GUS	PAT	PIN (antifungal protein from prawn) *bar* gene	Shoot tip	Biotic stress tolerance (leaf blast disease)	Latha et al. (2005)
Agrobacterium-mediated gene transfer	Maize ubiquitin promoter *PDH*45	*hph*	*Chi* 11 (rice chitinase gene)	Shoot apex	Biotic stress tolerance (leaf blast disease)	Ignacimuthu and Ceasar (2012)

2.3 Genetic Transformation in Finger Millet for Abiotic Stress Tolerance

Genetic engineering tools offer several opportunities in the improvement of finger millet for abiotic stress tolerance. During the last two decades, very few attempts have been made for the development of abiotic stress tolerance in finger millet (Table 3). Most of the efforts have been made on the development of salt stress-tolerant transgenics in finger millet as compared to other environmental stresses.

The development of transgenic lines of finger millet for abiotic stress tolerance was first reported by Mahalakshmi et al. (2006). They transferred serine-rich protein (*PcSrp*) gene from *Porteresia coarctata* under Actin-1 promoter from rice for salinity stress tolerance using the biolistic method in embryonic calli derived from shoot tips. Tungsten particles of size 1.1 μm were bombarded at 15 kg/cm^2 helium gas pressure at 7 cm target distance along with 4 h of pre- and post-bombardment treatment with 0.2 M sorbitol and mannitol. Selection of transformants was carried out on a medium supplemented with 250 mM NaCl.

An attempt for in vitro plant regeneration and transformation in finger millet was carried out by Babu et al. (2012). For induction of salt tolerance, a binary vector pCAMBIA carrying *PDH45* gene was transformed using *Agrobacterium*-mediated gene transfer. Embryonic seed callus was used as the explant for the transformation. The transformation was confirmed by PCR using *hptII* primers and expression of the transgene was confirmed using RT-PCR.

Bayer et al. (2014) developed the transgenic lines resistant to herbicides of dinitroaniline family on the application of both biolistic and *Agrobacterium*-mediated approach to transfer the genes. For gene transfer using biolistic gene gun method, plasmid pAHTUAml containing mutant α1-tubulin gene from *Eleusine indica* and β1-tubulin gene from *Hordeum vulgare* was used. The plasmid construct contained phosphinothricin resistance (*bar*) gene as a selectable marker, maize ubiquitin promoter (*PUbi*), and nopaline synthase (*NOS*) as a terminator. A binary vector pBIT-UBA8 with *TUBm1* and *HvTUB1* genes under CaMV35S promoter was used for gene transfer using *Agrobacterium*. The transformants were selected by growing on a medium containing trifluralin in in vitro stages. The use of activators of virulence genes for *Agrobacterium* amplified the frequency of transformation in finger millet.

Anjaneyulu et al. (2014) reported the introduction of vacuolar H^+-pyrophosphatase (*SbVPPase*) gene from *Sorghum* in finger millet using *Agrobacterium*-mediated gene transfer and enhanced the salt stress tolerance capacity of finger millet. Embryogenic calli of finger millet was transformed with pCAMBIA1301 plasmid vector containing *SbVPPase* gene placed under CaMV35S promoter. The transformation was confirmed using GUS assay and PCR. Furthermore, the expression of the transgene was tested by growing control and transgenic plants in 100 mM and 200 mM NaCl and testing certain biochemical and physiological parameters of growth. The transgenic plants exhibited tolerance to salt compared to controls.

Similarly other genes, Na^+/H^+ antiporter of *Pennisetum glaucum* (*PgNHX1*) and H^+-pyrophosphatase (*AVP1*) from *Arabidopsis thaliana*, was transformed using *Agrobacterium*-mediated transformation (Jayasudha et al. 2014). Using the plant

Table 3 Genetic transformation in finger millet for abiotic stress tolerance

Method of transformation	Promoter/reporter used	Selection marker	Transgene	Target tissue/Explant	Outcome	References
Biolistic	Actin-1 promoter from rice	–	*PcSrp*	Embryonic callus derived from shoot tip	Salt tolerance	Mahalakshmi et al. (2006)
Agrobacterium-mediated gene transfer	CaMV35S GUS	*nptII*, *hptII*	*PDH45*	Embryonic seed calli	Salt tolerance	Babu et al. (2012)
Biolistic and *Agrobacterium*-mediated gene transfer	*PUbi* CaMV35S GUS	Phosphinothricin resistance (*bar*) gene	*HvTUB1*, *TUAml*	Embryogenic callus	Herbicide tolerance	Bayer et al. (2014)
Agrobacterium-mediated gene transfer	CaMV35S GUS	–	*SbVPPase*	Embryogenic calli	Salt tolerance	Anjaneyulu et al. (2014)
Agrobacterium-mediated gene transfer	CaMV35S GUS	*hptII*	*PgNHX1*, *AVP1*	Embryogenic calli	Salt tolerance	Jayasudha et al. (2014)
Agrobacterium-mediated gene transfer	CaMV35S	*hptII*	*mtlD*	Embryogenic calli	Drought and salt tolerance	Hema et al. (2014)

binary expression vector pCAMBIA 1301, both the transgenes were transferred to finger millet using *Agrobacterium tumefaciens* strain EHA105. The transfer of transgene was confirmed by physiological analysis and PCR. The transgenic plants displayed enhanced salt stress tolerance capacity than respective wild types.

Hema et al. (2014) attempted a stable expression of bacterial mannitol-1-phosphate dehydrogenase (*mtlD*) gene in finger millet. The present study *Agrobacterium*-mediated gene transfer technique was used to produce transgenic plants. The transgenic plants showed tolerance to drought as well as salinity stress as compared to their respective wild types. Under drought stress, transgenic lines also showed a higher retention of chlorophyll than wild type (Hema et al. 2014).

3 Conclusion

Finger millet is a nutritionally ironic crop serving as a major source of nutrition especially for the economically weaker sections of society in developing countries. The development of transgenic finger millet is a challenging task due to the limited availability of reports at the genome and transcript level. In the present study, a summary regarding the successful development of transgenic finger millet is discussed. This study will certainly help in exploring further opportunities for the development of finger millet research involved in key nutrient management, abiotic and biotic stress tolerance, genetic diversity analysis, gene expression analysis, and use of genome editing tools like CRISPR/Cas9, etc. Presently limited attempts with few genes have been made for the development of transgenic finger millet. Targeting key genes in nutrient management and stress tolerance via emerging biotechnological techniques will help in the generation of bio-fortified crops.

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Transgenic Pigeonpea [*Cajanus cajan* (L). Millsp.]

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Abstract

Pigeonpea is a multipurpose legume crop that grows in many tropical and subtropical regions. Its properties such as high protein content, deep root system, fast growth and ability to withstand drought condition make it an economically important crop. With an increase in population, there is a huge gap in the demand and supply of the pigeonpea as the crop is affected by various biotic and abiotic stresses. Insect pests like *Helicoverpa armigera* and *Maruca vitrata* and fungal diseases like *Fusarium* wilt are a few yield-limiting factors which lead to stagnated productivity. Among abiotic factors, cold sensitivity, water logging and salinity affect the crop. To overcome these problems and increase the production of pigeonpea to meet societal requirements, biotechnological approaches can be employed. Transgenic technology has emerged as one of the successful biotechnological approaches used in crop improvement programmes. Various genes have been identified, validated and deployed in crop species through transgenesis towards their improvement. However, a prerequisite is successful transformation of plants through high-throughput tissue culture methodologies. However, genotypic dependent response and/or difficulty to regenerate make pigeonpea a recalcitrant crop. To tackle these problems, alternate non-tissue culture methods have been used to transform pigeonpea. In this chapter, we present various aspects of stress mitigation in pigeonpea through transgenesis and their utility in crop improvement.

Keywords

Pigeonpea · Transgenics · Biotic stress · Abiotic stress · *Bacillus thuringiensis*

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1 Introduction

Pigeonpea (*Cajanus cajan* (L.) Millspaugh) is a diploid crop which belongs to subtribe Cajaninae and tribe Phaseoleae, under sub-family Papilionoideae of the family Fabaceae (Leguminosae) (Upadhyaya et al. 2013). The tribe Phaseoleae also includes other edible beans like soybean, mungbean and field bean. Pigeonpea is also known by different vernacular and trade names such as red gram, arhar, tuar/tur (in Hindi), Angola pea, Congo pea, yellow dal (Hindi) and oil dal. This legume crop is a perennial shrub that is grown in tropical and subtropical regions of many countries like Asia, Latin America, Africa, and the Caribbean region. Though the origin of pigeonpea is disputable, the gene pool and some archaeological evidences show that the most probable primary centre is India, and then it spread to Africa (van der Maesen 1980).

Pigeonpea has an important role in sustainable and eco-friendly agriculture. It is a multi-purpose crop that has a wide variety of applications. The most common use is for human consumption and income generation. The seeds of pigeonpea contain 20–30% of protein (Salunkhe et al. 1986; Singh et al. 1990; Saxena et al. 2002) and other essential amino acids, making it a principal source in providing dietary protein to vegetarian population. The main use of the seeds is for cooking dal for which dry, dehulled, whole or split seeds can be used. The green seeds can also be canned, frozen or cooked as a vegetable. The other parts of pigeonpea like seed husk, green leaves and pod walls are used as cattle feed. Pigeonpea plant has many miscellaneous uses. The stem is used for producing baskets and fuel, different parts are used for medicinal and cosmetic purposes, tall perennial plants are used as live fences, wind breakers and for soil conservation. The fallen leaves are an excellent source of organic matter and symbiotic nitrogen fixation adds value to the soil and increases soil fertility. In maintaining food security, pigeonpea is best suited crop due to its deep rooting system and ability to grow in places with low-rainfall and withstand the drought condition. The fast growth and adaptability to wide environmental conditions makes it an economically important crop.

Globally, pigeonpea is cultivated in an area of 7 Mha with the 6.8 metric tons (MT) of production. Among global producers, India accounts for more than 80% of the total production by producing 4.8 MT of pigeonpea. The other main producers are Burma (0.79 MT), Malawi (0.47 MT), Tanzania (0.27 MT) and Kenya (0.20 MT) (FAOSTAT 2017).

With burgeoning population, present production of pigeonpea is inadequate for the domestic demands. There is a large gap between the demand and the supply of the crop due to its static production. Despite having high-yielding cultivars, productivity still remains stagnant. The huge variation is observed in the potential yield of the crop and the yield obtained by the farmers. There are a number of factors that are responsible for the low yield of pigeonpea. Poor production practices such as low-density varieties, low soil fertility, inappropriate use of fungicides and herbicides and insufficient weeding affect the yield. There are biotic and abiotic constraints that damage the crop on the field that results in yield loss. The biotic stress factors include fungal, bacterial and viral diseases and insect pests. Most

widespread disease is *Fusarium* wilt (*Fusarium udum* Butler), a fungal disease that can affect the plant at all developmental stages and cause major loss in productivity (Lateef and Reed 1990). Another bacterial pathogen *Xanthomonas cajani* also damages the crop by causing diseases such as leaf spot and stem canker (Sharma et al. 2008). Another economic threat of pigeonpea is sterility mosaic disease caused by pigeonpea sterility mosaic virus (PPSMV) that can result in entire crop failure (Lateef and Reed 1990). Among insect pests, the pod borer *Helicoverpa armigera* is the most common insect, as it attacks the pods at an early stage (Shanower et al. 1999). *Maruca vitrata* is another pod borer that reduces the productivity of pigeonpea (Taylor 1978; Shanower et al. 1998). Furthermore, abiotic stresses such as water-logging, drought, low temperature and salinity are also responsible for substantial yield reduction.

Pigeonpea is often considered as an 'orphan crop'. Despite having very diverse applications, very little effort has been made towards the improvement of pigeonpea. In the present scenario, when the food has become a luxury due to high-cost products, it is the need of the hour to pay extra attention to this economically important crop. Pigeonpea can provide proper nutrition and balanced diet to the population living in poverty. This will help in alleviating poverty and fighting against the problem of malnutrition which has become a major concern in many countries. Pigeonpea requires minimum input for its growth and has the ability to produce good amount of food and forage, thus making it an agriculturally important crop. The economic growth of the country can be enhanced by doing some serious efforts in boosting the production of this protein-rich legume.

The traditional method to improve any crop is plant breeding, but it mostly depends on phenotypic characters, and it is usually laborious and time consuming. However, integration of molecular and genomic aspects in agriculture has led to the improvement in the conventional breeding strategy. Along with the technological development, this method is not able to produce the required quality and quantity of the crop. There are many limitations such as narrow range of germplasm, domestication effect, incompatibility and linkage drag, which makes it difficult to improve pigeonpea through breeding strategies (Venkata et al. 2018). These shortcomings can be overcome by introducing alien genes through genetic engineering. With the advancements in biotechnological research, the strategies like genetic transformation, gene-editing and RNAi might help in improving the crop by introducing agronomically important traits (Rashid et al. 2017). In this chapter, we discuss the improvement of pigeonpea by biotechnological approaches. The technological advances are briefly discussed along with the case studies of transgenic pigeonpea.

2 Conventional vs. Modern Strategies

Since the beginning of agriculture, farmers practiced crop improvement for the benefit of humanity. Historically, there are two main techniques adopted by farmers for engineering the plants. The first is selection, which involves the screening of plants based on the genetic variability. Plants are selected which have better quality traits

like big fruit or grain size or easy to harvest. This method follows Darwinian theory of natural selection; the plant that is best fitted in the environment is chosen and cultivated. The seeds from the selected plants were saved for the next year's cultivation. The second method is plant breeding, in which the farmers or scientists cross pollinate two varieties to obtain the desired trait. Such efforts led to the development of improved varieties that have agronomically important traits such as high yield, big grain size, insect resistance, early maturity, or disease resistance. Along with the plant breeding technology, other factors such as the improved agricultural practices, soil and water management and the use of agrochemicals contributed significantly towards the enhancement of agricultural output (National Research Council 1984).

Breeding in pigeonpea is complex and challenging due to some crop-specific traits. The first constraint is the narrow genetic diversity due to the loss of many genes in the course of artificial selection (Venkata et al. 2018). This leads to the exploitation of wild relatives of the crop for the development of improved varieties. The wild relatives of pigeonpea are known for pest and disease resistance, and higher genetic diversity is present in comparison to their domesticated descendants (Zhang et al. 2017). Despite rich germplasm, its utilization is very limited due to the lack of information on the presence of useful traits. The incompatibility of the wild-type further lowers the chances of breeding in pigeonpea. The wild relatives are classified into three groups on the basis of gene pool that are primary, secondary and tertiary (Zhang et al. 2017). The crossing between the primary and secondary relatives is easy, but gene exchange with tertiary is usually difficult. Due to lack of interspecific crossing, the utilization of wild relatives in developing improved cultivars is limited.

Photosensitivity is another factor that limits breeding in pigeonpea (Venkata et al. 2018). The flower induction in pigeonpea usually requires photoperiod which includes shorter days and long hours of darkness. The traditional cultivars of pigeonpea are medium to long duration that are harvested in 6–12 months of sowing. Recently short and extra short duration cultivars have been developed, which mature in 90 days. The long duration pigeonpea is well suited for intercropping due to reduced competition, whereas short duration is advantageous in monocrop system for high yield. It has been reported that long duration genotype is more sensitive to photoperiod than short-duration genotypes of pigeonpea. Linkages of genes also cause hindrance in breeding (Venkata et al. 2018). Transfer of undesired traits along with the desired ones due to close association also narrows down the option of plant breeding in crop improvement.

Agricultural biotechnology plays a crucial role in meeting food demand. Today with the help of modern biotechnology tools, we are able to modify the genetic structure of crop and introduce economically important traits utilizing genetic engineering methodologies. The utilization of genetic engineering in crop improvement helps us in easily manipulating the crop according to the need. Many novel characters can also be introduced in plant species without any undesirable effects.

3 Genetic Engineering in Pigeonpea Crop Improvement

The sustainable agriculture can only be maintained with the involvement of new technologies. At the time of 'Green Revolution' also, it was observed that the improved varieties with the support of technology resulted in boosting the yields. However, the potential of technology is limited in plant breeding due to linkage drag and lack of genetic diversity among the cultivated germplasm. Genetic engineering provides the opportunity for introgression of traits from distantly related plant and other sources.

Genetic engineering includes techniques like recombinant DNA technology, gene-editing, and RNAi, which enables the reshuffling and editing of genes thus opening new sources of genetic diversity for crop improvement (National Research Council 1984). In contrast to plant breeding where the outcome can be uncertain due to the close association of many genes, genetic engineering is very specific in transferring only the desired gene to the recipient plant. There is no barrier in selecting the donor for the gene, the genetic engineer can borrow from outside the plant kingdom and introduce into the desired recipient. Improvement in transgenic technology makes the manipulation of plant genome more precise and helps in the improvement of crop genetic diversity, thus having a great contribution in crop improvement. In general, tissue culture-based methods are widely used for making transgenics. In the tissue culture approach, the whole plant is regenerated from different explants for example leaf, whole seed, cotyledon and embryonic structure. The regeneration of plants is carried under controlled conditions, and the best suitable transformed plants are then selected for further testing. Regeneration of transgenic plants can be obtained either through direct organogenesis or indirect organogenesis. In pigeonpea, various explants and transformation strategies were attempted for regeneration (Table 1). The explants like leaves (Geetha et al. 1998; Dayal et al. 2003), cotyledons (George and Eapen 1994; Naidu et al. 1995; Geetha et al. 1998), epicotyls (George and Eapen 1994; Naidu et al. 1995), shoot apices (Geetha et al. 1999) and axillary meristems (Sharma et al. 2006) were reported for regeneration. The successful development of transgenics will be dependent upon selection of suitable transformation vector, transformation method and explants. Many research groups exploited the genetic engineering technology for pigeonpea crop improvement, and various transformation methods and strategies have been utilized (Table 1). In the last two decades, many traits have been incorporated in pigeonpea through genetic engineering to manage various biotic and abiotic stresses.

3.1 Transgenics for Biotic Stress Tolerance

Biotic stress in pigeonpea is caused by various pest and pathogens, which cause reduction in quality and yield of pigeonpea. Among the biotic stresses, the insect pests are the most problematic. They attack and feed on all parts of the plant which leads to major yield losses. There are more than 200 species of insects that attack pigeonpea from germination to harvest (Shanower et al. 1999). The inappropriate

Table 1 Various studies demonstrating genetic transformation in pigeonpea

Cultivar	Explants	Transgenic trait	Gene	Transformation technique	Reference
Hyderabad C	SA and EA	Standardization	*uidA*	*Agrobacterium*	Geetha et al. (1999)
ICPL 88039	CN	Standardization	*gus*	*Agrobacterium*	Ramchandar (1999)
ICPL 88039	LE	Standardization	*uidA*	Biolistics	Dayal et al. (2003)
T 15-15	EA	Standardization	*uidA, gfp*	*Agrobacterium*	Mohan and Krishnamurthy (2003)
LRG 30	CN	Standardization	*uid A*	*Agrobacterium*	Kumar et al. (2004b)
TTB7	CN	Standardization	*uidA*	*In planta*	Rao et al. (2008)
ICPL87, ICPL85063, LRG30	EA and CN	Transformation variation	*uidA, GS-TAPI*	*Agrobacterium*	Surekha et al. (2007)
Hyderabad	EA and CN	Edible vaccine	*RVPV*	*Agrobacterium*	Satyavathi et al. (2003)
Hyderabad	CN	Edible vaccine	*PPRV-NH*	*Agrobacterium*	Prasad et al. (2004)
ICPL 87	CN	Nutritional improvement	*dhdps-r1*	Biolistics	Thu et al. (2007)
TTB7	Seedlings	Nutritional improvement	*DsCs*	*In planta*	Hussain et al. (2016)
LRG 30	CN	Disease resistance	*gfp, Rchit*	*Agrobacterium*	Kumar et al. (2004a)
ICPL 87	EA	Pest resistance	*cry1E-C*	*Agrobacterium*	Surekha et al. (2005)
PAU 881	EA	Pest resistance	*cry1Ac*	*In planta*	Kaur et al. (2016)
TTB7	EA	Pest resistance	*cry1AcF*	*In planta*	Ramu et al. (2012)
ICPL 87	Seedlings auxiliary bud	Pest resistance	*cry1Ab*	*Agrobacterium*	Sharma et al. (2006)
Pusa 992	EA	Pest resistance	*cry2Aa*	*In planta*	Singh et al. (2018)
UPAS 120	EA	Pest resistance	*cry2Aa, cryAc*	*Agrobacterium*	Ghosh et al. (2017)

EA embryonal axes, *CN* cotyledonary node, *SA* shoot apices

use of pesticides by farmers and ability of insects to evolve and develop resistance against these insecticides are matters of great concern. Moreover, these pests are motile which leads to inter-mating of pests present in different geographic areas, thus increasing the genetic variability within the population. Due to high genetic variation, the insects evolve and adapt to different environmental conditions. The

best way to control insect pests is the development of resistant cultivars and germplasm.

The pigeonpea pest can be divided into three categories: flower and pod feeding Lepidoptera, pod-sucking Hemiptera, and seed-feeding Diptera and Hymenoptera (Shanower et al. 1999). The pod borer *Helicoverpa armigera* and *Maruca vitrata* are the two important Lepidopteran species that are responsible for most of the damage (Shanower et al. 1999; Taylor 1978). These two are the most damaging pests, and their occurrence often results in complete crop failure. The pod fly, *Melanagromyza obtusa* is another insect that attacks pigeonpea. At appropriate conditions, pigeonpea has the ability to tolerate and recover from the early season damage (Shanower et al. 1998). Thus, the pests that attack at the middle or end of the life cycle are economically important. A number of studies identified genotypes of pigeonpea that show the resistance against the pests (Choudhary et al. 2013). However, the source of resistance is found to be more in wild relatives than in the cultivated genotypes of pigeonpea (Zhang et al. 2017). Host resistance is an important factor to control in insect infestation.

Pigeonpea breeding usually focuses on morphological and biochemical aspects of plant to enhance resistance against insect pests. The morphological traits associated with the resistance in pigeonpea include structure of the pod wall, pod toughness and trichomes (Choudhary et al. 2013). The thickness of the pod wall is directly proportional to the host plant resistance. The cultivars with thick pod wall have more resistance against pod fly. The orientation, length and density of trichomes on the pod contribute to resistance towards the pod borer (Peter et al. 1995; Choudhary et al. 2013). The biochemical production of the plant is also important for the resistance. In pigeonpea, biochemical traits of pod wall like wax content, total phenols, low levels of reducing and non-reducing sugars and total amino acids are responsible for resistance against pod borer as well as pod fly (Choudhary et al. 2013).

3.1.1 Genetic Engineering for Insect Resistance

The main reason of insufficient and unstable production of pigeonpea is due to its susceptibility to insect pests. The pod borer *Helicoverpa armigera* is one of the insect pests which causes significant damage to pigeonpea (Shanower et al. 1999). *H. armigera* larvae feed on nitrogen-rich part of the plants such as reproductive structures and growing tips (Fitt 1989). The four features of *H. armigera* that makes it a serious insect pest are high fecundity, strong flying ability, extensive polyphagy and facultative diapauses. The pest prefers cotton, tomato, sorghum and maize as its hosts (Ramnath et al. 1992). Pigeonpea is also a suitable host for *H. armigera* in terms of ovipositional response that is required by moth for reproductive growth (Tripathi and Singh 1989). *Maruca vitrata* is another pest whose host range is restricted to legumes (Taylor 1978). The larval feeding is the major complication of this pest. The larvae feed from inside a webbed mass of leaves, pods and flowers, which causes difficulty for pesticides and natural enemies in penetrating the shelter.

Lack of resistance sources for both the pests and limited genetic variation in pigeonpea germplasm minimize the option for conventional breeding for the development of protection strategies. In this situation, the transgenic approach for insect

resistance is pertinent with the *cry* genes of *Bacillus thuringiensis* widely used to develop insect-resistant crops. Many insecticidal crystal proteins (ICPs) from the bacteria *Bacillus thuringiensis* are reported to provide insect-resistance in different crops (Bravo et al. 2017). *Bt* cotton is one of the significant achievements of transgenic research where the *cry1Ac* gene insertion resulted in resistance to bollworm (Chakrabarti et al. 1998). *Cry* genes are also used in improving other crops like maize, brinjal, tomato, etc., against Lepidopteran pests (Kumar and Sharma 1994; Chakrabarti et al. 1998; Kumar et al. 1998; Bravo et al. 2017). After achieving positive results in many crops, *Bt* genes have also been used in pigeonpea to develop resistance against its major pest, pod borer *H. armigera*. Successful development of pigeonpea transgenics against *H. armigera* was accomplished by various transformation protocols using different explants (Singh et al. 2018).

In pigeonpea, when *Bt* genes such as *cry1Ab, cry1Ac*, and *cry2Aa* (Sharma et al. 2006; Kaur et al. 2016; Ghosh et al. 2017; Singh et al. 2018) were successfully transferred into different susceptible cultivars, they exhibited resistance against *H. armigera*. Apart from the utility of single ICP genes, gene pyramiding and protein domain swapping in transgenic plants could be a viable strategy for delaying the evolution of resistant pests to *cry* genes (Greenplate et al. 2000; Cao et al. 2002; Rathinam et al. 2019). Interestingly, studies have shown that the synergistic effect of *cry*1Ac and *cry*1F increases the toxicity of the protein 26 times to *H. armigera* (Chakrabarti et al. 1998). Based on the synergistic effect observed against *H. armigera*, the chimeric *Bt* gene, *cry1*AcF was developed by domain swapping between Cry1Ac (D-I and II) and Cry1Fa1 (D-III). The chimeric *cry1AcF* gene overexpressed in pigeonpea exhibited 100% resistance against *H. armigera* (Ramu et al. 2012). It proved that the exploitation of chimeric genes for crop improvement against insect herbivore will provide enhanced toxicity. It could be a viable strategy for delaying pest resistance against Cry proteins.

3.1.2 Genetic Engineering for Disease Resistance

One of the main factors of yield loss is a fungal wilt disease, caused by *Fusarium oxysporum* and *Fusarium udum*. In nature, plants accumulate pathogenesis-related (PR) proteins in response to fungal infection, which prevents the fungal invasion and resists pathogen attack. However, the effect of natural defense varies across the plant species. In general, most of the available pigeonpea germplasm is susceptible to *Fusarium* wilt disease. To develop *Fusarium* wilt resistance, rice chitinase gene was introduced in pigeonpea through genetic engineering (Kumar et al. 2004a). However, the efficiency of the transgenic lines against the *Fusarium* wilt needs to be validated at the field level. There are some pigeonpea wild accessions with resistant gene(s). Therefore, efforts need to be made to exploit such resistance genes from pigeonpea wild relatives towards crop improvement.

3.1.3 Genetic Engineering for Abiotic Stress Tolerance

Pigeonpea is a hardy plant species cultivated in rain-fed conditions, and most of the cultivars are moderately drought tolerant. However, pigeonpea is highly susceptible to salinity that affects various morphological, physiological and biochemical

processes of the plant (Rahneshan et al. 2018). Excess of salt causes toxicity and ion imbalance leading to decrease in the growth of plants. Various genes that confer salt tolerance have been identified and validated. Among them, helicases have been shown to play an important role in plants against salt stress. The DEAD motif of the helicase is believed to play a crucial role in plant growth and development (Linder and Jankowsky 2011). There are many reports in other transgenic crops like cotton, soybean and rice (Chen et al. 2016; Kim et al. 2017; Tuteja et al. 2013) where overexpression of *helicase* improves tolerance to different abiotic stresses. This gene has been introduced in legumes as well, and novel marker-free Psp68 helicase family gene was used to develop transgenic pigeonpea (Neha 2019). The transgenic pigeonpea confirmed improved tolerance against salinity stress. The transgenic plants exhibited higher antioxidant activity in response to salt stress.

Salinity stress management in plants is a complex process, and maintaining cellular osmotic balance under salt stress is important for cellular homeostasis and rigidity (Sairam and Tyagi 2004). Proline acts as an osmoprotectant as well as redox regulator. This amino acid exhibited scavenging activity against singlet oxygen, hydroxyl ions and other free radicals. Thus, proline helps to maintain plant homeostasis and protect plant cell structure under stress condition (Matysik et al. 2002). Overexpression of *D1-pyrroline-5-carboxylate synthase* (*P5CS*), a rate-limiting enzyme in proline biosynthesis enhances tolerance to oxidative stress caused by drought and salinity (Yamchi et al. 2007; Anoop and Gupta 2003). The site-directed mutation in *P5CS* replaced Phe residue at 129 position of the polypeptide with Ala. The conformational change in P5CS129A stopped the feedback inhibition of proline thus allowing its accumulation in the transgenic plant. The investigation in pigeonpea with *Vigna aconitifolia P5CSF129A* gene demonstrated over-production and accumulation of proline in transgenic as compared to the wild-type (Surekha et al. 2014). The transgenic pigeonpea plants performed better under salinity conditions and exhibited lower rate of lipid peroxidation. Both *DEAD-box helicase* and *P5CSF129A* genes overexpression in pigeonpea suggested potential utility of these genes in stress tolerance against salinity.

3.1.4 Genetic Engineering for Nutrient Uptake

Pigeonpea is known to improve soil fertility because of its ability to fix nitrogen in roots. However, availability of phosphorous (P) is limited by its low mobility and excessive fixation in soil. As an adaptive strategy, plants change lateral root numbers, produce excess root hair and release the exudation of organic acids to change the rhizosphere (Shen et al. 2005). Majority of plants produce citric acid to improve nutrient uptake. Increased citrate synthase production and its correlation with citrate production is reported. To improve P uptake in pigeonpea, *Daucus carota citrate synthase* (*DcCs*) was overexpressed in pigeonpea (Hussain et al. 2016). The transgenic pigeonpea overexpressing *DcCs* gene displayed improved root development in P deficient and P available conditions. Higher expression of *citrate synthase* gene was observed in transgenic plant roots. This provided the evidence for utility of genetic engineering in the improvement of plant nutrient uptake.

3.1.5 Genetic Engineering for Nutritional Quality Improvement

Pigeonpea is known to be one of the rich sources of protein for the predominantly vegetarian population in India. However, it is still nutritionally poor among legumes due to lower amount of sulphur-containing amino acids and higher content of anti-nutritional factors. The legume protein is usually abundant in lysine and complements the protein in cereals, but most of the lysine and tryptophan are lost during processing of the crop (Singh and Eggum 1984). In addition, feedback inhibition mechanism in plants also restricts the synthesis of lysine. There is a very limited variation in nutritional quantity in the existing gene pool which makes breeding strategy inapplicable. The goal of improving the nutritional quality of pigeonpea can be fulfilled only with the help of genetic engineering. One approach to do that is by increasing the synthesis and accumulation of lysine in the edible parts of the plants. It was reported that the overproduction of lysine can cause abnormalities in plants, sterility and yield loss. So, it is important to target only the edible parts like seeds to accumulate the lysine.

Aspartate kinase (AK) and dihydrodipicolinate synthase (DHDPS) are the two key enzymes of the lysine biosynthetic pathway that regulate feedback inhibition process (Dewaele et al. 2002). Experiments were performed in tobacco, potato, barley and soybeans (Perl et al. 1992; Karchi et al. 1994; Falco et al. 1995; Brinch-Pedersen et al. 1996) where overexpression of mutated DHDPS gene resulted in the plants being insensitive to lysine feedback inhibition showed an increase in lysine content. The same strategy was applied to increase lysine content in pigeonpea. A mutant DHDPS gene from *Nicotiana sylvestris* that encodes lysine insensitive enzyme (Ghislain et al. 1995) was introduced in the pigeonpea genome under the regulation of phaseo linseed specific promoter. The transgenic pigeonpea plants produced 1.6–8.5 times more free lysine content in seeds (Thu et al. 2007). Improved lysine in pigeonpea seeds is an evidence for the utility of transgenic technology in plant quality improvement.

3.1.6 Genetic Engineering for Edible Vaccine Production

The advances in genetic engineering technology allowed the development of transgenic plants that can express recombinant biopharmaceutical compounds such as viral and bacterial antigens, antibodies and various therapeutic proteins. The concept of ‘edible vaccine’ is now extensively used in research. The major issues with current vaccines are a high risk of contamination, high cost and need for refrigeration, which can be resolved by edible vaccines if developed. The plant expression system can be utilized to produce immunogenic proteins, which will be an economical alternative. Limited reports regarding edible vaccines in pigeonpea are known (Satyavathi et al. 2003; Prasad et al. 2004).

There are few reports of transgenic pigeonpea where the plant system is manipulated to produce vaccines. A report on the development of pigeonpea transgenic expressing protective antigen, hemagglutinin protein (H protein) of Rinderpest virus (RPV) has been published (Satyavathi et al. 2003). RPV is a highly contagious disease and causes mortality of wild and domesticated ruminants in parts of Africa, the Middle East, and South Asia. The drawback of present live-attenuated vaccine is

its heat-liability, and the edible vaccine can be advantageous in this situation. Similarly, transgenic pigeonpea was developed expressing the hemagglutinin-neuraminidase protein of *Pes des petits* ruminant's virus (PPRV) which can provide protection to goats and sheep against PPR disease (Prasad et al., 2004). As pigeonpea is also used as cattle feed, production of vaccine for PPR disease is more economical and suitable.

4 In Planta Transformation

Despite immense efforts towards the development of transgenic pigeonpea, success has been sparse. Successful transformation requires totipotent recipient cells, a medium for transferring genes into the plant genome and a proper method for the selection of transformants (Somers et al. 2003). In the case of pigeonpea, the major challenge has been its recalcitrance to in vitro regeneration.

Low frequency of transformation is another factor that hinders the development of transgenics. To increase the efficiency of transformation, *Agrobacterium*-mediated and microprojectile bombardment-based protocols were utilized. For in vitro regeneration, different explants were cultured via organogenesis as well as somatic embryogenesis. However, very few genotypes of pigeonpea respond to tissue-culture-based regeneration.

To tackle issues with regeneration, alternate non-tissue culture-based methods are in place. These techniques are currently used in many laboratories to develop transgenics for crop improvement as they minimize or totally avoid tissue culture steps (Table 2). The major disadvantage of tissue culture-based transformation protocols is that they are laborious and time consuming. These shortcomings can be overcome by in planta transformation strategy (Rao et al. 2008) as large number of transformants can be developed in short time with minimal labour and reagents.

Though the non-tissue culture-based strategies were initially developed for recalcitrant plant species, these are being used for other plants as well. Various types of in planta transformation protocols have been developed and validated that target

Table 2 Utility of in planta transformation strategy in crop improvement

Crop	Gene	Reference
Peanut	*uidA*	Rohini and Rao (2001)
Field bean	*cry1AcF*	Keshamma et al. (2012)
Castor	*cry1AcF*	Kumar et al. (2011)
Sunflower	*uidA*	Rao and Rohini (1999a)
Safflower	*uidA*	Rao and Rohini (1999b)
Medicago truncatula	*uidA*	Trieu et al. (2000)
Cotton	*uidA*	Keshamma et al. (2008)
Chili	*PDH45*	Shivakumara et al. (2017)
Rice	*AtPCS*	Venkataramaiah et al. (2011)
Groundnut	*epsps*	Manjunatha et al. (2008)
Chickpea	*cry1AcF*	Neelima et al. (2008)

Fig. 1 Tissue culture-independent apical meristem targeted in planta transformation strategy

meristems, seeds, fruits and flowers. Many leguminous crops and other economically important crops have been demonstrated to be transformed using these strategies.

Our group standardized one such in planta strategy to develop the transgenics (Figs. 1 and 2). The technique involves in vitro inoculation of the differentiating apical meristem and allowing them to grow ex vitro. The method is based on the hypothesis that transformation of L3 layer cells of the differentiating apical meristem facilitates the inheritance to the next generation. This strategy has been successfully used in our laboratory to develop stable and commercially viable transgenics in a large number of economically important crop species. Apical meristem targeted in planta transformation strategy has been used to develop transgenics in pigeonpea (Rao et al. 2008; Kesiraju and Sreevathsa 2017). Two-day-old seedlings were targeted with virulent *Agrobacterium.* To increase the virulence of A*grobacterium* and improve the transformation efficiency, wounded tobacco leaf extract was added to the culture. After infection, seedlings were transferred to soilrite for 7–8 days under photo-period of 16 h light and 8 h darkness. Plants were then transferred to the soil for further development. Seeds produced in this generation are chimeric in nature and hence are screened by suitable antibiotic selection for the detection of transformants in T_1 generation. Molecular analysis was performed in T_1 generation plants. To avoid the problem of handling large number of plants,

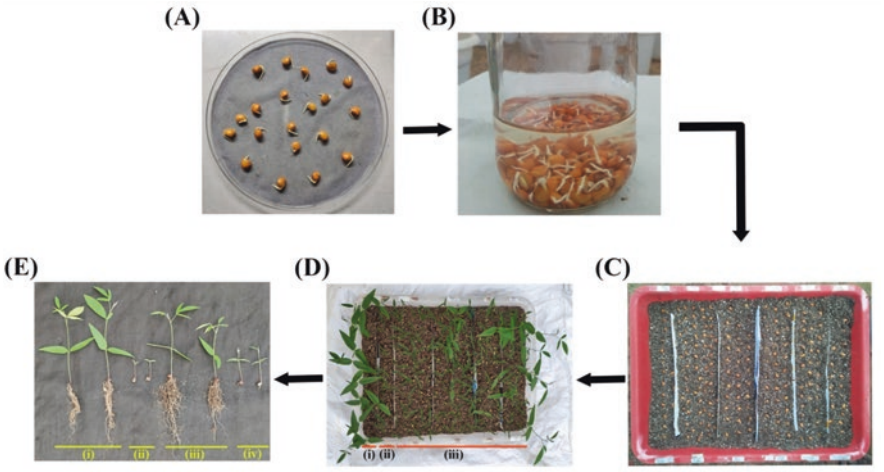

Fig. 2 Screening for the identification of positive transformants: (**a**) Two-day-old seedlings for antibiotic treatment; (**b**) Antibiotic treatment in jam bottle; (**c**) Transformants were recovered on soilrite after antibiotic treatment; (**d**) Differential growth of transformants after antibiotic treatment: (i–iii) Absolute control, treated control and putative transformants, respectively; (**e**) (i and iii) Absolute control and positive transformants established completed root formation; (**e**) (ii and iv) Treated control and non-transformants show stunted growth with no root formation

high-throughput screening strategies were adopted for identification of the transformants (Kesiraju and Sreevathsa 2017).

Using in planta strategy, our laboratory has successfully developed pigeonpea transgenics against the devastating herbivore, *H. armigera*. Independent transgenics expressing two *Bt*-ICPS, *Cry1AcF* and *Cry2Aa* (Ramu et al. 2012; Ghosh et al. 2017; Singh et al. 2018), were developed. These transgenics have been demonstrated to be quite effective against pod borers resulting in 80–100% of the larvae dying upon feeding on the leaves and pods. Stringent molecular and bio-efficacy analysis in advanced generations have identified a set of promising transgenic events. These events are ready to be evaluated under confined field conditions for their performance against the herbivore.

5 Conclusions

The major concern bothering pigeonpea breeders and growers worldwide is the stagnation of productivity. The crop battles various biotic and abiotic stresses during the growth period resulting in the inability of pigeonpea to attain its yield potential. Therefore, crop improvement is the need of the hour to combat escalating population or climate change as well as evolution of pests and pathogens. Tackling these problems successfully through several biotechnological approaches would be a worthwhile contribution from scientists globally. Novel techniques and strategies have continued to emerge and are being exploited for crop improvement. Integration

of various strategies in a meaningful manner can lead to sustainable agriculture. Use of transgenic technology in case of non-availability of resistance sources and conventional/marker-assisted breeding in the presence of resistance source has been in vogue towards successful crop improvement. Further, another potential option for crop improvement can be the exploitation of wild relatives of pigeonpea that are known to be bestowed with a galore of useful agronomic traits like resistance to various pests, diseases and abiotic stresses. Recently, successful sequencing of pigeonpea genome has resulted in the identification of several hundreds of genes that impart resistance to biotic as well as abiotic stresses. Similarly, comparative genomics approaches embarking on the differences in the genes pertaining to resistance mechanisms in the wild relatives can be a worthwhile option towards crop improvement. Rapid progress of advanced biotechnologies that can bridge genotype–phenotype gaps can facilitate the successful utilization of crop wild relatives in pigeonpea improvement programmes. Use of molecular techniques like genome editing to edit the genes implicated in stress response in the cultivated pigeonpea would lead to the production of resistant lines to both biotic and abiotic stresses. It can be presumed that the future of crop improvement in pigeonpea could be promising with the amalgamation of genomics, molecular tools coupled with conventional breeding programmes.

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Genetically Engineered Chickpea: Potential of an Orphan Legume to Achieve Food and Nutritional Security by 2050

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Abstract

Chickpea is an important legume, and its protein-rich seeds make it a healthy alternative to meat for humans. Furthermore, the low glycemic index of carbohydrates in the grain is considered healthy for humans. Chickpea is a cheap source of protein for the people in India, Bangladesh, Pakistan, Africa, and the Mediterranean region. Therefore, the improvement of the orphan chickpea is necessary to achieve food and nutritional security in those countries. The potential of the chickpea improvement was impeded by the green revolution, and the consequence was a slow (<1% per annum) increase in the global chickpea yield which was recorded since 1990. The lack of availability of high-yielding varieties with adequate protection from various stresses is the reason for low yield. The International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) and a few other national institutions have been releasing improved varieties; however, abiotic stresses, pests, diseases, and weeds remain challenging to manage in the field and storage conditions. Water and salinity stresses are significant in Asia, Australia, and the Mediterranean regions, while *Helicoverpa armigera*, aphids, and *Ascochyta* are predominant in Asia, Australia, and Canada. In the Middle-East, weeds compete with chickpea for water and nutrition. For many of these constraints, conventional or advanced breeding approaches are limited due to the lack of resistant/tolerant sources within the gene pool. Genetic engineering has the potential to address some of these constraints; however, it needs adequate resources to achieve significant impacts. In the past decade, efforts have been made to genetically modify the chickpea genome using either *Agrobacterium*-mediated or biolistic method. In a few instances, success has been documented; for example, genes from *Bacillus thuringiensis* have been introduced for com-

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plete resistance to pod borers (*Helicoverpa armigera*). Also, the drought-tolerant trait has been incorporated using transgene. These traits were either tested in the greenhouse or approved for field trials; however, yet to be commercialized. The possibility to save the yield losses by genetic engineering is immense and has been successful in other legumes such as soybean, common bean, and cowpea. Thus, a second green revolution may be implemented to improve the potential of chickpea and other grain legumes to attain food and nutritional security of the growing population.

Keywords

Chickpea · Genetic modification · Transgenics · Drought resistance · Helicoverpa resistance · Aphids · Nutritional improvement

1 Introduction

Genetic engineering is a modern crop breeding technique that facilitates the improvement of various traits in crops when the application of conventional or advanced molecular breeding to improve those traits is not feasible. It offers an opportunity to introduce desired gene(s) along with the regulatory elements from various sources into crops, and subsequently, the improved or engineered crops or varieties are released into the field after elaborate and rigorous bio-safety assessments. The application of this modern breeding technique was successfully used to generate genetically modified (GM) crops, and to date, more than 25 GM crops were approved for commercial cultivation, including three legumes, soybean, common bean, and cowpea (ISAAA 2018, 2019).

Genetic improvement of all the legumes has been slow or neglected over the past few decades, except soybean. Soybean improvement by breeding or release of GM soybeans was mainly driven by the high demand for soy-based feeds for the livestock industries. Herbicide-tolerant and pod borer-resistant soybean are the first achievements of using genetic engineering tools in legumes, and GM soybean was widely adopted in various countries (ISAAA 2018). A similar approach was successfully adopted for other legumes, for example, virus-resistant beans and pod borer-resistant cowpeas which are now approved for commercial cultivation in Brazil and Nigeria, respectively (ISAAA 2019). Therefore, genetic engineering of chickpea holds promise to gain yield advantage by protecting the crop from biotic and abiotic constraints as well as by improving its nutritional quality.

Chickpea is an important crop grown widely in India and to a lesser extent in Australia, Canada, the Mediterranean regions, and the USA. For many Indians and people in Pakistan and Bangladesh, chickpea is a cheap or affordable source of protein compared to animal-based ones. It is considered as one of the healthy sources of protein for the vegetarian populations in those developing countries (Sarmah et al. 2004; Acharjee et al. 2010).

Chickpea is also preferred in an integrated cropping system to enhance soil fertility due to its ability to fix atmospheric nitrogen with the help of *Rhizobium*. It plays a vital role in increasing the productivity of intercropping systems; mainly sequential and intercropping. In addition, intercropping of chickpea reduces the application of fertilizers, consequently lowering carbon emission from the farm environment (Reckling et al. 2016).

Chickpea is often called an orphan legume because no significant change in productivity was achieved in the past 30 years. Based on the FAO statistics on chickpea, a >1% increase in the yield per hectare (from 0.9 to 1.0 T/ha) was recorded since 1990. The slow growth in productivity indicated that adequate attention is required to improve yield per plant and resistance to significant biotic and abiotic stresses to avoid annual yield losses.

Based on the seed size, color, thickness, and the seed coat, chickpeas are grouped into two types, desi and kabuli. Desi chickpea seeds are smaller in size with thick seed coats that range in color from light tan to dark brown. This type of chickpea is widely grown in India, Bangladesh, Pakistan, Ethiopia, Mexico, and Iran. Kabuli types have larger seed size with thin seed coats and white to pale cream color. Kabuli chickpeas are mainly cultivated in Southern Europe, Northern Africa, Afghanistan, Pakistan, and Chile (Gaur et al. 2015)

Chickpea seeds are a rich source of proteins, dietary fiber, various minerals, and vitamins. Carbohydrates and proteins together constitute about 80% of the total dry seed mass (Jukanti et al. 2012). The low glycemic index of the carbohydrates in the protein-rich seeds of chickpea makes it a healthy option for both vegetarian and non-vegetarian diets (Mudryj et al. 2013). Globally, seeds are consumed raw after soaking overnight or used in several different preparations. In India, chickpea seeds are used in various forms, such as whole, split, roasted, ground into flour, and boiled. The young green leaves are also consumed as cooked green leafy vegetables. Chickpeas are also used as the animal feed in various livestock industries (Acharjee and Sarmah 2013).

In 2017, the global yield of chickpea was 14.7 MT, and >80% contribution to the global yield was from India. Chickpea is an important grain legume in India and was gown over an area of 9.5 M ha with a production of 9 MT in 2017 (FAO 2017). Pakistan and Australia follow by producing about 3 MT and 2 MT, respectively. Although the crop is widely grown in India, no significant increase in productivity was recorded since 1990. In 1990 the productivity was 0.6 T/ha, while it was 0.9 T/ha in 2017. Over the past 15 years, several improved varieties were released; however, due to low levels of resistance to several pests and diseases, global yield losses are unavoidable (Singh 1997). The application of modern breeding techniques to improve chickpea was neglected compared to cereals due to the green revolution impetus. Therefore, chickpea falls under the category of orphan legumes despite its potential to attain food and nutritional security. The lack of adequate resources led to the cultivation in resource-poor conditions in the Indian continent (Srivastava et al. 2017)

1.1 Limitations of Chickpea Production

Chickpea is cultivated as a winter crop in many countries. A few major production constraints in those countries are pests, diseases, drought, and salinity. Pod borers are one of the major pests of chickpea followed by bruchids. Among the diseases, *Ascochyta* blight, *Fusarium* wilt, and Botrytis gray mold cause infection to the crop (Acharjee and Sarmah 2013).

1.2 Pod Borers

In the field conditions, a lepidopteran insect pest, *Helicoverpa armigera* (pod borer) is the most significant pest of chickpea in Asia, Africa, and the Mediterranean region. The adult female lays eggs on the dorsal surface of the leaves, and the emerging larvae (early to middle instar) feeds on the young leaves, while the late instar larvae prefer flowers and pods. During pod development stage, the larvae bore inside the pod and consume the protein-rich developing cotyledons (Giri et al. 1998). The pod borers cause >40–60% yield losses worth $325 million annually (Sharma 2001). *H. armigera* is a polyphagous insect and feeds on several other crops, both leguminous and non-leguminous; therefore is difficult to manage. The low efficacy of microbial formulations, such as *H. armigera* nuclear polyhedrosis virus (HaNPV) and *Bacillus thuringiensis*, and crop husbandry practices led to more reliance on insecticides to manage this pest in the field. The current method of its management relies on the application of insecticides, and over-spraying also results in the emergence of resistant insect population and frequent replacement of pesticides to avoid yield losses (Gujar et al. 2000). One of the most cost-effective and environmentally sustainable methods is to release GM chickpea expressing the *B. thuringiensis* genes.

1.3 Black Aphids

Aphis craccivora, also known as black aphid, is a serious pest of chickpea in Asia and Ethiopia, the Pacific Northwest of the USA (Dhingra 1994). Viral diseases of chickpea such as *soybean dwarf virus* (SbDV), *beet western yellow virus* (BWYV), and *chickpea stunt disease-associated virus* (CpSDaV) are carried by aphids. Aphids are controlled by the application of insecticides, such as methomyl, oxydemeton-methyl, and monocrotophos, which are found to be effective to a certain degree.

1.4 Bruchids

The second significant pests are bruchid beetles (*Callosobruchus* spp.), which cause significant damage by reducing both quantity and quality of the stored chickpea

seeds for sowing, food, and marketing (Dias and Yadav 1988; Hariri and Tahhan 1983). Both *Callosobruchus chinensis* and *C. maculatus* are common stored grain pests of chickpea. The primary infestation begins in the field when the adult female lays eggs on the mature pods. The larvae bore inside the seeds and rapidly multiply in the storage conditions causing significant damage to the quality and quantity of the stored chickpeas. One of the standard practices widely adopted by the farmers is the spray of insecticides during the reproductive stage in the field. The dry seeds which are stored for subsequent planting are fumigated or dusted; however, the application of these chemicals on chickpeas sold for consumption is banned due to health hazards.

1.5 Water and Salinity Stress

The abiotic stresses such as terminal drought during reproductive stages and salinity cause more than 50% yield losses in chickpea globally (Varshney et al. 2009). Chickpea suffers terminal water-deficient stress because it is cultivated in the arid and semi-arid regions of the world (Kashiwagi et al. 2015). The mechanisms by which plants adapt to water and salinity stresses are adequately documented. Therefore, efforts are being made to use genomic resources to generate tolerant varieties to water-deficit stresses (Varshney et al. 2019).

2 In Vitro Regeneration of Chickpea

Both *desi*- and *kabuli*-type chickpeas were grouped as recalcitrant to in vitro manipulations; however, several laboratories standardized in vitro regeneration protocol of chickpea over the past few decades. The first attempt to regenerate chickpea was made in 1979 using shoot tip meristem (Bajaj and Dhanju 1979). Later, several methods were published using explants such as hypocotyl and shoot tip (Neelam et al. 1986), immature cotyledons with excised embryonal axis (Shri and Davis 1992), immature seeds (Malik and Saxena 1992), whole seed and cotyledons (Prakash et al. 1992), leaflet (Barna and Wakhlu 1993, 1994; Rizvi and Singh 2000), shoot tips (Fontana et al. 1993; Chandra et al. 1993; Polisetty et al. 1996, 1997), embryonic axis without apical root and shoot part (Kar et al. 1996), epicotyl (Vani and Reddy 1996), single cotyledon with half embryo (Sarmah et al. 2004; Chakraborti et al. 2009; Bhowmik et al. 2019), root tips from a day old seedlings (Paul et al. 2000), embryonic axis (Jayanand et al. 2003), and mature or immature embryo (Chauhan et al. 2003).

Till 2000, the generation of multiple shoots from explants excised from various chickpea cultivars was successful; however, the establishment of tissue culture-derived plants in the soil was challenging. The first successful method which led to the establishment of plants in the greenhouse were derived from embryonic axes (without shoot or root apex) of desi chickpea cultured on cytokinin- and auxin-containing medium (Kar et al. 1996). Later, tissue culture-derived plants were also

established from using various explants cultured on medium containing either cytokinin or auxin or combination of both (Polisetty et al. 1996; Paul et al. 2000; Rizvi and Singh 2000; Rizvi et al. 2002; Chauhan et al. 2003; Jayanand et al. 2003; Sarmah et al. 2004; Chakraborti et al. 2009).

The methods described above are based on either direct or indirect shoot organogenesis; however, the in vitro regeneration method using the cotyledons with half embryonic axes was adopted for the generation of stable transgenic plants (Sarmah et al. 2004; Chakraborti et al. 2009).

3 The First Transgenic Chickpea

A group in Italy (Fontana et al. 1993) were the first to publish their work to transform chickpeas in 1991. They cultured embryo axes without apical meristem as explants derived from overnight-soaked seeds. The explants were infected with *Agrobacterium tumefaciens* harboring a binary plasmid (pBI121) having neomycin phosphotransferase II (*npt*II) and β-glucuronidase (*GUS*) genes in the transfer DNA (T-DNA) region. After 2–3 weeks of co-cultivation on MS medium, only 10% of the multiple shoots emerging from each explant survived on kanamycin (50 mg/L) containing selection medium. These shoots were rooted on MS (Murashige and Skoog 1962) medium without kanamycin because root morphogenesis was inhibited by the presence of kanamycin in the medium. The frequency of transformation was reported to be 4%; however, the data was based on the number of whole plants transformed, and the total number of embryos infected. GUS and NPT II activities were observed in the leaves of three primary transformed plants, of which two plants showed the integration of the transgene in Southern analysis. Although the number of transformed plants generated was low to perform detailed molecular analysis, the protocol was the first to document the use of seed-derived embryonic axes as explant, growth hormones (BAP, Kinetin, NAA) appropriate for multiple shoot induction from the embryo axes, use of *Agrobacterium* for the transformation of chickpea, and suitability of *npt*-II as a selectable marker. It was the first successful protocol on genetic transformation chickpea, and later, significant improvement was made in this protocol by various laboratories to generate transgenic chickpea.

4 Improved Genetic Transformation Protocol of Chickpea

After the first successful report on *Agrobacterium*-mediated genetic transformation of chickpea (Fontana et al. 1993), several groups improved this method to obtain a large number of stable transgenic chickpea lines (Fig. 1). In 2000, a group in India described an *Agrobacterium*-mediated technique using embryo axes of four different cultivars (PG1, Chafa, Turkey, and PG12) of chickpea (Krishnamurthy et al. 2000). They tested the efficiency of various *Agrobacterium* strains (C58C1, GV2260, and EHA101), harboring a *GUS* gene. Also, two different selectable marker genes, phosphinothricin acetyltransferase (*PAT*) and *npt*-II, were tested. The

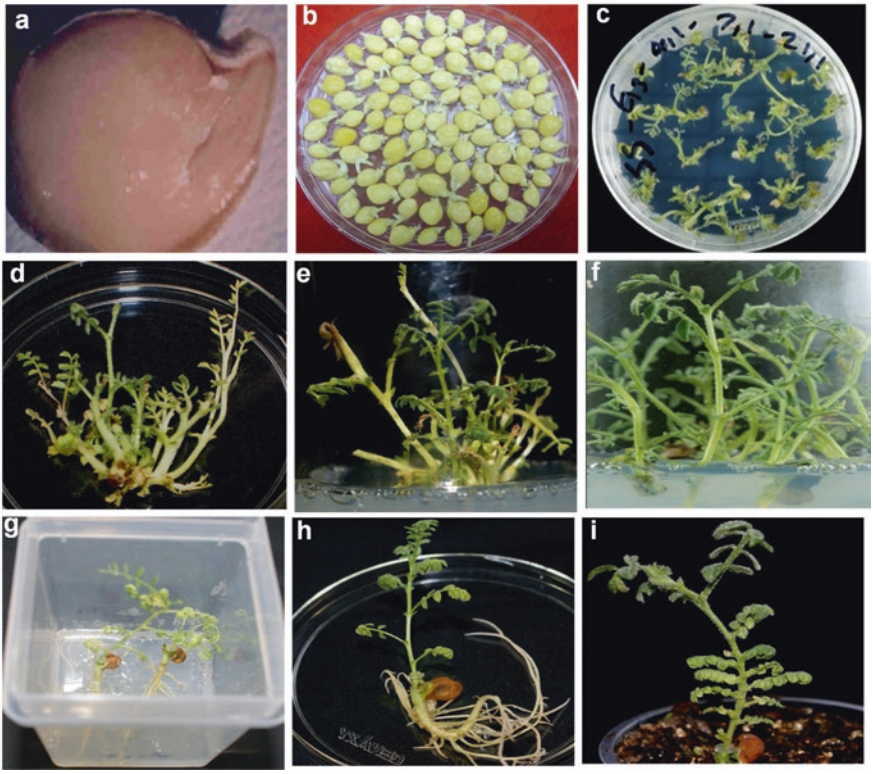

Fig. 1 *Agrobacterium*-mediated genetic transformation of chickpea. (**a**) The explant is a cotyledon with half embryonic axis attached (indicated by arrow). (**b**) *Agrobacterium*-infected explants on B5 co-cultivation medium. (**c**) Regeneration of shoots on RS1 after 5 days. (**d**) Regeneration and selection of shoots on RS3 (arrow shows a bleached shoot). (**e**) Regeneration and selection of single, healthy, green shoots in the fourth Regeneration Cycle 4 (bleached shoot indicated by an arrow). (**f**) Regeneration and selection of individual shoot in Regeneration Cycle 5. (**g**) In vitro grafting of a healthy, green shoot onto a non-transgenic rootstock (arrow indicates graft union held in place by silicon ring). (**h**) Grafted shoots in magenta box. (**i**) In vitro grafted plantlet established in the soil

results showed MS medium with a kanamycin dose of 100 mg/L, which was higher than the previous report (Fontana et al. 1993) was useful in screening the transformed shoots, while 10 mg/L of PPT was suitable for selection of shoots transformed with *PAT* as a selectable marker gene. The transformation efficiency ranged from 0.4 to 1.5%, and the highest transformation efficiency was observed in cultivar Turkey. A total of 36 plants were established in the glasshouse of which only five plants were fertile and set seeds (T_1 progeny). Only four T_1 progeny showed an expected amplicon for *npt*-II gene by PCR; however, no *GUS* activity was observed in those plants. The study included various *Agrobacterium* strains and two different selectable marker genes but did not summarize the best combination for the transformation of chickpea embryo axes. Therefore, a clear conclusion on the

transformation protocol could not be reached due to the lack of detailed analyses on segregation and transmission of the transgene, which was limited by reduced seed set of transgenic lines.

An attempt was also made to optimize an *Agrobacterium*-mediated protocol using longitudinal slices of embryonic axes derived from overnight-soaked seeds of *kabuli*-type chickpeas (Polowick et al. 2004). The *Agrobacterium* strain (LBG66) was different from the previous reports; however, the binary plasmid had similar genes (*GUS* and *npt*-II). The co-cultivation of explants was performed on B5 medium (Gamborg et al. 1968) without growth hormones, whereas multiple shoot induction was observed in B5 medium-supplemented with high dose of BAP (3 mg/L) and kanamycin (concentration 50 mg/L). The multiple shoots were elongated on MS medium composed of B5 vitamins with a low (1 mg/L) level of BAP and a low (50 mg/L) dose of kanamycin. The concentration of kanamycin was increased to 75 mg/L in the final two subculture cycles for shoot elongation followed by rooting on B5 medium with NAA and 150 mg/L of kanamycin. The transformation efficiency of this protocol was lower (3.1%) than the previous report (Fontana et al. 1993).

In 2004, an *Agrobacterium*-mediated genetic transformation method was also described for chickpea, and an insecticidal gene (α-amylase inhibitor, α-AI) for resistance to bruchids (Sarmah et al. 2004) was successfully introduced. Several transgenic lines were generated after cotyledons with half embryonic axes explants were infected with an *Agrobacterium* strain (AGL1) followed by co-cultivation on the B5 medium supplemented with BAP, NAA, and 100 μM of acetosyringone for 3 days. The regeneration and selection of the explants were carried out on MS medium supplemented with BAP, KN, NAA, and a very high (200 mg/L) dose of kanamycin. The explants were selected on kanamycin-containing medium for six subculture cycles of 10–14 days each before grafting on to the root stock. The protocol was also adopted to generate transgenic chickpea expressing a *cry2Aa* (Acharjee et al. 2010) and *cry1Ac* (Hazarika et al. 2019) by the same group and, later, generation of transgenic lines expressing a Cry1Ab/Ac in the pods by another group in India (Ganguly et al. 2014). The method helped generate stable transgenic lines; however, the frequency of transformation was low ranging from 0.5 to 1%.

A different type of explants was also utilized for the generation of transgenic lines. Matured seeds were soaked overnight in MS liquid with BAP followed by germination on MS solid medium with slight modification for the isolation of cotyledonary node (CN) explants from 10- to 30-day-old seedlings. The initial protocol tested various parameters such as growth stages to excise CNs, pre-conditioning of explants, *Agrobacterium* strains, acetosyringone concentration, sonication, kanamycin dose, and others (Sanyal et al. 2005). Based on the results CNs excised from 20-day-old seedling when pre-conditioned for 24 h followed by co-cultivation with *Agrobacterium* strain, LBA4404 on medium containing 100 μM of acetosyringone and selection on medium with 100 mg/L kanamycin for first and second selection and adding 150–200 mg/L of kanamycin in the third and fourth cycle was found useful to recover transformed shoots. The transformed shoots were rooted or grafted on to wild-type root stock. The method was used for establishing transgenic

chickpea lines expressing a *cry1Ac* gene with a transformation efficiency of 1.12%. This protocol was also used by another group (Mehrotra et al. 2011) to generate chickpea lines expressing a *cry1Ac* gene, and they reported a high (1.69–2.77%) transformation frequency.

For the genetic transformation of chickpea, immature cotyledons excised after 21 days of pollination were exploited. Shoot morphogenesis from these immature cotyledons was successful on medium with BAP, TDZ, kinetin, zeatin, and 2-iP either alone or in combination with IAA or NOA (elaborate). The explants were infected and co-cultivated with *Agrobacterium* strain, LBA4404 having plasmid carrying *GUS* and *npt*-II genes. The in vitro selected shoots were rooted on MS medium with IBA and established in the glasshouse. The transformation efficiency of the protocol was reported to be 2.08%; however, the protocol is yet to be used for the introduction of any agronomically important transgene (Tripathi et al. 2013).

Recently, transgenic chickpea expressing a *cry1Ac* gene regulated by homologous ubiquitin and Rubisco promoter (Chakraborty et al. 2016) was generated using the protocol described by Sarmah and his coworkers in 2004. Later, the same protocol was also modified slightly to improve the transformation efficiency by performing microinjury in the embryonic axes and culturing them under light-emitting diode (LED). The LED lights helped reduce the duration of the culture cycle to 9 weeks (Bhowmik et al. 2019) from 15 to 18 weeks (Sarmah et al. 2004). The comparative study showed that microinjury and LED lights successfully improved the transformation efficiency by onefold. The protocol was also used for the generation of stable transgenic lines expressing stress tolerance and iron-fortification genes.

The above *Agrobacterium*-mediated protocols are reasonably similar in terms of choice of explant, selection of growth hormones for regeneration, choice of the selectable marker gene, and the dose of kanamycin for selection of transformed shoots under in vitro conditions.

5 Genetically Engineered Chickpea with Resistance to *H. armigera*

Pod borers are one of the notorious pests of chickpea, causing significant damage to the crop. The major pod borers of chickpea are *H. armigera,* which is distributed in Asia, Africa, and the Mediterranean region, and *H. punctigera* is dominant in southern Australia. At the ICRISAT, screening of germplasm or accession for resistance was attempted, and accessions such as ICC 506, ICC 10619, and ICCL 84205 were identified to have low levels of resistance to pod borers. The selection was useful to identify parents to breed new varieties with less damage by pod borers, but a complete or high degree of resistant lines was not available within the gene pool. Resistance to pod borers is a polygenic trait. Also, resistance to pod borers was found to be linked with susceptibility to wilt caused by *Fusarium oxysporum.* Conventional breeding was mostly unsuccessful due to narrow genetic pool, nonsynchronous flowering, sexual incompatibility, and autogamy (Somers et al. 2003).

Therefore, an alternative approach to impart resistance by introducing transgene(s) appeared suitable to complement the breeding.

Genetic engineering to develop elite chickpea varieties with complete resistance to pod borers was successful by using insecticidal genes of *B. thuringiensis*. The Bt genes encoding proteinaceous endotoxins are used as bioformulation to protect chickpea for pod borer damage. Two major Bt proteins, Cry1Ac and Cry2Aa, were found to be very toxic to *H. armigera.* The mode of action of these toxins is dependent on the specific receptors of the pod borer midgut, making them unique and selective. The specificity of these proteins makes them an ideal candidate for gene pyramiding through genetic engineering. The mechanism by which Bt protein kill larvae have been well documented (Jurat-Fuentes and Adang 2001). The Bt proteins have three domains that interact with the pod borer midgut leading to pore formations. The α-loop of the domain II binds to the midgut membrane receptor, which results in creating pore or channels in the midgut membrane followed by osmotic lysis of the midgut epithelium cells. The altered membrane structure retards digestive activity leading to larval death. These Bt genes have been successfully used in many commercial GM crops such as cotton, maize, and soybean. Therefore, both the *cry1A* and *cry2Aa* genes were used for the generation of transgenic Bt chickpeas.

The chickpea cultivars (ICCV-1 and ICCV-6) were used for the genetic transformation using a *cry1Ac* gene and *npt*-II as a selectable marker gene. Embryo axes derived from matured seeds were transformed by the biolistic method (Kar et al. 1997). A total of 24 kanamycin-resistant plants were transferred to soil, with a genetic transformation efficiency of 45.8% based on the Southern analysis. In all, five plants were found to carry a single copy of the transgene, of which only two plants showed detectable levels of Cry1Ac protein by ELISA and Western blot. The growth of the larvae was reduced when fed with young shoots from a single positive plant; however, complete mortality was not observed. The progeny of the one positive plant showed transmission of the transgene to the next generation; however, no further study was carried out. The number of transgenic lines expressing the Cry1Ac protein was less to carry out further investigations.

In 2005, a similar gene (*cry1Ac*) was introduced into various chickpea cultivars such as C 235, BG 256, P 362, and P 372 (Sanyal et al. 2005). The genetic transformation efficiency of 1.12% was recorded in the case of BG 256. Eighteen transgenic lines having a single copy of the transgene expressed high (>3.0 ng/mg) levels of Cry1Ac protein. However, when these lines were subjected to leaf-feeding bioassays, none were found to be fully resistant to *H. armigera*. Even 10% of the larvae survived on a chickpea line expressing a high (14.5 ng/mg) level of Cry1Ac protein. Lines expressing high levels of Cry1Ac protein appeared promising for introgression breeding to develop elite chickpea varieties; however, detailed molecular characterization on transgene stability, zygosity, and vector backbone integration would have been appropriate to initiate the breeding process.

A codon-optimized Bt *cry2Aa* gene was also introduced into chickpea (Acharjee et al. 2010) to complement the above Cry1Ac lines. It was known that the neonate larvae are a voracious eater of green leaves; therefore, an *Arabidopsis rubisco small subunit* gene promoter (AtSSU) was used to regulate the *cry2Aa* gene. Several

transgenic lines were generated, and a few lines expressed high levels of Cry2Aa protein in leaves and stem throughout the crop development as well as in the reproductive organs. Southern blot hybridization of nine lines showed the presence of a single copy of the *cry2Aa* gene in five lines, and segregation analyses on the T_1 progeny confirmed that these lines behaved as a single dominant locus of *cry2Aa* gene. Insect bioassays on selected lines showed complete protection to *H. armigera* larvae. In the greenhouse, the phenotypic aberration of lines expressing a very high level of Cry2Aa protein was observed. A few homozygous lines expressing a high level of Cry2Aa protein were used for introgression breeding after several rounds of greenhouse bioassays. After the approval of the regulatory body on the GM crops in India, confined field trials were conducted in 2015 and 2016. The field trials showed a yield advantage of about 25% with reduced insect damage in the GM chickpea varieties (S Acharjee and B.K Sarmah, pers. comm.).

A pyramided approach was also adopted for enhanced insect resistance management in chickpea by Mehrotra et al. (2011). Though the aim was justified by the selecting genes (modified *cry1Ab,* and *cry1Ac*) but not inappropriate for pyramiding because the mode of action of both these genes is similar. The group generated 118 stable independent lines. Most of the transgenic lines carried a single copy of the *Cry1Ab* or *Cry1Ac* gene or both and segregated in a Mendelian inheritance pattern of a single gene. The expression of *Cry1Ab* or *Cry1Ac* ranged from 5 to 40 ng/mg of total soluble protein, and lines expressing about 40 ng/mg of Cry protein showed complete protection to *H. armigera* damage. However, no further study was carried out using these lines.

Targeting the *cry1Ac* gene to plastids with a signal peptide and a homologous Ubiquitin and Rubisco small subunit gene promoter was also documented (Chakraborty et al. 2016). A total of 46 transformed plants with normal phenotypes were established. The transformation efficiency varied within the binary vectors ranging from 0.80 to 1.7%. Southern hybridization showed a single copy of the transgene in most of the transgenic lines with a Mendelian fashion of segregation for a single gene. The homologous green tissue-specific promoter resulted in the expression of very high levels of Cry1Ac proteins (25–40 ng/mg of total soluble proteins). The level of expression was higher than that of the commercial Bt soybean; however, complete insect mortality was not observed.

The group at the Assam Agricultural University in collaboration with the Commonwealth Scientific and Industrial Research (CSIRO) also introduced a second Bt gene, tr*Cry1Ac* in chickpea (Hazarika et al. 2019). They used a similar Bt gene construct that was reported previously (Acharjee et al. 2010) except for the gene. A large number (57) of independent lines expressing >50 μg/g of trCry1Ac protein were established. Two high expressing lines, having a single copy of the transgene, attained homozygosity in the T_2 generation. The lines were completely (100%) protected from the insects, and the whole plant bioassays of the T_4 progeny in the greenhouse revealed only 1.4% pod damage. In addition, the phenotype of the plants was indistinguishable from the controls. These lines are ideal for crossing with the above Cry2Aa lines to generate elite pyramided chickpea varieties with enhanced protection to insect pests in the field.

6 Aphid-Resistant Chickpea

In the field conditions, chickpea plants are damaged by a sap-sucking pest known as aphid (*Aphis craccivora*). This phloem sap-sucking pest also transmits chickpea stunt virus (Reddy and Kumar 2004).

Sugar-linked protein or glycoproteins such as the *Galanthus nivalis* agglutinin (GNA) (Hilder et al. 1995; Rao et al. 1998) and *Allium sativum* leaf agglutinin (ASAL) (Dutta et al. 2005a, b; Sadeghi et al. 2008; Saha et al. 2007) when expressed in the plant can confer resistance to aphid. Transgenic chickpea lines expressing ASAL in a phloem-specific manner were generated by the *Agrobacterium*-mediated method. The lines expressing ASAL were used for in planta bioassays in the greenhouses, and T_1 progeny of transgenic lines showed the reduction of aphid infestation by 11–26%, but this reduction in the aphid population would be useful to control damage, and virus spread remains to be studied.

7 Engineering Bruchid Resistance in Chickpea

Bruchids are economically important pests of stored pulses (Clemente and Cahoon 2009; Sharma 2001). Both *C. maculatus* and *C. chinensis* cause significant (up to 30%) damage to stored chickpeas. The most common method used to protect the chickpeas kept in storage for propagation is dusting of seed using insecticides; however, insecticides are not used in seeds used for consumption.

Interestingly, among the legumes, *Phaseolus vulgaris* is resistant to bruchid infestation due to the presence of a gene encoding an α-amylase inhibitor 1 (αAI1), and the presence of this inhibitor reduced bruchid multiplication in the storage conditions. Therefore, this gene was isolated from *P. vulgaris* and expressed in other pulses, such as peas (Shade et al. 1994; Schroeder et al. 1995) and azuki bean (Ishimoto et al. 1996). The gene was also used for resistance to bruchids in chickpea (Sarmah et al. 2004). Several transgenic lines expressing this gene in the seeds were generated by *Agrobacterium*-mediated method. Lines accumulating αAI1 up to 4.2% of seed protein strongly inhibited the development and emergence of *C. chinensis* and *C. maculatus* in insect bioassays.

The αAI1 chickpea lines appeared to be very promising for field trials; however, a study of transgenic pea expressing the same gene showed altered immunogenic activities in BALB/c mice. This initial result appeared to be due to alteration in the post-translational modification of the αAI1 protein in pea, and interestingly, this modification of the protein appeared to vary within legumes species (Prescott et al. 2005). This warrants elaborate investigation on transgenic legumes expressing this protein, which led to more exciting results (Lee et al. 2013). Lee et al. (2013) could not confirm the previous work of Prescott et al. (2005), and the immunogenicity of bean and pea version of the αAI1 protein showed no difference. In the report, it was concluded that the mice model of testing the allergenic response of transgenic crops might not be perfect due to a general unexpected immunogenic response in mice fed

with plant-derived food. Thus, it is very unlikely that chickpeas expressing a bean αAI1 will pose any risk to human health beyond that expected of common beans.

8 Transgenic Chickpea for Water and Salinity Stresses

Water and salinity stresses are prevalent in many legumes, mostly during the reproductive stages. An osmoregulatory gene (*Δ^1-pyrroline-5-carboxylate reductase*; *P5CR*), was expressed in chickpea which resulted in overexpression of proline in chickpea report to improve water use efficiency. However, screening in transgenic lines in the greenhouse for water stress tolerance revealed no significant yield advantage (Bhatnagar-Mathur et al. 2009). In 2015, a new report was published on use of RNAi to impart abiotic tolerance in chickpea was published (Hajyzadeh et al. 2015). The micro RNA named miR408 was overexpressed in chickpea, and lines were screened for tolerance to water deficit. A few selected lines overexpressing the micro RNA could tolerate the water deficiency regime for more than 17 days. The lines were found to express high levels of transcription factor, DREB, and other drought-responsive genes. These RNAi lines were tested in the field.

Recently, a gene from *Arabidopsis* (AtBAG4) and *Tripogon loliiformis* (T1BAG) were also introduced in chickpea for tolerance to various abiotic stresses (Bhowmik et al. 2019). The significant role of these two proteins is to act as adopter proteins by forming complexes with various signaling molecules and molecular chaperones, which are involved in programmed cell death pathways. Selected transgenic chickpea lines will be tested in the field in Queensland in 2019 (Brett Williams, pers. comm.).

9 Chickpea: An Orphan Legume in Nutritional Security

Grain legumes are consumed for at least 10,000 years and grown widely for their nutritional value. The recent Dietary Guidelines of Americans (DGA 2015–2020) recommends eating healthy food that includes legumes. Chickpea is an excellent source of dietary protein. Chickpea provides protein and fiber, as well as a significant amount of several vitamins and minerals. The major proteins in chickpea are albumins and globulins; however, smaller amounts of glutelins and prolamins are also present. In western cuisines, chickpea is mainly included in the form of salad or hummus (chickpea paste), whereas, in Asian and the Mediterranean regions, chickpea is consumed both raw and cooked, used as flour, or included in processed foods. It is also considered as poor man's meat due to its high protein content and other nutritional values.

Chickpea seed carbohydrate is high (about 50–56%) yet low in the glycemic index (GI) scale. However, the GI depends on the cooking and processing of chickpea; for example, the GI index of chickpea is 10, while the canned form has a GI of 38 (Atkinson et al. 2008). Chickpea is also a source of high fiber comprised of both insoluble and soluble forms. Chickpea, which is rich in lysine but deficient in

cysteine and methionine, complements cereals, which are low in lysine but high in cysteine and methionine. Therefore, combining chickpea with cereals grains is a healthy or nutritionally superior choice.

Chickpea is also an excellent source of micronutrients and high in thiamin, niacin, folate, riboflavin, pyridoxine, and several vitamins (Wallace et al. 2016). Micronutrients such as iron chickpea is bound to phytate, which reduces its absorption in the body (Sandberg 2002). In addition, chickpea is a source of phytochemicals, saponins, and tannins, which are considered as antioxidants and anti-carcinogenic. Consuming legumes such as chickpea in the diet has a positive effect on health, such as it could lower the risk of cardiovascular disease, blood pressure, and diabetes (Mudryj et al. 2013).

Legumes were underutilized for the past few decades due to the high-intensity cultivation of cereals due to the impetus of the green revolution. The low productivity of chickpea led to an increase in the price, making them less affordable to the poor, especially in India and other Southeast Asian countries. The UN celebrated the year 2016 as the International Year of Pulses to encourage the stakeholders to work on legume improvement to attain food and nutritional security by 2050. Pulses, including chickpea, hold immense promise to provide nutritious food to the under-nourished people in the highly populated counties. According to FAO (2017), the global demand for legumes is projected to increase by three- to fourfold by 2050 and achieving this would be challenging unless adequate resources are facilitated to improve global pulse production.

10 Economic Benefits of GM Legumes

Legumes are an essential part of a healthy diet for both humans and animals. There is a growing concern about achieving food and nutritional security by 2030 due to rate of population growth, erratic rainfall patterns, more incidences of pests and diseases, and the effect of climate change on crops. In 2017, the United Nations (UN report 2017) reported that the world would be about 9.8 million by 2050, and many will live in disadvantaged conditions. Therefore, producing more food to feed the growing population can only be achieved by judicious use of resources, high-intensity crop cultivations, and adequate technological interventions to mitigate losses of food grains due to biotic and abiotic stresses.

Modern breeding technology may help in reducing the crop yield losses to a great extent as well as create a sustainable farm environment. For example, GM soybean is an excellent innovation to reduce the cost of farming without damaging farm health. Since 1996, the cultivation of glyphosate-tolerant soybean has led to an increase in farm income by more than $38.1 billion (Brookes and Barfoot 2017). This increase in the farm income is due to the reduction in the cost of production, mainly fewer herbicide sprays and other weed management practices. In many countries such as Mexico, Bolivia, and Romania, the increased farm income was also contributed by the increase in the yield per hectare of soybean. Although the herbicide application was increased (4.1%) in GM soybean, it did not impact farm

environment (Brookes and Barfoot 2017). A similar trend was observed when a stacked soybean having Bt and herbicide-resistant traits was commercialized in 2013. The farm income for a stacked GM soybean was $1.23 billion in 2015/2016, which was due to the reduction in insecticidal and herbicide sprays. Also, less fuel consumption was reported due to reduced number of sprays and zero tillage, this was useful to lower down greenhouse gas emissions. Therefore, this farm economics study helped to conclude that GM soybean increased farm income and lowered down the carbon footprint.

11 Conclusion

The review highlighted the progress made on developing the GM chickpea to improve useful traits. In chickpea, the modern breeding tool known as genetic engineering was successful; therefore, now insertion of the novel trait(s) into existing high-yielding chickpea varieties appeared to be feasible. Moreover, exploiting new tools such as Zinc Finger Nucleases (ZFNs), TALENS, and CRISPR-CAS for precise modifications of the chickpea genome is achievable. The case study on the farm economics of GM soybean suggests that the release of GM chickpea having insect resistance would have similar impacts in India and other countries. GM chickpea would be useful not only to save yield losses but also to help create an environmentally sustainable farm.

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Progress in Genetic Engineering of Cowpea for Insect Pest and Virus Resistance

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Abstract

Cowpea [*Vigna unguiculata* (L.) Walp.] is an important staple legume, grown in many parts of the world for human and animal consumption. However, its production is severely constrained by infestation of insect pest and viruses. Genetic resources in cowpea are very limited, and they lack sufficient level of resistance to pathogens, especially the most damaging insect pests and viruses. Breeding insect pest and virus resistance have met with limited success. Biotechnological intervention for improving host plant resistance has been the most attractive for sustainable management of *Maruca* pod borer, storage pest bruchid species, and cowpea infecting Begomoviruses. In this review, we describe the major insect pests and viral diseases of cowpea, and the molecular basis of transgenic resistance. We highlight the recent successes in developing transgenic cowpea for both insect pest and virus resistance. Evaluation of Bt cowpea through multilocation field trials in sub-Saharan African countries with observed high insect mortality brings in renewed expectations among poor farmers in enhancing cowpea yield and income through better insect pest management. RNA silencing technology has been successfully implemented in developing cowpea resistant to MYMIV (Mungbean yellow mosaic virus), CPSMV (Cowpea severe mosaic virus), and CABMV (Cowpea aphid-borne mosaic virus). We also discuss the opportunities lie in precision genome editing and RNAi to confer cowpea molecular immunity against viruses and insect pests.

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Cowpea · Insect pest · RNAi · Virus resistance · Genome editing

1 Introduction

Cowpea [*Vigna unguiculata* (L.) Walp] is the most widely cultivated grain legume in sub-Saharan Africa, because of its ability to grow in hot and drought-prone area and thrive in poor soils (Ehlers and Hall 1997; Timko et al. 2007; Boukar et al. 2018). It is also cultivated in the tropical and subtropical regions of Asia, Middle East, and South America. Cowpea is a very important crop for low input and fragile agriculture system due to its capacity to grow in nutrition poor soil, wide range of soil pH, at relatively high temperature, and water-deficit conditions (Dadson et al. 2005). However, cowpea productivity in typical farmer's field in sub-Saharan Africa is abysmally low (less than 600 kg/ha) as compared to potential grain yield (over 2000 kg/ha) (Boukar et al. 2018). The principal factors responsible for yield and quality losses in cowpea are insect pests (aphids, legume pod borer, and bruchids), viral and fungal diseases, nematodes, parasitic weeds (*Striga*), and plant nutritional deficiencies. Biotic stresses reduce the overall grain yield in cowpea to 0.37 ton/ha (Waddington et al. 2010). Cowpea is the most sensitive to soil moisture stress, during the seedling and reproductive growth stages (Agbicodo et al. 2009; Alidu et al. 2013). Under water-deficit stress conditions, cowpea is highly vulnerable to diseases, insect pests, and parasite attacks.

Among the diseases, infestation by the legume pod borer, *Maruca vitrata*, causes severe yield loss amounting to 80% (Bett et al. 2017). The damage is primarily caused by the feeding of cowpea flower buds, flowers, green pods, and young leaves by *M. vitrata* larvae (Agunbiade et al. 2014). The yield can be stabilized with repeated spraying of insecticides; however, bulk use of insecticides is beyond reach of poor subsistence farmers in sub-Saharan Africa and Asia. These insect species survive on multiple host plants when cowpea is not in cultivation, posing more difficulties in controlling them by cultural practices and insecticidal spray (Taylor 1978; Bottenberg et al. 1997; Arodokoun et al. 2003). Moreover, excessive use of insecticides poses unintended detrimental effects on soil quality, agricultural water runoff, beneficial insects, and human health. Biological control of these insects by microbial formulations have had limited success because of their differentiation in alternative host plant habitats (Agunbiade et al. 2014). Virus infection in cowpea incur 10–100% yield loss (Kareem and Taiwo 2007). More than 140 viruses are reported in cowpea, of which 20 viruses are known to have widespread distribution (Thottappilly and Rossel 1992). Insect vector control, cultural management by using virus-free seed stocks (Biemond et al. 2013), and virus-resistant cultivars (Hampton and Thottappilly 2003) can reduce the incidence, spread, and damage to cowpea crops. However, insect control measures by using pesticides and cultural

measures other than growing virus-free seed are seldom feasible for smallholder farmers.

Breeding for host plant resistance to insect pests and viruses is the most attractive option to stabilize the yield. However, it has met with limited success in cowpea due to the absence of sufficient level of resistance to insects and viruses in primary gene pool, narrow genetic base, and barriers in sexual hybridization with distant wild species, which are reservoirs of resistance genes (Bakshi and Sahoo 2013; Kumar et al. 2017). Genetic diversity, population structure, and outcrossing studies revealed an extensive gene flow from domesticated to wild cowpea, suggesting a narrow cultivated genetic base (Kouam et al. 2012; Xiong et al. 2016). Consequently, transfer of insect and virus resistance genes by transgenic technologies holds the key to accelerate the development of resistant cultivars for resistance breeding and improved yield.

In this review, we discuss the major insect pests and viral diseases of cowpea, limitations in molecular breeding, the molecular basis of transgenic resistance to major insect pests, and Begomoviruses. We describe the recent successes in the development of transgenic cowpea resistant to major field insect (*Maruca* pod borer), storage pest (Bruchid species), and Begomoviruses. Advancement of insect-resistant cowpea through multi-location field trial in sub-Saharan Africa and application of RNA silencing in the development of Begomovirus-resistant cowpea are also discussed. We highlight the future directions in trait improvement through precision genome editing and RNAi.

2 Economic Importance of Cowpea

Cowpea is popular among rural and urban populace of developing nations (Singh 2002; Diouf and Hilu 2005; Xu et al. 2010), due to its high nutritional value. The seeds contain 25% of protein, rich in essential amino acids such as tryptophan and lysine, minerals (iron and zinc), and vitamins (folic acid and vitamin B) (Gonçalves et al. 2016). Cowpea seeds have carbohydrate content, with starch being the main component and energy source. Like other legumes, cowpea starch is more slowly digested than cereal starch, producing less abrupt changes in plasma glucose and insulin upon ingestion, which is extremely beneficial to human health (Gonçalves et al. 2016). Fresh leaves are consumed as pot herbs in East Africa as they contain seven times more calcium and three times more iron than cooked seeds. In developed countries, cowpea is considered as a healthy alternative to soybean for having low fat and high fiber, besides having other health benefits (Timko and Singh 2008a). Cowpea haulm (dried leaves, stems, and pod walls) contain up to 18.6 g protein per 100 g dry weight, and an invaluable source of fodder for cowpea farmers in the dry savannah areas that keep livestock (Samireddypalle et al. 2017). Being a dual-purpose crop, cowpea provides vital support to livelihood of small sustenance farmers contributing to nutritional security, livestock management, income generation, and enhancement of soil fertility (Boukar et al. 2016).

The bulk of cowpea production is confined to West Africa of which Niger, Nigeria, and Burkina Faso together contribute 77% of the total production (FAOSTAT 2017). Nigeria produces the most of cowpea grains and is also the largest consumer. Cowpea is known to be originated and first domesticated in Southern Africa and later spread to east and West Africa and Asia (Boukar et al. 2016). Commonly known as black-eye pea, cowpea is an annual herbaceous crop belonging to the family Fabaceae and genus *Vigna* (Maréchal et al. 1978). Cultivated cowpea comes under subspecies *unguiculata* and is divided into five major cultivar groups, namely *unguiculata* (commonly cultivated cowpea), *biflora*, *sesquipedalis* (yard-long bean), *textilis* (characterized by long peduncles and grown for fiber in Nigeria), and *melanophthalmus* (Pasquet 2000). Cowpea has a relatively small genome of size 620 Mb, consisting of $2n = 22$ chromosomes (Arumuganathan and Earle 1991). The maximum diversity of land races and cultivated cowpeas is present in West and Central Africa (Padulosi and Ng 1997).

3 Insect Pests in Cowpea

Insect pest's infestation and damage represent the major limiting factor in cowpea production worldwide. Several insect pests attack cowpea plants in the field at various stages of their growth (Togola et al. 2017). The yield loss due to insect pest infestation is around 50–80% (Singh 2014). Though insect pests are active at all growth stages of cowpea, the most susceptible stage for insect damage is during flowering and pod formation (Alghali 1992; Kyamanywa 1996). Among the field insect pests, aphids (*Aphis craccivora*), flower bud thrips (*Megalurothrips sjostedti*), legume pod borers (*Maruca vitrata*), and diverse pod sucking bugs (*Clavigralla* spp., *Anoplocnemis* spp., and *Riptortus* spp.) are the most prevalent. Overlapping incidence of these field insect pest infestation can cause complete yield loss (Jackai and Adalla 1997; Singh and Jackai 1985). Aphids feed at all developmental stages of plant from seedlings to flowers and pods. It sucks sap from petioles, leaves, flowers, and pods causing stunting, distortion of leaves, delayed flowering, and reduced fruit setting (Ofuya 1997; Qin et al. 2017). Aphids transmit non-persistently cowpea aphid-borne viruses which alone can cause 10–100% yield loss (Orawu et al. 2013). More than 20 viruses (about 14 of which from Africa) have been identified as cowpea aphid-borne viruses worldwide (Rossel and Thottappilly 1990). Aphids can be more devastating when drought occurs shortly after seedling emergence in the field. Thrips feed on flower buds causing their premature abortion, flower drooping eventually inhibiting pod formation.

Maruca vitrata, commonly known as *Maruca* pod borer (MPB) or legume pod borer, is the major insect pest of cowpea (Jackai and Daoust 1986; Ba et al. 2019). Larvae of this pest attack cowpea at all growth stages, but are particularly destructive when they infest reproductive organs at their early stage, including flower buds, flowers, and green pods (Ba et al. 2019). Presently, there are no known cowpea varieties resistant to *M. vitrata*, and therefore, farmers regularly spray insecticides five to eight times in a season to control this and other insect pests (Murdock et al.

2008). Conventional insecticides, however, are not very effective against *M. vitrata* larvae once they bore into the cowpea floral parts and pods. The inability to control these *M. vitrata* larvae is a serious challenge to cowpea production (Abudulai et al. 2017; Ba et al. 2019). A number of pod-sucking bugs (including *Clavigralla tomentosicollis*, *Riptortus dentipes*, and *Anoplocnemis curvipes*) feed on young pods and suck saps from the seeds while still developing within the pods, resulting into seed deformity, shrinkage, and non-viability rendering them unfit for human consumption. On the other hand, storage pests, cowpea weevil (*Callosobruchus maculatus*) which often accompanies seeds from the field, cause severe damage to stored seeds by the insect's larvae, that feed and develop inside, and eventually emerge through the holes bored by their adults. Up to 100% infestation of the stored seeds has been reported within 3–5 months of storage conditions (Redden et al. 1984; Singh 1980). Economic losses due to *Callosobruchus maculatus* infestation is estimated to be US$30 million annually in Nigeria alone (Ogbuinya 1997). The potential yield of cowpea is not realized unless effective chemical control measures are followed. Farmers apply insecticides 8–10 times in a season to increase the yield by several folds (Omongo et al. 1998). Development of cowpea varieties with host resistance to insect pests is the most viable way of sustainable cultivation.

4 Viral Diseases in Cowpea

More than 140 viruses have been reported to infect cowpea globally. In sub-Saharan Africa, Cowpea chlorotic mottle virus (CPCMV), Cowpea severe mosaic virus (CPSMV), Cowpea mosaic virus (CPMV), Cowpea mild mottle virus (CPMMV), Cucumber mosaic virus (CMV), Cowpea aphid-borne mosaic virus (CABMV), and Cowpea chlorotic mosaic virus (CCMV) are the most prevalent (Alegbejo and Kashina, 2001). The cowpea viruses are transmitted by aphids (BCMV-BlCM, CABMV, and CMV), by beetles (CCMV, CPMV, CPSMV, CPMoV, and SBMV), or by whiteflies (CPMMV and CGMV). With the exception of CGMV and CCMV, all the other viruses are known to be seed-transmitted at a variable rate between none to 55% depending on virus strain, cowpea genotype, and time of infection (Salem et al. 2010). The BCMV-blackeye cowpea mosaic strain (BCMV-BlCM), CABMV, and CMV were mostly detected in the cowpea-producing countries (Cruz and Aragão 2014). In India, cowpea golden mosaic disease (CGMD) and severe leaf curl diseases caused by different isolates of Mungbean yellow mosaic India virus (MYMIV) are the most severe (Kumar et al. 2017). The non-seed-borne viruses, cowpea golden mosaic virus, cause one of the most destructive diseases in the world, and cowpea chlorotic mottle bromovirus causes disease losses either alone or in combination with other viruses (Kuhn 1990). Besides infections caused by isolated viruses, mixed infections with more than one virus are frequently observed with relative frequency in cowpea under field conditions. Some of the viruses in combination (*BICMV + CMV*; *BICMV + CPSMV*; and *CMV + CPSMV + SBMV*) can cause severe disease symptoms and crop loss (Anderson et al. 1994). These

viruses are widespread in cowpea-growing areas of the world frequently appearing in infected commercial seeds, germplasm repository, and variety trial locations.

5 Genetic Resources for Insect Pest Resistance

Host resistance is the most cost-effective, eco-friendly, and easy means for control of insect pests and viruses in cowpea (Tamò et al. 1997). Insect pest- and virus-resistant cultivars provide substantial benefit due to minimal insect damage and reduced cost of insecticidal applications (Fernandez-Cornejo et al. 2003; Gatehouse 2011). Progress in the development of insect pest- and virus-resistant varieties depends on the availability of germplasm with desired traits. The International Institute Tropical Agriculture (IITA), Ibadan, Nigeria, conserves over 14,000 accessions of cultivated cowpea in its gene bank, and the United States Department of Agriculture (USDA), Griffin, USA, and the University of California, Riverside, California, have duplicates of most of the IITA cowpea lines for safe keeping (Boukar et al. 2013; Dumet et al. 2012). Besides cultivated cowpea lines, some accessions of wild cowpea relatives are also conserved in the gene bank at IITA. However, there are not many reports in literature on the use of wild cowpea relatives for the genetic improvement of cultivated varieties for insect pest and virus resistance (Boukar et al. 2013). Numerous studies have attempted to identify cowpea genotypes resistant to various insect pests such as aphids (Ofuya 1993; Huynh et al. 2015), flower thrips (Togola et al. 2019; Omo-Ikerodah et al. 2009), storage pests (Kpoviessi et al. 2019; Arotolu et al. 2018), and viruses (Lima et al. 2011).

Host plant resistance (HPR) is a heritable trait associated with the ability to avoid, withstand, and recover from the damage caused by pests (Kogan 1994). In general, HPR involves three different mechanisms, including strategies that repel the insects (non-preference/antixenosis), affect the biology of insect pest, alter its development (antibiosis) or enable the plant to recover from the damage caused by the insects (tolerance) (Rubaihayo 1996; Goggin et al. 2015). In cowpea, both antibiosis- and antixenosis-based insect resistances have been observed (Jackai et al. 1996). Antibiosis is primarily governed by plant biochemical compounds that induce increased larval mortality, low growth index, and decreased fecundity or that attract insect herbivores (Lattanzio et al. 2000; Togola et al. 2017). Cowpea genotype, IT86D-716 induces impaired development of pod sucking bug, *Clavigralla tomentosicollis*, and such antibiosis is attributed to the presence of unusual polyphenols (Dabire-Binso et al. 2010). Some cowpea genotypes, including IT86D-716, exhibit antixenosis by maintaining as non-preferential host with the help of emission of volatile odors from pods (Koona et al. 2003). Chiang and Jackai (1988) noticed that cowpea cultivars have resistance to pod sucking bugs (PSBs) due to the presence of phenols and tough pod walls. Pod wall trichomes in some resistant cowpea cultivars restrict legume pod borer infestation. This suggests that trichomes act as the first line of defence against insects (Oghiakhe et al. 1992; Jackai et al. 2001; Koona et al. 2002). The cowpea genotypes TVU-1509, Sanzi, and IT2841*Brown exhibit resistance to thrips by accumulating flavonoids, total reducing sugars and

total carbon in the stipules, floral buds, and flowers, whereas soluble amino acids accumulated higher in susceptible cultivar (Agbahoungba et al. 2018). Cowpea varieties TVu 4080p2, TVu 410, and Ife pose resistance to aphid infestation through antixenosis and antibiosis (Karel and Malinga 1980). Seven cowpea genotypes, IT85 F-2687, MN05-841 B-49, MNC99-508-1, MNC99-510-8, TVu 1593, Canapuzinho-1-2, and Sanzi Sambili exhibit non-preference-type resistance to the oviposition and feeding of *C. maculatus* (de Castro et al. 2013).

Inheritance of *M. vitrata* resistance in cowpea is reported to have involvement of one or multiple epistatic interactions, and such additive gene effects are highly significant in plant height, peduncle length, flowering, and pod and seed formation (Philip 2004). *M. vitrata* resistance in cowpea flowers and pods is reported as heritable and controlled polygenically by alleles showing partial dominance (Woolley and Evans 1979). However, no clear information is available about the number of genes, their positions in the genome, and their inheritance pattern in cowpea (Sodedji et al. 2020). Host plant resistance can be either polygenic (several minor genes) or monogenic (single dominant gene). Over the years, extensive evaluation of cowpea for insect pests has identified 8500 accessions for resistance to pod borer and pod sucking bugs and 4000 accessions for resistance to flowering thrips and bruchid (Boukar et al. 2013). Studies on genetics of *Maruca vitrata* resistance in cowpea suggest high heritability of the trait (Sodedji et al. 2020). Most of the insect resistance traits are polygenic in nature, and therefore, it is tedious to introgress them to cultivars. Polygenic resistance is highly influenced by the environment and the genotypes, and thus cannot be directly inferred from the phenotype.

Monogenic resistant individuals are easy to identify among the breeding populations, but establishing monogenic insect resistance is complex as the varieties can be easily overcome by new biotypes of the insect pests. A well-known example is transfer of a single dominant gene to confer resistance to aphids in cowpea variety TVu-3000, but it has become no longer effective due to unknown gene resistance break down (van Emden 1991; Ofuya 1993). Introducing single genes responsible for absolute or superior levels of resistance through backcrossing is simpler than quantitative resistance controlled by multiple minor genes/quantitative trait loci (QTL). However, the major constraint in developing resistant varieties is absence of good and reliable source of resistance. The level of resistance to insect pests identified in the cowpea genotypes is very low, defeating the purpose of resistance breeding. Cowpea germplasm lacks *Maruca* and bruchid resistance genes. The related or wild species like *Vigna vexillata* and *Vigna oblongifolia* that contain resistance genes to *Maruca* and bruchids are sexually incompatible with cultivated cowpea (Fatokun 2002). Developing host plant resistance through incorporating desired genes is the most promising approach to protect cowpea from field insects and storage pests (Boukar and Fatokun 2009).

6 Genetic Resources for Virus Resistance

Host plant resistance is viewed as the most practical, economical, and environmentally friendly option for the management of viral diseases (Bashir and Hampton 1996). Several sources of genetic resistance to viruses have been identified in certain cowpea cultivars or landraces (Bashir and Hampton 1996; Umaharan et al. 1997). However, resistance to multiple virus infection is scarce in cowpea, and recent studies are putting greater emphasis on multiple virus resistance. In the majority of cases, the resistance identified is not immunity but tolerance. High levels of resistance to cowpea viruses, especially multiple virus infections, are limited in cowpea germplasm. Recent studies are giving major emphasis to identify durable resistant cowpea varieties, find out the genetic determinants of virus resistance, and focus on multiple virus resistance. However, development of transgenic plants with resistance, through induced post-transcriptional gene silencing (PTGS) or RNA interference (RNAi), is considered to be more robust and durable to control cowpea infecting DNA or RNA viruses.

7 Genetic Engineering for Insect Pest Resistance in Cowpea

Transgenic approach has emerged as a powerful means for the development of insect pest-resistant varieties for sustainable and ecofriendly crop improvement. Consequently, transfer of insect resistance genes by transgenic technologies holds the key to accelerate the development of resistant varieties for improved yields. Cultivation of insect pest-resistant varieties is more attractive to farmers due to reduced input costs and enhanced profits (Sahoo and Jaiwal 2008).

7.1 Bt Toxin Expression for Insect Resistance

The crystalline (Cry) proteins of *Bacillus thuringiensis* (Bt), produced during sporulation, are insecticidal in nature and work against a specific group of insects. Bt spore formulations are widely used as biopesticides to control insect pest infestation, but their application is limited due to their short half-life, non-persistence (easily washed off by rain or wind), and inability to reach to the burrowing insects. Therefore, expressing insecticidal *cry* genes is regarded to confer the most viable and durable insect pest resistance in plants. Several Cry proteins have been isolated and characterized, and among them, the Cry1Ab is found to be the most potent against early instar larvae of *M. vitrata*, followed by CryAa, Cry2Aa, and Cry1Ac (Srinivasan 2008). Cowpea transgenic lines were developed at CSIRO, Australia, expressing a chimeric Bt *cry1Ab* gene, similar to that used in maize event MON 810 of Monsanto company (Ishiyaku 2010) and later a chimeric *cry1Ab* gene driven by sub-clover stunt virus promoter which conferred protection against *M. vitrata* in a cowpea cultivar of West Africa (Higgins et al. 2012). Multiyear Confined Field

Trials (CFT) of these transgenic cowpea lines in Burkina Faso, Ghana, and Nigeria under the flagship of African Agricultural Technology Foundation (AATF) confirmed complete protection of *Bt* cowpea plants from MPB (Ba et al. 2018). A single homozygous cowpea transgenic line with consistent field protection to MPB was used in breeding programs with locally preferred cowpea genotypes (Higgins et al. 2013; Mohammed et al. 2014, 2015). Recently, a *Bt cry2Ab* gene was introduced into this transgenic line for the management of MPB (Ba et al. 2018). The *Bt* Cry1Ac protein is also effective against *M. vitrata*. We generated transgenic cowpea that stably expressed and inherited the *cry1Ac* gene, in an Indian cultivar Pusa Komal (Bakshi et al. 2011; Bakshi and Sahoo 2013). Some of these transgenic lines were evaluated up to T_4 generations, and they showed complete and consistent protection against *M. vitrata* (unpublished data).

7.2 Expression of Plant Lectin for Insect Resistance

Plant lectins are known to have insecticidal activities against insect pests. Evaluation of 25 lectins from 15 plant families tested against *M. vitrata* larvae in artificial diet assays showed *Listera ovata* agglutinin (LOA) and mannose binding specific *Galanthus nivalis* agglutinin most effective against MPB larvae at 2% level (Machuka et al. 2003). These plant lectins have been successfully expressed in a variety of crops to confer high resistance against their target pests (Vandenborre et al. 2011). However, Cry protein expression at a level of 2% in cowpea is difficult to attain through current transformation protocols, and moreover, such high expression of insecticidal proteins will be undesirable for host plant (Machuka et al. 1999). Vegetative insecticidal proteins (Vips) synthesized during the vegetative growth phase of the *Bacillus thuringiensis* show unique spectrum of insecticidal activity compared to other insecticidal proteins. Vips require many folds lesser dose than Cry proteins to achieve 100% insect mortality (Estruch et al. 1996). Vips have no amino acid sequence similarity to Cry proteins and have a different mechanism of action (Lee et al. 2003). Among the Vip toxins, Vip3 is highly potent against lepidopteran insects and could be a potential candidate for MPB management (Estruch et al. 1996). The BtVip3Ba expression in transgenic cowpea was found to confer complete protection against MPB larvae in feeding trials (Bett et al. 2017). Insect pests including *Maruca vitrata* tend to develop field-evolved resistance to the transgenic plants, particularly in those expressing a single type of insecticidal protein. Therefore, pyramiding two or more insecticidal genes, with differing mode of action can reduce the likelihood of insects evolving resistance. The combination of *Vip3Ba* and *cry1Ab* genes is proposed for pyramiding in cowpea in order to delay the resistance evolvement in MPB to transgenic plants as the encoded proteins are structurally different with binding affinities to different sites in insect midgut, and have no cross-resistance between them. Gene stacking of Vip and Cry toxins has enabled to control a variety of pests in different crops (Adamczyk and Mahaffey 2008; Palekar et al. 2011).

7.3 Storage Pest Resistance

The storage pests, Coleopteran Bruchid species (*Callosobruchus maculatus* and *C. chinensis*) cause severe post-harvest loss to stored cowpea seeds through consumption, qualitative deterioration, and reduced stock viability. Females lay large number of eggs on the seed surface and the larvae burrow into the seed, where they feed and complete their development in 30 days (Southgate 1979). The bruchids usually continue to multiply during seed storage and can lead to extensive damage to stored seeds or even inflict total losses if the seeds are stored for long periods. Treatment of stored seeds with surface and fumigant chemicals is effective for managing bruchid infestations, but prohibitive cost and adverse impact on human health have limited their large-scale applications. Protection of stored seeds from bruchid infestation can be better managed by developing cowpea varieties with inherent seed resistance to bruchid beetles. Screening of more than 8000 cowpea lines for resistance to the *C. maculatus* revealed moderate levels of resistance in only three lines, including the landrace TVu 2027 (Singh 1977; Singh et al. 1985). Moreover, moderate resistance in TVu 2027 lasted only for about 90 days post-infestation, whereas subsistence farmers need to store the seeds for at least 9 months for sowing in next season (Murdock et al. 2008). Absence of strong bruchid resistance available in cowpea germplasm has prompted sourcing bruchid resistance from other grain legume seeds. Transgenic approaches make feasible to develop varieties with substantially higher resistance than that available in the crop germplasm resources. The common bean and other *Phaseolus* species possess a family of evolutionary related defense proteins including phytohemagglutinin (PHA), arcelin (Arc), and α-amylase inhibitors (αAI) (Chrispeels and Raikhel 1991) featuring different modes of action and insecticidal properties against bruchids (Leavitt et al. 1977; Osborni et al. 1988; Janarthanan et al. 2008). The genes encoding these three proteins are located in a single locus in the *P. vulgaris* genome (Nodari et al. 1993), and these homologous genes have possibly arisen by duplication of an ancestral gene. These seed αAIs display strong inhibitory activity against gut α-amylases of bruchid species that feed starchy seeds of grain legumes and depend on α-amylases for survival (Franco et al. 2002). This feature of αAIs makes them attractive candidates for genetic engineering for seed storage pest resistance. The two different isoforms of *P. vulgaris* αAIs differing in their specificity toward α-amylases include αAI-1 (Moreno and Chrispeels 1989) and αAI-2 found in some wild accessions of the common bean that contain Arc as the major storage protein instead of phaseolin (Suzuki et al. 1993). They have very strong activity against midgut amylases of major bruchid pests found worldwide (Ishimoto and Kitamura 1992; Franco et al. 2002). The successful transfer of common bean αAI-1 and/or αAI-2 genes under the control of seed-specific promoter of common bean phytohemagglutinin (*dlec2*) into several grain legumes has conferred complete protection against respective bruchid species in peas (Shade et al. 1994; Schroeder et al. 1995), cowpeas (Solleti et al. 2008b; Lüthi et al. 2013), azuki beans (Ishimoto et al. 1996), and chickpeas (Morton et al. 2000; Sarmah et al. 2004; Lüthi et al. 2013).

7.4 RNA Interference (RNAi) for Insect Resistance

In plants, RNA silencing has emerged as a novel strategy and powerful means for developing insect resistance by downregulating the expression of vital insect genes through genetic transformation. For this purpose, gene constructs with sense, antisense, or self-complementary hairpin RNA (hpRNA) containing sequences homologous to the target gene were used (Helliwell and Waterhouse 2003). The potential of RNA interference (RNAi) for developing crop resistance against lepidopteran insects has been previously reported (Terenius et al. 2011). Downregulation of vital insect metabolism genes such as *TPS* (trehalose phosphate synthase), *AChE* (involved in nerve transmission and other metabolic processes), and CHS (involved in the synthesis of chitin molecules) through small interfering RNA (siRNA)-mediated specific silencing has arrested the growth of major lepidopteran insects like *Helicoverpa armigera* and *M. vitrata* (Kumar et al. 2009) in host plants. We recently developed transgenic cowpea expressing hairpin RNA (hpRNA) of *M. vitrata* specific *TPS* gene. Insect bioassay showed high mortality of *M. vitrata* larvae, and the metabolite analysis of transgenic cowpea seeds revealed no off-target effects of the transgene expression on host plant metabolism (Unpublished data).

7.5 microRNA Regulation for Insect Resistance

MicroRNAs (miRNAs) are non-coding small RNAs (18–25 nt) that play crucial roles in various biological processes, including development and regulation of gene expression. There are four more classes of small RNAs besides siRNA, which include microRNAs (miRNAs), transacting siRNAs (ta-siRNAs), natural antisense siRNAs (nat-siRNAs), and Piwi-interacting RNAs (Meins et al. 2005; Vaucheret 2006). These endogenous small RNAs have important regulatory roles in gene development and programming. In response to pathogen attack, plants express the regulatory genes such as microRNA (miRNAs), which control the expression of defense responsive genes. The plant miRNA expression is either induced or overexpressed upon pathogen attack. The insect pests also express miRNAs, which interfere with the expression of host plant's genes involved in defense. In resistant plants, the miRNAs associated with positive regulation of the resistant genes are overexpressed in response to pathogen attack. Since miRNAs play critical roles in insect development (Chawla and Sokol 2011), their suppression or overexpression may interfere with the normal development of insects with potentially fatal consequences. Therefore, the miRNAs have emerged as good candidate for the control of insect pests for crop improvement and potential exists in future for the development of transgenics using this technology. However, no attempts have been made to exploit miRNA-mediated insect pest resistance in cowpea.

8 Pathogen-Derived Resistance for Viral Disease Management

Pathogen-derived resistance in crops is routine, and the expression of varied viral gene products has proved to be effective in preventing or reducing infection by diverse plant viruses. RNAi method has emerged as an efficient means to confer resistance against plant viruses, including control of Begomovirus infection in legumes (Aragao et al. 1998; Bendahmane and Gronenborn 1997; Day et al. 1991; Haq et al. 2010; Patil et al. 2016; Zhang et al. 2005).

8.1 RNA Interference for Virus Resistance

RNA silencing specifically degrades target RNA in a sequence-specific manner via formation of double-stranded RNA (dsRNA), that are diced into short RNA fragments known as siRNAs, which are hallmarks of RNAi. A significant feature of RNA silencing technology is the presence of double-stranded RNA (dsRNA), which is not only the product of RNA silencing but also the potent trigger of RNAi. Considerable progress has been made in developing virus resistance in transgenic plants by selective suppression of viral genes, thus exploiting the phenomenon of RNAi. In India, different isolates of MYMIV cause golden mosaic disease (CGMD) and severe leaf curl diseases in cowpea. MYMIV belongs to the genus Begomovirus and is transmitted by whitefly (*Bemisia tabaci*). Their genome consists of two circular single-stranded DNA molecules (bipartite, ~2.7 kb), referred as DNA-A and DNA-B. The DNA-A component has seven ORFs, encoding *AC1*, *AC2*, *AC3*, *AC4*, and *AC5* on the complementary strand and *AV1* and *AV2* on the virion strand, which are needed for replication and encapsidation. The DNA-B component is composed of two ORFs, *BC1* and *BV1* essential for inter- and intracellular movement of the viral genome in the host (Hanley-Bowdoin et al. 1999; Jeske 2009). There are no known natural sources of resistance to MYMIV in cowpea. Therefore, we need to fall back on newer technologies for developing resistance. Accordingly, RNAi-derived virus resistance has been accomplished by targeting the *AC1* of geminiviruses, including bean golden mosaic virus (BGMV) (Bonfim et al. 2007), tomato yellow leaf curl virus (TYLCV) (Bendahmane and Gronenborn 1997), maize streak virus (MSV) (Owor et al. 2011), and African cassava mosaic virus (ACMV) (Chellappan et al. 2004; Vanderschuren et al. 2009). Suppression of the common/intergenic region has resulted in complete arrest of MYMV (Pooggin et al. 2003) and ACMV (Vanderschuren et al. 2007). The AC2 protein [transcriptional activator protein (TrAP)] is a multifunctional protein encoded by both monopartite and bipartite Begomoviruses. It activates the viral late gene promoters (Rajeswaran et al. 2007; Shivaprasad et al. 2005), suppresses RNA silencing (Trinks et al. 2005), and determines pathogenicity. Molecular basis of the AC2 suppressor activity revealed that the AC2 protein inhibits both RDR6 and AGO, the key players of host RNA silencing (Kumar et al. 2015). RNA silencing of TrAP or AC2 effectively controlled viral titer and infection in transgenic tobacco (Shanmugapriya et al. 2015). RNA

silencing of *AC4*, an important viral gene embedded within *AC1* ORF, has resulted in the resistance to cassava-infecting geminiviruses (Vanitharani et al. 2004). Both *AC2* and *AC4* are considered as the most potential targets for RNA silencing-mediated geminivirus resistance in grain legumes (Kumar et al. 2017). They have generated transgenic cowpea plants expressing hpRNA, specific to conserved regions of *AC2*, *AC4*, and *AC2 + AC4* genes of seven cowpea-infecting Begomoviruses. The hpRNA cowpea lines accumulated viral gene-specific siRNAs, exhibited nearly 100% resistance against MYMIV infection in lines that expressed *AC2*-hp and *AC2 + AC4-hp* RNA. These *AC2*-hp and *AC2 + AC4-hp* RNA lines displayed zero or very low titers of viral DNA and presented normal phenotype with no yield penalty. In Latin America and Africa, *cowpea severe mosaic virus* (CPSMV) and *cowpea aphid-borne mosaic virus* (CABMV) are more severe limiting the production of cowpea. Expression of RNA silencing of the CPSMV proteinase cofactor gene and the CABMV coat protein gene in cowpea enhanced the resistance to both the viruses (Cruz and Aragão 2014).

8.2 Genome Editing for Virus Resistance

Genome editing technologies have emerged, especially CRISPR/Cas, as powerful tools to elucidate the gene function as well as to generate new and valuable variations in important traits for improving biotic and abiotic stress tolerance in plants. Precision editing has recently been made possible through the use of programmable, sequence-specific nucleases (SSNs), designed to bind and cleave a specific nucleic acid sequence by introducing double-stranded breaks (DSBs) at or near the target site which is core to this technology (Piatek and Mahfouz 2016). Four major classes of SSNs, including the meganucleases, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced palindromic repeats/CRISPR-associated 9 (CRISPR/Cas9) are implicated in this process (Stella and Montoya 2016). The CRISPR (clustered regularly interspaced palindromic repeats)/CRISPR-associated 9 (CRISPR/Cas9) system has received special interest because of its simplicity, efficiency, and reproducibility. Recent studies have used CRISPR/Cas9 to engineer virus resistance in plants, either by directly targeting and cleaving the viral genome or by modifying the host plant genome to impart viral immunity. These concepts have been harnessed to improve crop plant resistance to viral diseases directly by targeting viral genomes or by targeting various host factors. The host plant factors that assist in virus replication, transcription, and translation are potential targets of genome editing to curb virus infection. Lack of ribosomes in virions makes them dependent on host translation machinery for viral protein synthesis. The eukaryotic initiation factor 4E (eIF4E) and its isoform (eIFiso4E) of host plants are essential for some viruses to initiate viral protein translation (Sanfaçon 2015). Although *eIF(iso)4E* translation initiation factors are important for growth and reproduction, CRISPR/Cas9-based knockout of *Arabidopsis eIF(iso)4E* has resulted in resistance to *Turnip mosaic virus* (TuMV) without having any penalty on plant growth (Pyott et al. 2016). The knockout of

cucumber *eIF4E* gene by CRISPR/Cas9 showed broad-spectrum resistance to *Cucumber vein yellowing virus* (*CVYV*), *PRSV*, and *ZYMV* (Chandrasekaran et al. 2016). Similarly, double knockout of Cassava-encoded *eIF4E* family genes, *nCBP-1* (novel cap-binding protein-1) and *nCBP-2* which interact with VPg (viral genome-linked protein) of *Cassava brown streak virus* (CBSV), showed delayed and attenuated symptoms in CRISPR/Cas9 edited lines (Gomez et al. 2019).

9 Conclusion and Future Prospective

Cowpea is an important warm-season legume grown for human and animal consumption by the low-subsidence farmers in the tropics and subtropics of the world. The production of cowpea is affected mostly by biotic stresses, of which insect pest infestation and viral diseases alone cause substantial yield loss. Breeding for pathogen resistance appears the most feasible and economical approach to insect pest management. However, absence of reliable and durable natural resistance in cowpea germplasm has impeded the precision introgression breeding for controlling insect pest and virus infestation. Consequently, application of emerging DNA technologies in cowpea for screening large-scale germplasm based on next-generation sequencing for high-throughput gene discovery, genetic engineering, and genome editing offers enormous opportunities for developing insect pest- and virus-resistance and enhanced yield. Cowpea is considered highly recalcitrant to genetic manipulation in vitro. Efficient genetic transformation methods in cowpea have been established in our laboratory (Solleti et al. 2008a; Bakshi et al. 2011, 2012a, b) and by other research groups (Popelka et al. 2006; Chaudhury et al. 2007; Ivo et al. 2008; Bett et al. 2019). These transformation protocols were successfully used to introduce candidate genes for various traits (Sindhu et al. 2019). Controlling legume pod borer, *Maruca vitrata*, infestation in cowpea is the major concern for poor farmers in sub-Saharan Africa, Latin America, and India. A significant advance in the management of *M. vitrata* is the recent success in multi-location field trials of transgenic cowpea expressing BtCry1Ab delta endotoxin in Burkina Faso, Ghana, and Nigeria, where high mortality of insect larvae in field conditions has been reported. Upon release to farmers, transgenic Bt cowpea should address yield loss in cowpea in sub-Saharan Africa resulting from *M. vitrata* feeding. However, potential effects of Bt Cry proteins on non-target organisms associated with cultivated and wild cowpea need to be assessed properly prior to commercial release of Bt cowpea. Gene pyramiding need to be explored to generate durable and multiple pest resistance. Application of next generation sequencing has enabled global transcription profiling of plant–pathogen interactions, narrowing on key pathways and candidate genes responsible for pathogen resistance. These findings create opportunities to make available novel pathogen-resistant genes for introgression into locally adapted cowpea cultivars by either precision breeding or genetic engineering. Application of RNAi has been conceptually successful in developing resistance to MYMIV, CPSMV, and CABMV. Application of RNAi and genome editing has great potential to control the viral diseases, particularly the mixed viral infection and complex viral

counter-defensive measures against RNA silencing. Genome editing can play a vital role in providing molecular immunity against the insect pest infestation and viral diseases by altering the essential host plant factors associated with pathogen susceptibility.

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Peanut (*Arachis hypogaea* L.) Transgenic Plants for Abiotic Stress Tolerance

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Abstract

Groundnut (*Arachis hypogaea* L.) is one of the important legume cash crops of tropical and semi-arid regions, where it provides a major source of edible oil and vegetable proteins. Abiotic and biotic stresses in groundnut negatively influence on survival, biomass production, and total crop yield. Breeding groundnut genotypes for abiotic stress tolerance will likely sustain groundnut production. Traditional approaches such as breeding for abiotic stress tolerance have been slow, due to the rare alleles implicated in abiotic stress tolerance in the existing groundnut germplasm. Hence, engineering for abiotic stress resistance is an important target for increasing groundnut productivity. The chapter focuses on the development of transgenic groundnut plants for abiotic stress tolerance and the constraints associated with it. This review also describes the recent progress in using genetic engineering approaches for the improvement of abiotic stress tolerance in groundnut.

Keywords

Peanut · Abiotic stress · Transgenic plants · In planta transformation · DREB1A · NHX

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1 Introduction

Peanut (*Arachis hypogaea* L.), also known as groundnut, is an important member of the legume family. It is grown not only as a source of vegetable oil but also as an important resource of protein, dietary fiber, vitamins such as vitamin B-complex and vitamin A, minerals like calcium, iron, potassium besides bioactive compounds (Desmae et al. 2017). Other parts of the crop such as foliage, stem, and cake (a by-product of seeds) serve as a source of animal feed. These multiple uses of groundnut make it an excellent cash crop for domestic markets as well as for foreign trade in several developing and developed countries. Most probably cultivated peanut has originated and domesticated ~6000 years back in southern Bolivia and northwest Argentina (Bertioli et al. 2019; Zhuang et al. 2019). Ethnological evidence suggest that probably Indian tribes of South America domesticated peanut long before the Spanish conquest. Subsequently, Spanish traders spread the peanut to Asia and Africa, and it is now grown in over 100 countries around the world (Gregory et al. 1980; Kochert et al. 1996). The cultivated peanut belongs to section *Arachis*, family Fabaceae, and subfamily Faboideae (Gregory et al. 1973). Based on the vegetative and reproductive characters, peanuts are grouped into two large groups, i.e., Virginia and Spanish Valencia. The cultivated species *Arachis hypogaea* L. consists of two subspecies, ssp. *hypogaea* and ssp. *fastigiata*. Each subspecies has botanical varieties, i.e., ssp. *hypogaea* has *hypogaea* (Virginia), *hirsuta* and ssp. *fastigiata* has *fastigiata* (Valencia), *vulgaris*, *peruviana*, *aequatoriana* (Krapovickas and Gregory 1994; Desmae et al. 2017). There were 80 species in the genus *Arachis*. The majority of *Arachis* species are diploid, having a $2n = 2x = 20$ complement of chromosomes. However, cultivated peanut is a self-pollinated, allotetraploid (AABB $2n = 4x = 40$), which is a hybrid of two wild species *Arachis duranensis* (AA-genome, $2n = 2x = 20$) and *Arachis ipaensis* (BB-genome, $2n = 2x = 20$), with a genome size of 2891 Mbp. Cytological characterization of *A. hypogaea* reveals two distinct pairs of chromosomes, one termed A genome, which is smaller than any other pair, and the other termed B genome, with a secondary constriction (Husted 1933, 1936; Desmae et al. 2017).

2 Area, Production, and Productivity

Peanut is grown in most of the tropical, subtropical, and temperate regions. The worldwide cultivation of peanut was 27.94 million hectares in the year 2017 and grown in 100 countries with China, India, the USA, Nigeria, and Sudan as major producers (FAOSTAT 2019). The worldwide total production of peanut in the year 2017 was 47.09 million tons with an average yield of 1686 kg/ha (FAOSTAT 2019). About 97% of peanut cultivation and 95% of total production come from the developing countries of Asia, Africa, and South America. Small hold farmers of Asia and Africa majorly cultivate the peanut under rainfed conditions with limited inputs. Asia alone accounts for 50% global cultivation and 62.5% global production followed by Africa with 40% of global cultivation and 26.1% of global production

(http://www.icrisat.org/what-we-do/crops/GroundNut/GroundNut.htm; FAOSTAT 2019). India is the second largest producer of peanut after China. In India, the peanut is cultivated in an area of 5.3 million hectares with a production of 9.179 million tonnes in the year 2017 (FAOSTAT 2019). In India, on an average, 85% of the peanut is cultivated under rainfed conditions in kharif season during June/July to October/November and the remaining 15% under irrigation in rabi season (October/November to February/March).

3 Abiotic Stress Effect on Peanut

Dryland regions of semiarid tropics (SAT) alone contribute relatively 70% of the world's peanut production. Peanut requires a well distributed rainfall ranging from 50 to 125 cm (Reddy 1988). The arid and semi-arid regions are highly prone to extremes of temperature, severe and frequent drought, low relative humidity, and high wind velocity. These climatic conditions at various growth stages of crop severely affect the growth and productivity of peanut. The global climate change predictions indicate that extreme weathers, particularly drought conditions, prevail more often in tropical and subtropical regions of the world, which would have major negative impacts on peanut productivity and food safety in peanut producing countries such as China, India, Nigeria, and the USA (Lobell et al. 2008; Battisti and Naylor 2009; Long and Ort 2010). Hence, it is very indispensable to put in place genetic and management interventions in an environmentally sustainable manner to combat the negative impacts of climate change on peanut. Drought and salinity are major abiotic factors limiting global peanut production (Stansell and Pallas 1985; Lamb et al. 1997).

3.1 Drought Stress

Drought stress alone accounts for an annual loss of over six million tons of peanut production (Gautami et al. 2011; Bhatnagar-Mathur et al. 2014). The loss of yields due to drought stress ranges from 5 to 75%, depending on timing, intensity, and duration of drought during the crop growth periods. The intensity of drought also depends on the water-holding capacity of the soil and other environmental factors such as high temperatures. Research studies on the effect of drought stress at various growth stages of peanut on yield revealed that drought stress at the early vegetative growth stage or pre-flowering growth stage enhanced the yield (Puangbut et al. 2010; Nautiyal et al. 1999; Rao et al. 1985).

Research studies on the effect of drought stress at various growth stages of peanut suggest that drought at early growth season or pre-flowering followed by recovery enhances the yield (Puangbut et al. 2009, 2010; Nautiyal et al. 1999; Rao et al. 1985); whereas prolonged drought stress during flowering stage or pod formation or seed maturation stages significantly decreases the yield (Nautiyal et al. 1999; Songsri et al. 2008; Awal and Ikeda 2002). The reason for enhanced yields at pre-flowering

stage could be perhaps due to two mechanisms: (a) deeper penetrations of roots into the soil to absorb the water and (b) unconstrained transpiration, which might increase photosynthesis and growth rate of the plant, and subsequently improve the yields (Rao et al. 1985; Nautiyal et al. 1999; Awal and Ikeda 2002; Puangbut et al. 2009; Jongrungklang et al. 2013). Drought stress during the flowering, pegging, and pod developmental stages affects the pod/seed yield. Usually, post fertilized peanut flowers convert into gynophores or pegs, elongate, penetrate into topsoil layers, and develop into pods (Haro et al. 2008, 2011). However, under drought stress conditions, hardness of soil inhibits or delays the elongation and penetration process of pegs and results in the reduction of the number of mature pods and seeds (Haro et al. 2010, 2011). Peanut-fertilized embryo has longer survival rates compared to other grain crops such as maize and soybean (Westgate and Boyer 1986). Thus, if drought stress-affected pegs are re-watered, viable pegs resume from the stress and penetrate into the soil layers to form pods. The resumption capacity is a drought-adaptive trait, and it varies between the genotypes.

Drought stress severely affects the broad-spectrum of physiological processes such as water relations by influencing the parameters like relative water content (RWC), leaf water potential, stomatal resistance, rate of transpiration, and leaf and canopy temperatures. Drought stress lowers the RWC of stress-imposed plants compared to non-stressed plants. RWC of non-stressed plants ranges from 85 to 90%, while in drought-stressed plants, it is as low as 30% (Babu and Rao 1983). Peanut genotypes display significant variation in leaf water potential and stomatal conductance under diverse water availability situations (Gautreau 1977; Black et al. 1985; Clavel et al. 2004; Nautiyal et al. 2008). Severity and duration of water stress in peanut gradually reduce the rate of photosynthesis, transpiration, and water potential (Subramanian and Maheswari 1990). Three weeks of water stress in peanut reduces the leaf area index, RWC, and transpiration rate (Clavel et al. 2004). Semi-arid environments are characterized with prolonged drought stress coupled with high temperatures, and peanuts cultivated under these conditions very often close their stomata, when the saturation vapor pressure deficit exceeds 3 kPa, i.e., usually mid-day, gradually decrease the rate of active gas exchange through stomata, and inhibit the rate of active photosynthesis. However, rapid recovery of stomatal conductance to normal status upon re-watering has been widely reported as a drought-adaptive strategy in peanut (Puangbut et al. 2009; Devries et al. 1989).

Numerous studies reported that drought stress severely affects the nitrogen fixation in peanut (Serraj et al. 1999; Reddy et al. 2003; Pimratch et al. 2008); however, when compared with other legume species, reduction in nitrogen fixation due to drought stress was less in peanut (DeVries et al. 1989; Venkateswarulu et al. 1989; Sinclair and Serraj 1995). Water potential levels lower than −1.4 Mpa in leaves and nodules, drastically reduce the activity of nitrogenase (Devries et al. 1989). Drought stress changes the leaf turgor pressure, which in turn changes the nodulation activity and N fixation. Under drought, a strong positive correlation was observed between biomass production and N fixation (Pimratch et al. 2008). Under unfavorable environmental conditions, plants synthesize and accumulate compatible solutes such as osmoprotectants to stabilize the cellular membranes and to

maintain the turgor as a stress-adaptive strategy. Accumulation of compatible solutes like high concentrations of total soluble sugars and reducing sugars after 15 days of drought stress in peanut genotype ICGV91114 correlated with the acquisition of drought tolerance (Hoekstra and Buitink 2001; Padmavathy and Rao 2013). Drought stress triggers antioxidative machinery in contrasting peanut genotypes, i.e., Florispan (drought tolerant) and Gazipasa (drought-sensitive) to combat reactive oxygen species-induced cell damage. However, in both the genotypes, peroxidase (POX) does not have a direct role against ROS-induced cell damage, while APX displays a role in protecting the drought-sensitive genotype Gazipasa during mild drought stress conditions. In contrast, the antioxidants CAT and APX display key roles against ROS-induced cell damage in drought-tolerant genotype Florispan (Akcay et al. 2010).

To uncover the molecular basis of drought tolerance in peanut, several studies were conducted related to gene expression levels, identification, and cloning of drought-responsive genes (Jain et al. 2001; Luo et al. 2005; Devaiah et al. 2007; Govind et al. 2009; Chen et al. 2012; Ranganayakulu et al. 2012; Sui et al. 2013; Dang Phat et al. 2013; Pruthvi et al. 2013; Li et al. 2014; Brasileiro et al. 2015; Zhao et al. 2018). The DDRT-PCR analysis of drought-tolerant and susceptible genotypes identified 43 differentially expressed transcripts under drought stress, and among them, three of the transcripts *PTRD-1*, *PTRD-10*, and *PTRD-16* were differentially expressed in the tolerant genotype (Jain et al. 2001). Gopala Krishna et al. (2001) identified a drought stress-induced gene flavonol 3-*O*-glucosyl-transferase (F30GT) from a subtractive cDNA library constructed from drought stress peanut leaves, which is involved in anthocyanin biosynthetic pathway that can protect the membrane lipids as an antioxidant.

Stress-responsive signaling molecules such as a serine-rich protein, a leucine-rich protein, and GTP-binding protein (*AhRabG3b*) were cloned from stress-tolerant genotypes (Devaiah et al. 2007; Sui et al. 2013). Expression analysis of *ERF* family transcription factors (*AhERF1–AhERF6*) suggests that these *ERF* proteins may have individual roles in stress tolerance and acclimation of peanut (Chen et al. 2012). Variations in the promoter regions of *ahERF7* gene levels in drought-tolerant (C76-16) and -sensitive genotypes (AP-3) alter the expression of *ahERF7* gene to 100-fold more in C76-16, compared to AP-3 under drought stress (Dang Phat et al. 2013). Enhanced accumulation levels of phospholipase D∞, late embryogenesis abundance (*LEA*), and reduced expression levels of serine protease minimized the loss of water from peanut under drought stress by protecting the cellular components from stress-induced damage and senescence (Drame et al. 2007). Different expression patterns of 19 LEA genes belonging to eight distinct groups in peanut suggested that these genes might play divergent roles in plant development and stress adaptation (Su et al. 2011). In an elegant study, Dang Phat et al. (2013) identified significantly higher levels of *HSP70*, *CuZnSOD*, drought protein, and *myo*-inositol phosphate synthase gene expression in the tolerant genotype C76-16. Especially, enhanced expression levels of two candidate genes, drought protein (*RD-22*) and *myo*-inositol phosphate synthase, were more associated with drought tolerance of C76-16 (Dang Phat et al. 2013). Subtracted cDNA library constructed

from a gradual drought-stressed peanut leaves yielded 700 differentially expressed genes, and among them 50% were unknown. Known stress-responsive genes in the subtracted library include auxin-repressed proteins (*ARP*), cytokinin-repressed (*CR9*), brassinosteroid-responsive (*BRHA1*), *LEA/dehydrins*, *HSP*s, *ADH*, proline-rich proteins, and metallothioneins (Govind et al. 2009). Several unknown, differentially expressed stress-responsive genes have been identified in subtracted cDNA constructed from a drought-tolerant genotype (cv. K-134) point out that tolerant genotypes might possess novel genes which play vital roles in stress tolerance (Ranganayakulu et al. 2012).

Peanut seedlings were subjected to drought stress with or without ABA pretreatment, and RNA samples were collected from leaves, roots, and stems. RNA sequence libraries were constructed. Transcriptome analysis yielded 100 putative transcription factors under drought and ABA pretreatment. However, only 22 transcription factors were identified under drought stress alone. These data indicate that peanut drought stress-responsive mechanisms respond through ABA-mediated pathway (Li et al. 2014). Leaves and roots of wild *Arachis* species, *A. duranensis* (AA genome) and *A. magna* (BB genome), were subjected to gradual drought stress, and the transcriptomic data analysis revealed that two of these species share a common transcriptional response to drought stress (Brasileiro et al. 2015). Transcriptome analysis of drought stress-induced root samples of drought-tolerant genotype J11 unveiled the candidate genes and metabolic pathways involved in the early stages of drought stress and drought tolerance. A large number of genes including transcription factors, associated with photosynthesis, carbon, and sucrose metabolism, were identified in drought-tolerant peanut genotype (Zhao et al. 2018). Drought stress severely affected the polypeptide composition (molecular weight between 10 and 70 kDa) of susceptible genotypes like Florunner and JL-24, but did not affect the drought-tolerant genotype TMV-2. Drought stress-induced several proteins such as ultraviolet-B (UV-B) repressive rubisco activase, glyceraldehyde-3-phosphate dehydrogenase, ribulose bisphosphate carboxylase, phosphoribulokinase, cytochrome b6-f complex, and oxygen-evolving enhancer protein (Katam et al. 2007). A proteomic approach was used to identify drought stress-tolerant genotype and to decipher the drought tolerant mechanism in genotypes COC041, COC166 and TMV2 (Kottapalli et al. 2009). Differentially expressed proteins involved in various metabolic pathways such as cell wall strengthening, signal transduction, energy metabolism, cellular detoxification, and gene regulation were identified (Kottapalli et al. 2009).

3.2 Salinity Stress

Similar to other legume crops, the peanut is also moderately susceptible to soil salinity and cultivated in low saline fields of China (Singh et al. 2008; Chakraborty et al. 2016). Peanut genotypes exhibit genotypic differences in saline tolerance (Sun et al. 2013). Salt stress significantly reduced the plant growth, leaf area, leaf biomass, and RWC of peanut seedlings (Nautiyal et al. 1989; Janila et al. 1999; Mensah

et al. 2006; Singh and Prasad 2009; Hammad et al. 2010; Parida and Jha 2013; Meena et al. 2016; Sui et al. 2018). To compensate for the salt stress-induced damage, peanut plants trigger the production of organic compatible solutes such as free proline, sugars, starch, and polyphenols, and as a result, cell membranes get protection from salt-induced damages as evidenced by the decrease in membrane lipid peroxidation and electrolyte leakage (Parida and Jha 2013). Salt stress increases relative electrolyte leakage (REL) of the leaf, in comparison with non-stressed conditions (Cui et al. 2018). Salt stress triggers the production of ROS, which causes damage to the photosynthetic apparatus (Qin et al. 2011; Sui et al. 2018). Salt stress also reduces the mineral nutrient concentrations such as Mg^{2+}, Ca^{2+}, P, K^+/Na^+, and Ca^{2+}/Na^+, and peanut genotypes exhibited genotypic variations in mineral nutrient partition under salt stress (Taffouo et al. 2010). Salt stress also affects pod development, pod, and haulm yield (Lauter and Meiri 1990; Girdhar et al. 2005; Hammad et al. 2010; Meena et al. 2016).

Information is scanty on molecular responses of peanut to salt stress. Microarray analysis of peanut roots revealed that genes involved in photosynthetic processes and phenylalanine metabolism were down-regulated along with genes involved in metabolic pathways, biosynthesis of unsaturated fatty acids, plant–pathogen interaction (Chen et al. 2016). Transcriptome and metabolite analysis of peanut salt-stressed leaves revealed that genes/proteins related to *HKT1*, *H^+-ATPases*, *H^+-pyrophosphatase*, *K^+-transporter*, *LEA* protein, aquaporin, and proline metabolic pathway play a major role in peanut salt stress tolerance (Cui et al. 2018). Salt stress decreases the unsaturated fatty acid content, and a strong relationship has been observed between salt tolerance and low expression of fatty acid metabolism genes like ω-3 fatty acid desaturase (Sui et al. 2018).

3.3 Heat Stress

During the growth period, peanut crop often encounters high day temperatures for a short or sometimes longer periods. Depending on the growth and developmental stages, heat stress affects the final yield. Pre-anthesis and anthesis stages were identified as the most sensitive stages of peanut for heat stress (Prasad et al. 2001). The temperatures ranging from 25 to 30 °C were identified as optimal temperatures for better pod yield and a temperature exceeding 33 °C substantially decreases the pod yield (Gillooly et al. 2001; Prasad et al. 2003). Peanut genotypes exhibit genotypic variation in heat stress tolerance/susceptibility and pod development (Awal et al. 2003; Craufurd et al. 2003; Prasad et al. 2003; Vu, 2005; Selvaraj et al. 2011).

4 Transgenic Approaches

Traditional crop breeding methods contributed very little toward the peanut crop improvement, due to incompatibility of wild species with cultivated genotypes, minor genetic variability among the available accessions, low recovery of hybrids,

and linkage of detrimental traits (Reddy et al. 1996; Garcia et al. 2006; Halward et al. 1993; Tallury et al. 2005). Marker-assisted breeding methods are also not successful in cultivated peanut due to its tetraploid nature and low genetic variability for important agronomical traits (Varshney et al. 2005; Gantait and Mondal, 2018). Advancement in technologies led to the development of transgenic approaches, which might aid in improving the peanut lines (Kavi Kishor et al. 2018). When compared to the breeding programs, transgenic approaches are quick, and transfer desired traits and characteristics into plants. But, successful genetic transformation depends on three important aspects, i.e., reliable regeneration system, production of more successful transgenic events, and efficient selection systems to avoid false positives.

5 Explants for Regeneration

Peanut tissue is recalcitrant for regeneration in tissue culture (Heatley and Smith 1996). However, several researchers tried to standardize the regeneration protocol using several explants and hormonal media combinations. Initially, several groups used immature leaflets of young seedlings as explants had found that these explants require a long duration of time for organogenesis, and also regeneration efficiency was very low (Mroginski et al. 1981; McKently et al. 1995; Cheng et al. 1992; Sukumar and Rangasamy 1984; Narasimhulu and Reddy 1983; Akasaka et al. 2000; Chengalrayan et al. 2001). Bud primordia and calli failed to regenerate shoots (Mroginski et al. 1981; Cheng et al. 1992; Sukumar and Rangasamy 1984); however, in some instances, only 19% of callus explants were able to produce shoots (Narasimhulu and Reddy 1983). Only 34.7% of shoot buds were converted into shoots, and several abnormalities in shoot development were observed (Akasaka et al. 2000). Several attempts were made to regenerate the shoots through somatic embryogenesis; however, due to low conversion rate of somatic embryos to plants, the efforts to use them as explants in transformation studies were withdrawn (Cucco and Jayme 2000; Gill and Saxena 1992; Zhang et al. 1999; Sellars et al. 1990; Chengalrayan et al. 1994, 1997; Wetzstein and Baker 1993). Finally, Sharma and Anjaiah (2000) were able to regenerate high frequency of shoots from mature cotyledonary explants of several genotypes, and subsequently, in most of the genetic transformation studies, cotyledonary explants were used.

6 Genetic Transformation Methods

Agrobacterium-mediated transformation method was successfully employed to develop transgenic peanut plants. Initially leaves from the young seedlings were used as explants and co-cultivated with *Agrobacterium* to develop transgenic plants; however, the primary transformants exhibited limited fertility (Cheng et al. 1994; Eapen and George 1994). Stable fertile plants were obtained from the leaf sections of New Mexico Valencia A when co-cultivated them with a tobacco leaf extract and

Agrobacterium strain *EHA105* (Cheng et al. 1996, 1997). These studies indicated that *Agrobacterium*-mediated transformation of peanut leaf explants is purely genotype-dependent. This remains the major restraint for the development of successful *Agrobacterium*-mediated transformation. A high-frequency (58%) genotype-independent *Agrobacterium*-mediated transformation was developed using cotyledonary node explants (Venkatachalam et al. 1998). Explants such as cotyledonary nodes, cotyledons, or hypocotyls of VRI-2 and TMV-7 genotypes were co-cultivated with *Agrobacterium* strain LBA4404 and shoot bud regeneration was achieved with antibiotic selection. A high percentage of transformation efficiency was observed in cotyledonary nodes compared to cotyledons or hypocotyls in both the genotypes (Venkatachalam et al. 1998). Cotyledonary node explants were successfully utilized for the transformation in other genotypes like TMV-2 and JL-24 (Venkatachalam et al. 2000; Sharma and Anjaiah 2000) (Fig. 1). Further, high-efficiency (90%) genotype-independent transformation was obtained from de-embryonated cotyledonary (DEC) explants of dry seeds (Tiwari and Tuli 2008). Compared to other explants, DEC explants from dry seeds have an advantage in storage, availability of the seeds throughout the year, and easy handling.

The most widely used genotype-independent DNA delivery method for peanut transformation was microprojectile bombardment. Leaflets from mature zygotic embryos were used as target tissues, and DNA was delivered in this method. Callus was obtained from transformed cells followed by the selection of hygromycin-containing medium (Clemente et al. 1992). Stable transgenic plants were recovered from the embryogenic tissue cultures of immature zygotic embryos (Ozias-Akins et al. 1993). Several genes like *cryIA(c)*, *GUS*, hydrolases, and *TSWVN* were effectively transformed using embryogenic tissue (Singsit et al. 1997; Wang et al. 1998; Chenault et al. 2002; Yang et al. 1998; Chenault and Payton 2003). The only constraint with the embryogenic tissue culture is the availability of immature zygotic embryos. To overcome this shortcoming, Livingstone and Birch (1999) developed a method to produce embryogenic callus from the plumule portions of mature seeds. Transgenics were developed by transforming *TSWVN* gene (Magbanua et al. 2000), oxalate oxidase gene (Livingstone et al. 2005), and several fluorescent reporter gene constructs (Joshi et al. 2005) into an embryogenic callus. The major constraint for the development of transgenics through microprojectile bombardment is the duration of time. From the generation of embryogenic tissue to the production of T_0 transgenic plants, it takes nearly 15–19 months.

To tackle the problems associated with *Agrobacterium*-mediated and microprojectile bombardment methods such as regeneration issues, time duration, and peanut transgenic groups explored for alternative methods. In planta transformation of *Arabidopsis* attracted scientific groups since it is tissue culture-independent, rapid, and a high-throughput method (Azipiroz-Leehan and Feldmann 1997). Taking advantage of this method, Rohini and Rao (2000, 2001) developed a genotype-independent procedure. Wounded apical meristems of differentiated seed embryos were used as target tissues for *Agrobacterium*-mediated transformation. The primary transformants (T_0) are chimeric. If the transgene is integrated into undifferentiated meristematic cells that are destined to develop into branches, seeds obtained

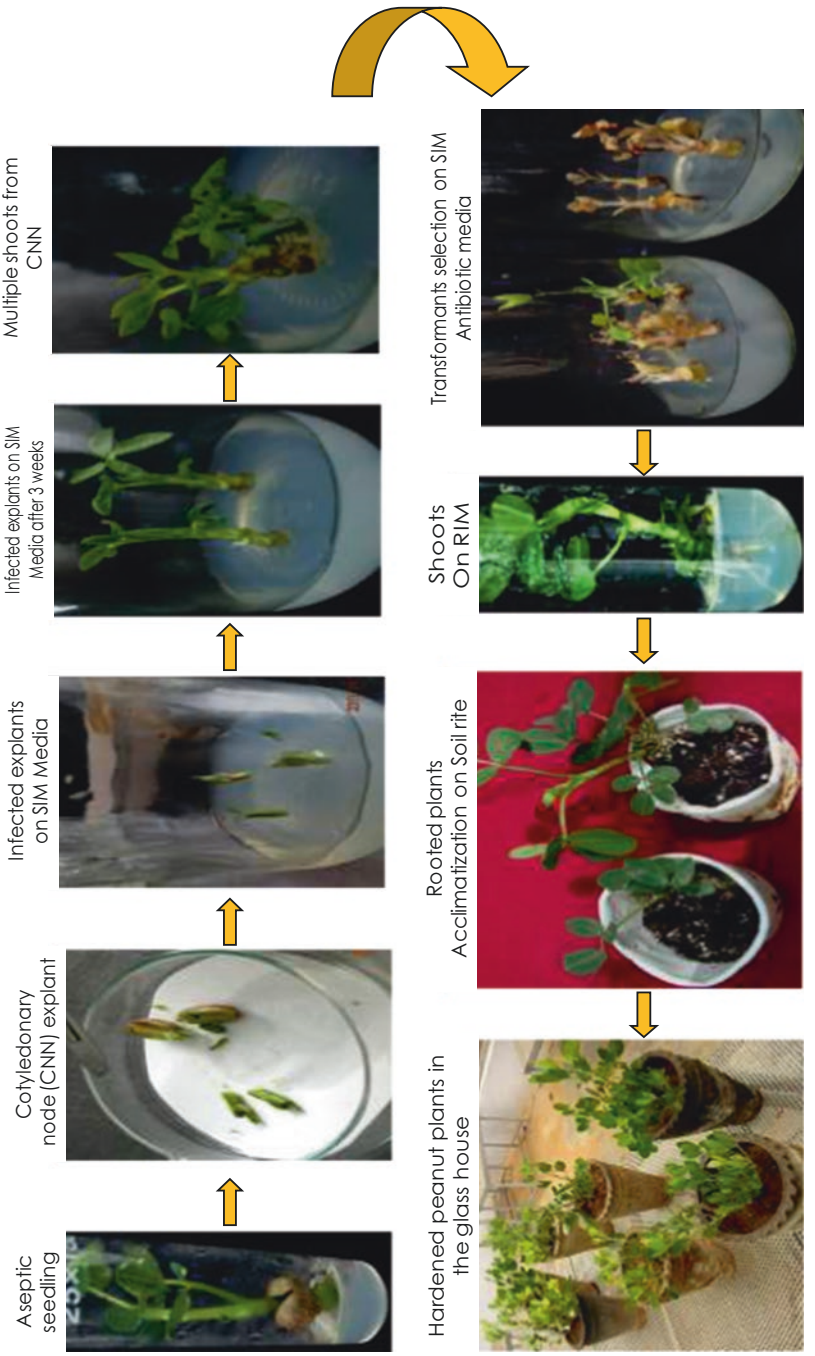

Fig. 1 Various stages involved in the development of transgenic peanut plants through *Agrobacterium*-mediated transformation using cotyledonary node of cv JL-24 as explant (Banavath et al. 2018)

from the reproductive structures of these branches are expected to give stable transformants in T_1. Using in planta transformation protocol, chitinase, chimeric *cry* gene, *cry1X*, and *pea DNA helicase* (*PDH45*) genes were introduced into groundnut, and stable transformants were recovered for fungal disease, insect pest resistance, and drought stress resistance (Rohini and Rao 2000; Entoori et al. 2008; Manjulatha et al. 2014; Pandurangaiah et al. 2014; Kiranmai et al. 2018; Lokesh et al. 2019).

7 Development of Transgenic Plants for Abiotic Stress Tolerance

Abiotic stress tolerance is a multigenic trait and involves changes in many physiological and biochemical processes. Transcriptomes of plant tissues exposed to abiotic stresses were analyzed which revealed both functional and regulatory genes. While functional genes protect the cells from abiotic stress-induced damage, regulatory genes regulate the signal transduction and modulate the expression of functional genes. Transcriptomic analysis of plants revealed that ~7% of plant genome comprises large families of transcription factors such as *AP2/EREBP*, *NAC*, *bZIP*, *MYB*, and *WRKY* (Udvardi et al. 2007; Golldack et al. 2011). Even though several transgenic plants were successfully developed using single functional genes, it is tricky to develop abiotic stress tolerance trait using a single functional gene. Therefore, from the last two decades, scientists have focused on transcription factors, which are master regulators and play a key role in abiotic stress response and tolerance (Bartels and Sunkar 2005; Chinnusamy et al. 2005; Yang et al. 2010; Lata and Prasad 2011) (Table. 1).

7.1 Regulatory Proteins:

The plant hormone ABA plays an important role in regulating several stress-responsive genes. But not all the stress-responsive genes are induced by ABA. The dehydration-responsive element-binding (*DREB*) transcription factors are important regulatory genes in ABA-independent signal transduction cascade, mainly induced by drought, low temperature, and salinity stress. The *DREB* family genes *DREB1* and *DREB2* are induced by cold, drought, and salt stress, interact with dehydration-responsive element/C-repeat element (DRE/CRT) and cis-acting elements in the promoter regions of several stress-responsive genes, and modulate their expression (Liu et al. 1998; Thomashow 1999; Dubouzet et al. 2003; Wang et al. 2008; Lata and Prasad 2011). *DREB* genes like *DREB1* and *DREB2* are initially isolated from *Arabidopsis* by yeast one-hybrid screening (Stockinger et al. 1997; Liu et al. 1998) and used for the development of transgenic plants in several models as well as in crop plants for abiotic stress tolerance under stress-inducible promoter *rd29A* (Nakashima et al. 2009; Kudo et al. 2017, 2019).

Table 1 Peanut transgenic plants developed for abiotic stress tolerance

S. no	Transgene	Transgene origin	Promoter	Variety/ cultivar	Explant tissue	Mode of gene transformation	Evaluation	Abiotic stress tolerance	Physiological change	References
1	*AtDREB1A*	*Arabidopsis*	*rd29A*	JL 24	Cotyledon	*Agrobacterium*-mediated	Green house, field	Drought stress	Superior transpiration efficiency; root traits, antioxidant composition, osmo-protection and higher yields under water limited conditions	Bhatnagar-Mathur et al. (2004, 2007, 2009, 2014), Vadez et al. (2007), Devi et al. (2011)
2	*AtDREB1A*	*Arabidopsis*	*rd29A*	GG20	Cotyledon	*Agrobacterium*-mediated	Glass house	Drought and salinity stresses	Enhanced accumulation of proline content, osmotic potential, RWC, electrolytic leakage, total-chlorophyll content, antioxidants, and gene expression under drought and salt stress	Sarkar et al. (2014, 2016), Bhalani et al. (2019)
3	*AtDREB2A, AtHB7, AtABF3*	*Arabidopsis*	*CaMV35S*	TMV2	Cotyledonary nodes	*Agrobacterium*-mediated	Growth chamber	Drought, salinity, and oxidative stresses	Improved membrane, chlorophyll stability, enhanced ROS, and osmotic adjustment. Several stress-responsive genes under stress	Pruthvi et al. (2014)

4	*DREB1A/CBF3*	*Arabidopsis*	*rd29A*	Smruti	De-embryonated cotyledon	*Agrobacterium tumefaciens*	Green house	Drought stress tolerance	Drought stress tolerance	Rana and Mohanty (2012)
5	*PDH45*	Pea	*CaMV35S*	K-134	Embryo in the mature seed	*Agrobacterium*-mediated in planta	Green house	Drought stress	Stay-green phenotype, increased WUE (low $\Delta^{13}C$) and improved growth rates and productivity	Manjulatha et al. (2014)
6	*Alfin1* *PgHSF4* *PDH45*	Alfalfa *Pennisetum* Pea	*CaMV35S* *rd29A* *2X CaMV35S*	GPBD4	Embryo in the mature seed	*Agrobacterium tumefaciens*	Green house	Drought Stress	Higher growth, productivity, RWC, expression of HSP, RBX, LEA5, PRP2	Ramu et al. (2016)
7	*MuNAC4*	*Macrotyloma uniflorum* (Lam) Verdc.	*CaMV35S*	Narayani	2-day-old groundnut seedlings	*Agrobacterium tumefaciens* in planta	Green house	Drought stress tolerance	Proliferated lateral root growth, reducing the damage to membrane structures and enhancing osmotic adjustment and antioxidative enzyme regulation under stress	Pandurangaiah et al. (2014)
8	*PgeIF4A*	*Pennisetum glaucum*	*rd29A*	JL-24	De-embryonated cotyledon	*Agrobacterium tumefaciens*	Growth chamber	Drought, salinity, and oxidative stress tolerance	Increase in oxidative stress markers and membrane stability	Rao et al. (2017)

(continued)

Table 1 (continued)

S. no	Transgene	Transgene origin	Promoter	Variety/ cultivar	Explant tissue	Mode of gene transformation	Evaluation	Abiotic stress tolerance	Physiological change	References
9	*AtHDG11*	*Arabidopsis*	*rd29A*	JL-24	De-embryonated cotyledon	*Agrobacterium tumefaciens*	Green house	Drought stress tolerance	Enhanced WUE traits, antioxidants, proline, known stress-responsive genes	Banavath et al. (2018)
10	*MuWRKY3*	*Macrotyloma uniflorum* (Lam) Verdc.	*CaMV35S*	K-6	Embryo in the mature seed	*Agrobacterium*-mediated in planta	Green house	Drought stress tolerance	Enhanced expression of stress-responsive genes, antioxidatants	Kiranmai et al. (2018)
11	*AtNHX1*	*Arabidopsis*	*CaMV35S*	Golden and BARI-2000	De-embryonated cotyledon	*Agrobacterium tumefaciens*	Growth chamber	Salt and drought tolerence	Increasing Na^+ accumulation and kept K^+/Na^+ balance, proline accumulation	Asif et al. (2011)
12	*AtNHX1*	*Arabidopsis*	*CaMV35S*	Flavor runner-458	De-embryonated cotyledon	*Agrobacterium tumefaciens*	Green house	Salt tolerence	Higher photosynthetic rates, more biomass accumulation	Banjara et al. (2012)
13	*SbNHXLP*	*Sorghum*	*CaMV35S*	JL-24	De-embryonated cotyledon	*Agrobacterium tumefaciens*	Green house	Salt tolerance	Higher chlorophyll content, superoxide dismutase, and catalase activities, accumulation of proline, and K^+ accompanied by lower Na^+ accumulation	Venkatesh et al. (2019)

14	*AVP1*	*Arabidopsis*	*CaMV35S*	New Mexico Valencia A	De-embryonated cotyledon	*Agrobacterium tumefaciens*	Growth chamber/ field	Drought and salt stress	Enhanced biomass and higher photosynthetic rates	Qin et al. (2013)
15	*SbpAPX*	*Salicornia brachiata*	*CaMV35S*	GG-20	Shoot apices	*Agrobacterium tumefaciens*	Growth chamber	Salt stress tolerance	Growth parameters, chlorophyll content, RWC, and EL	Singh et al. (2014)
16	*SbASR-1*	*Salicornia brachiata*	*CaMV35S*	GG-20	De-embryonated cotyledon	*Agrobacterium tumefaciens*	Growth chamber	Salinity and drought tolerance	Increases the expression of *LEA*, *APX*, *SOD*, and *CAT* gene	Tiwari et al. (2015)
17	***AhRabG3f***	*Arachis hypogaea*	*CaMV35S*	Xuzhou 68-4	–	*Agrobacterium tumefaciens*	Growth chamber	Drought and salinity stress	Expression of several stress-responsive genes	Song et al. (2012)
18	*AhKCS1*	*Arachis hypogaea* L.	*CaMV35S*	K-6	Embryo in the mature seed	*Agrobacterium*-mediated in planta	Green house	Drought stress tolerance	Enhanced epicuticular wax accumulation, reduction in cuticular transpiration, lower membrane damage, high cell membrane stability, and high free proline content	Lokesh et al. (2019)

The first peanut abiotic stress-tolerant transgenic plants were developed using *DREB1A*. The *DREB1A* TF from *Arabidopsis* was cloned under a stress-inducible promoter *rd29A* and was transformed into a drought-sensitive cv. JL-24. Transgenic peanut plants exhibited an array of physiological and biochemical traits under water limited conditions. The *DREB1A* peanut transgenic plants due to their low leaf stomatal conductance maintained higher transpiration efficiency (TE) under well-watered conditions and recorded 40% higher TE under limited moisture conditions compared to wild-type plants (Bhatnagar-Mathur et al. 2007). Under progressive water stress, transgenic plants also recorded higher antioxidants and osmoprotectants compared to their non-transgenic counterparts (Bhatnagar-Mathur et al. 2009; Bhalani et al. 2019). However, these enhanced antioxidants have no role in increased TE (Bhatnagar-Mathur et al. 2009). But, surrogate traits such as chlorophyll meter readings (SCMR) and specific leaf area (SLA) showed a significant positive correlation with enhanced TE, whereas $\Delta^{13}C$ did not show any correlation with TE (Devi et al. 2011). The *DREB1A* transgene also enhanced root length, biomass, and water extraction capacity of transgenic plants compared to wild-type plants under water-limited conditions (Vadez et al. 2013). Under various field drought stress regimes, *DREB1A* transgenic plants produced 24% more yield and higher harvest indices (HI) compared to their wild-type counterparts (Bhatnagar-Mathur et al. 2014). The *DREB1A* peanut transgenics also exhibited tolerance to salinity stress by improving various physio-biochemical traits (Sarkar et al. 2014).

In another study, simultaneous transgenic overexpression of *Arabidopsis* transcription factors *DREB2A,* ABA-responsive element-binding factor 3 (*ABF3*), and Homeodomain leucine zipper-binding family gene (*HB7*) under a constitutive promoter *CaMV35S* in peanut cv. TMV2 improved drought, salt, and oxidative stress tolerance (Pruthvi et al. 2014). The interaction of TF involved in ABA-independent (*DREB2A*) and ABA-dependent (*ABF3*) pathways enhanced the expression levels of several downstream functional genes of both the pathways such as Lea4, *Ring box 1 protein (Rbx1), HSP70, aldehyde reductase, glutaredoxin,* and *proline amino peptidase. Drought-induced protein* and improved physio-biochemical traits like antioxidant activities, osmotic adjustment, cell cycle regulation, and protein turnover contributed to cellular tolerance (CT) under drought and salt stress conditions (Pruthvi et al. 2014). This study suggests that co-expression of multiple genes would be a promising strategy for the developing of transgenic plants for complex traits like abiotic stress tolerance.

Similarly, a multigene cassette consisting of three regulatory genes *Alfin1* (Alfaalfa zinc finger 1), *PgHSF4* (*Pennisetum glaucum* heat-shock factor 4), and *PDH45* (Pea DNA helicase 45) was simultaneously mobilized, and transgenic peanut plants exhibited drought-tolerant traits. The Alfin1, a plant-specific transcription factor belongs to the plant homeodomain (PHD) subfamily, which involves root growth and development, nitrogen metabolism, and salt stress tolerance (Nethra 2010; Winicov 2000). Plant heat shock factors (*HSF*) are key players in plant abiotic stress signal transduction pathways and regulate the expression of several stress-responsive genes especially heat shock proteins (HSP), involved in protein turnover

and protection and enhanced plant abiotic stress tolerance (Guo et al. 2016). Peanut transgenic plants developed with these multigene cassette, maintained greater RWC, profuse root growth and greater seedling survival rates under drought stress conditions. Overexpression of multigene TF enhanced the expression levels of several stress-responsive genes such as *HSP*s, *LEA*s, RING box proteins, aldose reductase, and proline-rich protein (Ramu et al. 2015).

The *NAC* family members are large plant-specific transcription factors and consists of *NAM* (no apical meristem), *ATAF* (*Arabidopsis* transcription activator factor), and *CUC* (cup-shaped cotyledon) with DNA-binding domains (Nakashima et al. 2012; Shao et al. 2015; Alshareef et al. 2019). To date, a large number of *NAC* family genes have been identified from plants, and these TFs play key roles in various biological processes such as the development of shoot, flower, cell division, the formation of secondary cell walls, and tolerance to biotic and abiotic stresses (Sablowski and Meyerowitz 1998; Takada et al. 2001; Olsen et al. 2005; Kim et al. 2006; Zhong et al. 2010; Christianson et al. 2010; Tran et al. 2010; Breeze et al. 2011; Nakashima et al. 2012; Shao et al. 2015). *MuNAC4* (*Macrotyloma uniflorum*; *MuNAC4*) is a member of the *NAC4* family and abiotic stress-responsive TF. It was cloned and introduced into peanut cv. Narayani under the influence of a constitutive promoter (*CaMV35*S). Stable transgenic plants were subjected to long-term drought stress. Transgenics performed better compared to wild-type plants under drought stress conditions with an improved shoot and root growth, antioxidative capacity, RWC, chlorophyll stability, and enhanced sugar and osmolyte levels. Overall, *MuNAC4* transgenic plants exhibited improved drought tolerance (Pandurangaiah et al. 2014). In another study, heterologus overexpression of *AtNAC2* in peanut improved final yield, enhanced drought, and salt stress tolerance (Patil et al. 2014).

The *WRKY* domain-binding TF are plant-specific, involved in various biological processes such as development of trichome, root, seed, leaf senescence, and abiotic stress tolerance (Miao and Zentgraf 2007; Jiang et al. 2012; Grunewald et al. 2013; Schluttenhofer et al. 2014; Ding et al. 2014; Bakshi and Oelmuller 2014; Rinerson et al. 2015). Several transgenic plants were developed by overexpressing *WRKY TF*, with improved abiotic stress tolerance (Wu et al. 2009; Niu et al. 2012; Yan et al. 2014; Chu et al. 2015; Zhou et al. 2015; Qin et al. 2015). The horsegram *MuWRKY3 (Macrotyloma uniflorum*; *MuWRKY3*) was cloned under the constitutive promoter CaMV35S and mobilized into a peanut. Drought stress was imposed on the transgenic and wild-type plants by withholding water. The performance of the transgenic peanut plants was assessed by measuring the morpho-physiological traits. Transgenic *WRKY3* peanuts displayed better growth rates and antioxidative enzyme activities, and several stress-responsive genes such as *CAT*, *SOD*, *APX*, *MIPS*, *LEA*, and *HSP*s were not activated (Kiranmai et al. 2018).

Development of peanut plants with drought tolerance and WUE is highly desirous as they not only improve drought tolerance but also enhance the yield. To achieve this goal, our group transformed an *Arabidopsis HDG-11* gene, *HD-START* family transcription factor gene into a peanut. Previous studies suggest that constitutive overexpression of *AtHDG11* improves drought and salt tolerance in several

plant species (Cao et al. 2009; Ruan et al., 2012; Yu et al. 2013; Li et al. 2016; Zhu et al. 2016). Similarly, transgenic overexpression of *HDG11* under a stress-inducible promoter *rd29A* into peanut cv. JL-24, improved WUE and drought and salt tolerance. Transgenic peanut plants displayed improved root lengths and lateral roots to extract water from deeper layers of the soil, reduced number of stomata which can minimize the water loss, improved photosynthetic traits, enhanced production levels of osmolytes, higher expression of ROS detoxification genes, and protein protection genes for cellular tolerance. This study demonstrated the successful development of WUE and drought and salt tolerance in transgenic plants containing the *HDG-11* gene (Banavath et al. 2018).

DEAD- box RNA helicase, a transcriptional activator, improved the cellular tolerance and drought and salinity tolerance of peanut (Manjulatha et al. 2014; Ramu et al. 2015; Rao et al. 2017). DNA helicases belong to the *DEAD* box family of proteins and like *eIF4A* involve in translation initiation and regulation process (Tuteja et al. 2008). Numerous studies reveal that DNA and RNA helicases such as *Arabidopsis LOS4*, pea *PDH47*, halophyte *Apocynum venetum AVDH1*, rice *BIRH1*, and pea *PDH45* play an important role in abiotic stress tolerance (Gong et al. 2002, 2005; Nakamura et al. 2004; Sanan-Mishra et al. 2005; Vashisht et al. 2005; Liu et al. 2008; Li et al. 2008a, b; Tuteja et al. 2008). The pea DNA helicase 45 (*PDH45*) was cloned under a constitutive promoter *CaMV35S* and transferred into a peanut cv. K-134 through in planta *Agrobacterium*-mediated transformation. Transgenic plants were assessed for their drought stress tolerance using PEG-mediated water stress as well as moderate drought stress by withholding water in a pot experiment. Transgenic peanut plants exhibited several drought-adaptive traits such as water conservation, WUE, water extraction capacity from the soil, osmotic adjustment, anti-oxidative stress tolerance, protein protection, and cell cycle regulation. This study reveals that *PDH45* acts as a multifunctional protein, under stress conditions, and acts as both helicase and topoisomerase and helps to protect the CT levels of peanut under drought stress (Manjulatha et al. 2014). Similarly, stress-inducible overexpression of a DEAD box RNA helicase family protein *PgeIF4A* (*Pennisetum glaucum* eukaryotic translational initiation factor 4A) improved drought, salinity, and oxidative stress tolerance in peanut transgenic plants (Rao et al. 2017). G proteins are a very important signaling molecule in the signal transduction pathway. The small G protein family contains several families such as Rab, Rho, Arf, and Ran homologs; however, plants do not have Ras family. In plants, small GTP proteins have several biological roles, involve in plant hormone signal cross talk, various biotic and abiotic stress responses, defense response, and growth and development of roots and shoots (Ma 2007). Environmental stresses such as drought trigger the production of Rab and Ran family proteins. Drought stress induces the expression levels of Rab7 (PgRab7) protein in *Pennisetum glacum* (Choudhary and Padaria 2015). Transgenic overexpression of abiotic stress-induced *Arachis hypogaea* RabG3f (*AhRabG3f*) gene improved the peanut plants to both drought and salt stresses (Song et al., 2012).

7.2 Plant Hormone Biosynthesis Genes

Besides overexpression of genes related to transcription factor or activator, genes involved in signal transduction pathways or hormone regulation have also been transferred into a peanut. The enzyme isopentenyltransferase (*IPT*) is a key player in the biosynthesis of plant hormone cytokinin as a rate-limiting enzyme. Cytokinin involves in organogenesis and meristem maintenance. Besides these activities under abiotic stress conditions, cytokinins also regulate the water balance of the cells, trigger the production of antioxidative enzymes, modulate several plant hormone levels, and protect the cells from stress-induced damage (Pavlů et al. 2018). The *IPT* gene was isolated from *Agrobacterium tumefaciens* and cloned under a senescence-associated gene promoter (*PSAG12*). The construct was transferred into tobacco to generate transgenic plants. Tobacco transgenic plants exhibited drought tolerance with enhanced cytokinin levels (Rivero et al. 2007, 2009, 2010). The *PSARK*:: *IPT* construct was mobilized through *Agrobacterium*-mediated transformation into peanut cv. New Mexico Valencia A. The *IPT* overexpressed transgenic peanut plants, recorded higher photosynthetic rates and stomatal conductance, and produced significantly higher biomass under reduced irrigation conditions in greenhouse and field conditions. Transgenic *IPK* plants produced much larger root systems under reduced irrigation in greenhouse conditions, which allowed them to use water more efficiently and produced 30–35% higher yields than that of wild-type plants based on 2 years of field data (Qin et al. 2011).

7.3 Antioxidative Enzyme Genes

Abscisic acid stress ripening-1 (*ASR-1*) is a plant-specific, nucleus-localized, biotic, abiotic, and hormone-induced protein and acts as a transcription factor. Amino acid analysis suggests that *ASR-1* protein functions as group-7 *LEA* protein. In vitro studies of *ASR-1* protein suggests that it has antioxidant property (Kim et al. 2012). Transgenic overexpression of *ASR-1* improved drought, salt, and oxidative stress tolerance (Jha et al. 2012; Hu et al. 2013; Liu et al. 2013). Transgenic overexpression of *SbASR-1* (*Salicornia brachiata ASR-1*) in peanut improved chlorophyll, RWC, proline, sugars, starch accumulation, and lower electrolyte leakage compared to wild-type plants. Transgenic plants displayed elevated levels of antioxidative transcripts such as *APX*, *SOD*, and *CAT* (Tiwari et al. 2015).

Abiotic stresses trigger the production of reactive oxygen species (ROS), and they damage various biological molecules by oxidation and degrade the biological membranes and enzymes (Janků et al. 2019). To combat ROS-induced damage, plant cells produce various enzymatic and non-enzymatic antioxidative machinery (Gill and Tuteja 2010). The enzymatic machinery includes SOD (superoxide dismutase), CAT (catalase), APX (ascorbate peroxidise), GR (glutathione reductase), MDHAR (monodehydroascorbate reductase), DHAR (dehydroascorbate reductase), and GST (glutathione-*S*-transferase). Among them, *APX* is an important player to detoxify H_2O_2, located in various cell compartments, i.e., cytoplasmic,

mitochondria, chloroplast, and peroxisomes (Singh et al. 2014). Numerous studies suggest that overexpression of cytosolic *APX*s and chloroplastic *APX* significantly enhanced the oxidative stress tolerance (Davletova et al. 2005; Yoshimura et al. 2000, Yabuta et al. 2002). Transgenic overexpression of peroxisomal *APX* improved drought and salt tolerance in tobacco (Li et al. 2009; Singh et al. 2014). Similarly, transgenic overexpression of *Salicornia brachiata* peroxisomal ascorbate peroxidase (*SbpPAX*) in peanut improved chlorophyll content, RWC, and tolerance to salt stress (Singh et al. 2014).

7.4 Wax Biosynthesis Genes

To cope up with abiotic stresses, plants develop several adaptive traits such as the development of deep root system, leaf modifications, and cuticular wax deposition on leaf surfaces (Samuels et al. 2008; Lee and Suh 2015). Among them, deposition of epicuticular wax is an important adaptive trait for drought tolerance in several plant species (Sanchez et al. 2001; Samdur et al. 2003; Burow et al. 2008). Waxes are very-long-chain fatty acids (C16–C18 to C32–C40) synthesized in the endoplasmic reticulum by a series of enzymes such as ß-ketoacyl Co-A synthase (*KCS*), ß-ketoacyl Co-A reductase (*KCR*), hydroxyacyl Co-A dehydratase (*HCD*), and eonyl Co-A reductase (*ECR*), excreted, and deposited on the leaves and stems in the form of crystals (Samuels et al. 2008; Kunst and Samuels 2009; Haslam and Kunst 2013; Lee and Suh 2015). *AhKCS1* gene from a drought-tolerant peanut genotype K9 was cloned under a constitutive promoter *CaMV35S* and introduced into a drought-sensitive peanut genotype K6. Transgenic plants deposited a greater amount of wax content on leaves. Under drought stress conditions, *AhKCS1* transgenic plants performed extremely well by minimizing the water loss, enhanced the levels of antioxidative enzymes and osmolytes, and improved drought stress tolerance (Lokesh et al. 2019).

7.5 Transporter Genes

Soil salinity alters the ratios of K^+/Na^+ and increases the accumulation of Na^+ and Cl^-, which further damages the plant cells. However, salt-tolerant plants maintain high ratios of K^+/Na^+ by discharging out the Na^+ from the cell or assortment of Na^+ in the vacuoles (Yamaguchi and Blumwald 2005). The plasma membrane antiporter (*NHX*) genes plays a key role in sequestration and extrusion of Na^+ in the cells, and it is very important for salt tolerance of plants, because sequestration of Na^+ ions into vacuole increases osmotic pressure of the cells, decreases the toxic effect of Na^+ ions, and protects the cells (Sottosanto et al. 2004). Initially, *NHX* gene was isolated from *Arabidopsis* and, later on from several plants and transgenic overexpression of it, led to the development of salt tolerance in several plant species (Zhang and Blumwald 2001; Zhang et al. 2001, 2015; Ohta et al. 2002; Chen et al. 2007, 2008; Yin et al. 2004; Xue et al. 2004; Tian et al. 2006; Xu et al. 2009;

Soliman et al. 2009; Leidi et al. 2010; Mishra et al. 2014; Yarra and Kirti 2019; Mushke et al. 2019). Transgenic overexpression of *AtNHX* in peanut under a constitutive promoter improved the Na^+ in the leaves, enhanced biomass production, greater chlorophyll contents, and photosynthetic rates, increased the production of proline levels, and improved drought and salt tolerance in comparison with wild-type peanut (Asif et al. 2011; Banjara et al. 2012). In a recent study, transgenic overexpression of sorghum *NHX* like protein (*SbNHXLP*) under a constitutive promoter in JL-24 improved the biochemical and yield traits such as antioxidative capacity, osmotic potential, lower levels of Na^+ accumulation and improved biomass and yield contents in transgenic plants compared to non-transgenic plants under salt stress conditions (Venkatesh et al. 2019).

The vacuolar osmotic pressure can also be improved by increasing the proton gradient on the vacuolar membrane. One of the genes identified to increase the proton gradient was vacuolar H^+-ATPase, an H^+ pump gene (Gaxiola et al. 2001). Transgenic overexpression of the *Arabidopsis* vacuolar H^+-ATPase (*AVP1*) improved drought and salt tolerance by improving the osmotic pressure, reducing the Na^+ toxicity and enhancing root development in several plant species (Gaxiola et al. 2001; Park et al. 2005; Li et al. 2005; Zhao et al. 2006; Gao et al. 2006; Lv et al. 2008; Li et al. 2008a, b; Pasapula et al. 2011). Constitutive overexpression of *Arabidopsis AVP1* gene in peanut plants improved growth, photosynthetic rates, yield, and drought and salt tolerance (Qin et al. 2013).

8 Challenges and Prospects

Transgenic studies for abiotic stress tolerance in peanut suggest that peanut genome can be efficiently engineered for multiple abiotic stresses with genes of signal transduction pathway and transcription factors. However, the pyramiding of multiple genes would be a better strategy with the advantage of GATEWAY technology. As most of the transgenic plants are evaluated only under controlled environmental conditions, the major concern is the performance of transgenics under field conditions, because, in the field, the plants have to cope up with multiple abiotic and biotic stresses. Moreover, the cumbersome regulatory procedures and subsequent release of transgenics for growing transgenics in the field take many years. Further, environmental issues/concerns need to be addressed before releasing the transgenics for commercial purposes. Genome-editing technologies such as CRISPR/Cas9 form an attractive and viable alternative that can address the difficulties associated with the conventional breeding (time-consuming) as well as the transgenic (unacceptability of the GMOs) approach. Genome editing or genome engineering using site-specific nucleases (SSN) to precisely target the desired region(s) of the genome to insert/delete (INDELS) and substitute nucleotides in both plants and animals (Jinek et al. 2012). These mutations can be used to develop non-genetically modified plants with improved traits such as enhanced yield under biotic and abiotic stress conditions (Jaganathan et al. 2018). However, due to the

complex nature of the abiotic stress, so far only a few genome-editing studies have been carried out in crop plants, and studies are yet to be initiated in peanut.

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Genetic Engineering of Sunflower (*Helianthus annuus* L.) for Important Agronomic Traits

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Abstract

Sunflower assumes importance both as an oilseed crop and for confectionery purpose, and the breeding programmes for both these types run in parallel. Genetic improvement programmes are mostly based on traditional breeding and introgressive breeding approaches. Sunflower is one of the crops that has been greatly benefitted through interspecific hybridization for several traits such as cytoplasmic male sterility, seed quality traits, and resistance to biotic and abiotic stresses. However, continuous cultivation of the crop, changing climatic conditions, emerging new diseases, evolution of new races of the pathogens, and changing pest scenario demand intensive efforts at genetic upgradation of the crop. Despite the existence of ~50 *Helianthus* species of varying plant habits (annual, perennial) and ploidy levels (diploid, tetraploid, hexaploid), introgression for some of the agronomically desirable traits into cultivar germplasm is hampered by either lack of resistance in the wild species or the ploidy differences and the associated chromosomal imbalances leading to sterility. Hence, intervention of biotechnological tools is required for introgression of beneficial traits into cultivated sunflower. Application of genetic engineering tools in sunflower is limited by several problems associated with tissue culture-based shoot regeneration. Nevertheless, attempts have been made to establish the protocols for genetic transformation predominantly through *Agrobacterium*-mediated methods using explants with pre-existing meristems as target tissues. Most of the published reports (~75%) are on optimization of variables for obtaining a high frequency of transient gene expression while the studies on introgression of desirable attri-

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butes are mainly focused at enhancing resistance to biotic and abiotic stresses. Field testing of the transgenic events and intellectual property protection of the protocols is essentially from the private firms. This chapter provides an overview of the challenges associated with the development of tissue culture and transformation protocols and the present status of transgenic development in sunflower.

Keywords

Abiotic stresses · *Agrobacterium tumefaciens*-mediated transformation · Biotic stresses · Genetic engineering · Interspecific hybridization · Transgenics

1 Introduction

Sunflower (*Helianthus annuus* L.), an ornamental crop species is mainly valued for its seed oil and subsequently has gained attention for its use for confectionery purposes as well. Globally, the crop with an area of 26.5 Mha, productivity of 1803 kg/ha, and production of 47.9 million tons stands fourth after soybean, rapeseed, and groundnut (FAOSTAT 2017). Ukraine and Russian Federation with a production of 12.2 million tons and 10.5 tons, respectively, are the leading sunflower-producing countries (FAOSTAT 2017). Sunflower is used for a variety of purposes including ornamental, vegetable oil, confectionery, animal feed, cosmetics, and pharmaceuticals but primarily for cooking purpose. The seed contains about 40–46% edible oil and contains large amounts of vitamin E. Traditional sunflower oil is composed of saturated C16:0 palmitic (7%) and C18:0 stearic (4%), monounsaturated C18:1 oleic (20%), and polyunsaturated C18:2 linoleic (69%) fatty acids. Sunflower oil is considered as premium quality compared to other vegetable oils because of high level of polyunsaturated linoleic acid, which can reduce the risk of cardiovascular disease. In addition, the oil contains tocopherols, which are the source of antioxidants that provide oxidative stability to the oil (Seiler and Jan 2010).

2 Major Breeding Objectives and Accomplishments

Breeding objectives in sunflower include high seed yield, early maturity, and plants with short stature, resistance to insect pests, diseases, herbicides, improved oil quality, protein content, and quality. In oilseed sunflowers, high oil content is desired while in confectionery sunflower, large seed size, a high kernel-to-hull ratio, uniform seed shape, size, and color assume importance. Sunflower is attacked by several biotic and abiotic sources during different crop growth stages causing economic yield losses, and breeding for yield traits coupled with resistance to these stresses is a constant challenge for the breeders. Sunflower is one crop where interspecific hybridization played a key role in genetic improvement of cultivated sunflower as wild *Helianthus* species represent a rich repertoire of genes for conferring resistance to major diseases like rust, downy mildew, wilt, powdery mildew, *Sclerotinia*

rot, and *Alternariaster* leaf spot; insect pests like head moth, stem weevil, and sunflower beetle; abiotic stresses like drought and salinity; herbicide tolerance, resistance to broomrape, and have desired genetic variability for oil content and fatty acid composition (Thompson et al. 1981; Seiler 1992; Kolkman et al. 2004). Besides, *Helianthus* species continue to serve as a source for novel cytoplasmic male sterile (CMS) sources and fertility restoration genes for exploitation in hybrid breeding programmes (Horn 2006). However, utilization of wild species is often hampered by genomic incompatibility, genetic distances, structural heterozygosity, ploidy differences, and cytoplasmic differences leading to F_1 hybrid sterility and the linkage drag.

The advent of molecular biology and genetic engineering tools have facilitated rapid development of genotypes that can tolerate biotic and biotic stresses besides widening the genetic base to several other agronomically desirable traits including seed traits in a more effective manner. Genetic improvement of sunflower through the use of biotechnological tools requires a reliable, efficient, and reproducible in vitro shoot regeneration and transformation methods. The need for genetic transformation in sunflower has been realized, and protocols for both tissue culture and genetic transformation were undertaken simultaneously in the early 1980s and the progress made during the past three and half decades is reviewed.

3 Sunflower Tissue Culture

Sunflower is considered to be refractory to in vitro manipulations, and hence, most of the initial regeneration studies were with shoot tips and explants with pre-existing meristems. The factors that favored caulogenesis included the genotype, media, culture conditions, tissue type, and age of the tissue. Somaclonal variations are reported to be of common occurrence even in shoots derived from meristematic tissues (Pugliesi et al. 1991, 2000), and hence, the in vitro culture should be very short. Regeneration from somatic tissues either through organogenesis or somatic embryogenesis has become necessary owing to the problem of chimeras in regenerants derived from meristematic tissues. Subsequently, plant regeneration from several tissues such as cotyledons derived from mature seeds, seedling cotyledons, cultured hypocotyls and thin cell layers of hypocotyls, cotyledonary petioles, leaf tissues, protoplasts, and immature embryos were attempted (Pugliesi et al. 1991; Ceriani et al. 1992; Nestares et al. 1996; Sujatha et al. 2012). These studies report regenerations through direct and callus-mediated shoot organogenesis (Lupi et al. 1987; Power 1987; Mc-Cann Wilcox et al. 1988; Witrzens et al. 1988; Espinasse et al. 1989; Knittel et al. 1991; Pugliesi et al. 1991; Ceriani et al. 1992; Chraibi et al. 1992). However, in most of the cases, regeneration was direct without an intervening callus phase. The factors controlling organogenesis included genotype, explant type, age, and physiological state of the cultured tissue, balance of exogenous and endogenous growth regulators, etc. Regardless of the organogenic pathway, regeneration was mainly dependent on the genotype. In the studies where different genotypes were tested for organogenic competence, only few genotypes were found to

be prolific (Everett et al. 1987; Sujatha et al. 2012). Among the different genotypes, hybrids including interspecific hybrids and wild *Helianthus* species are reported to possess superior ability to shoot regeneration and transformability (Binsfeld et al. 1999; Pugliesi et al. 1993; Weber et al. 2000; Sujatha et al. 2012).

Tissue culture-based regeneration protocols described so far are beset with problems of genotype dependence and low rate of plant regeneration and poor reproducibility (Greco et al. 1984; Paterson and Everett 1985; Power 1987; Witrzens et al. 1988; Espinasse and Lay 1989; Pelissier et al. 1990; Chraibi et al. 1992; Sarrafi et al. 1996). Regardless of the explant type, precocious flowering was a major issue (Finer 1987; Lupi et al. 1987; Power 1987; Mc-Cann Wilcox et al. 1988; Witrzens et al. 1988; Knittel et al. 1991; Ceriani et al. 1992; Alibert et al. 1994; Nestares et al. 1996; Baker et al. 1999) which could to a certain extent be circumvented with the use of 2-iP (Sujatha et al. 2012). Further, vitrification and poor rooting (Burrus et al. 1991; Knittel et al. 1991; Ceriani et al. 1992; Nestares et al. 1996; Baker et al. 1999; Mayor et al. 2003; Abdoli et al. 2007), lengthy time in culture, abnormal morphogenesis (Freyssinet and Freyssinet 1988), proliferation of callus along with shoot bud induction which inhibits shoot development and rooting (Knittel et al. 1991; Ceriani et al. 1992), etc. are some problems encountered in shoot cultures. Shoot regeneration is reported to be stimulated through the use of ethylene inhibitors like cobaltous chloride ($CoCl_2$) and silver nitrate ($AgNO_3$) molecules (Chraibi et al. 1992). Recently, refinements for obtaining good quality shoots were made through preconditioning of explants. Zhang and Finer (2015) developed adventitious shoots from primary leaves of 7-day-old sunflower seedlings, and the highest number of developed shoots was obtained from leaves preconditioned with 5 mg/L BAP, with 1.9% developed shoots per explant. Studies of Zhang and Finer (2016) demonstrated the need for short pulse treatments and micrografting techniques for the development of shoots. Short pulse treatments (4 days and 8 days) on medium containing 1.5 mg/L 6-benzylaminopurine and 0.2 mg/L 1-naphthaleneacetic acid followed by culture on elongation medium containing 0.1 mg/L gibberellic acid generated well-developed shoots that were more responsive to micrografting.

Somatic embryogenesis from immature zygotic embryos, mature seeds, and seedling tissues is also reported (Finer 1987; Mc-Cann Wilcox et al. 1988; Espinasse and Lay 1989; Pelissier et al. 1990; Jeannin and Hahne 1991; Fiore et al. 1997; Sujatha and Prabakaran 2001). Immature zygotic embryos (IZEs) were the explants of choice due to their regeneration response either through organogenesis or somatic embryogenesis, use of same cytokinin (BAP) for both regenerative pathways, high frequency of regeneration with good repeatability. The only decisive factor is the concentration of sucrose wherein low concentrations of sucrose-induced organogenic response while high concentrations (>12%) evoked embryogenic route for regeneration (Chandler and Beard 1983; Finer 1987; Charriere and Hahne 1998; Sujatha and Prabakaran 2001). Pretreatments that enhanced embryogenesis include pretreatment in darkness and etiolated seedlings responded well to somatic embryogenesis (Carola Fiore et al. 1997).

Shoot regeneration is reported to be mostly consistent and reproducible with immature embryos and cotyledons from mature seeds. Despite the repeatability

with IZEs, it is laborious to isolate the embryos from fertilized ovules and time consuming, there is possibility of getting the same stage, and it requires continuous source of flowering plants. Regeneration from cotyledons derived from mature seeds is highly repeatable, and the age of seedlings from which the cotyledons is derived was found to be critical. Best results were obtained from split cotyledons from mature seeds (Sujatha et al. 2012), 2-day-old seedlings (Chraibi et al. 1992), and 4- to 5-day-old seedlings (Knittel et al. 1991). Nevertheless, cotyledons from mature seeds constitute an excellent and convenient choice of explant for routine plant regeneration of sunflower plants due to their year-round availability, ease of cultivability and applicability to a wide range of genotypes (Baker et al. 1999). Regeneration through both direct organogenesis and somatic embryogenesis is reported from mature seed cotyledons. This is probably due to adequate metabolite levels in the cotyledons to support the processes of seed germination and stimulate organogenetic potential (Pugliesi et al. 1991).

4 Genetic Transformation

Sunflower is reported to be naturally susceptible to *Agrobacterium tumefaciens* infection, and hence, transient expression is observed at a high frequency in most of the transformation experiments. This evoked interest for designing transformation experiments for the transfer of desirable genes in sunflower. A genotype-independent regeneration system is the bottleneck through which transgenics in sunflower could be produced. Owing to the difficulties in shoot regeneration through adventitious organogenesis, most of the transformation experiments have relied on the use of shoot apices and cotyledonary nodes. However, the efficiency of stable integration was rather low besides leading to chimeras (Schrammeijer et al. 1990; Bidney et al. 1992; Knittel et al. 1994; Malone-Schoneberg et al. 1994; Grayburn and Vick 1995; Laparra et al. 1995; Burrus et al. 1996; Alibert et al. 1999; Rao and Rohini 1999; Muller et al. 2001; Weber et al. 2003).

Transgenic sunflowers were produced from explants such as mature embryos (Bidney et al. 1992; Alibert et al. 1999; Rao and Rohini 1999), immature embryos (Lucas et al. 2000; Muller et al. 2001), cotyledons from mature seeds (Sujatha et al. 2012), shoot apices (Weber et al. 2003; Bidney et al. 1992; Knittel et al. 1994; Grayburn and Vick 1995; Schrammeijer et al. 1990), and direct transfer of transgenes to protoplasts (Moyne et al. 1989; Kirches et al. 1991). Although stable transformation occurred, involvement of pre-existing meristems invariably resulted in chimeric shoots. Use of mature and immature embryos as target tissues for transformation resulted in stable transformation but the efficiency of conversion of transient expression to stable integration was rather low. Regeneration of viable shoots from the transformed cells/calli was another major limiting factor for the overall efficiency of the transformation system (Moyne et al. 1989; Burrus et al. 1996). Along with the lack of an efficient adventitious shoot regeneration system, other limitations include low *Agrobacterium* virulence, low transformation rates, lack of a stringent selection system for transformants, unusual sensitivity to antibiotics, genotype

dependence of regeneration efficiency, and lack of stable transmission of the introduced gene.

Effective delivery of *Agrobacterium* to regenerable plant tissue is essential which is possible by adopting different wounding procedures. Wounding of plant tissues leads to release of phenolic compounds and monosaccharides which in turn trigger the *vir* gene induction resulting in enhanced expression. Attempts were made to enhance the transformation efficiency by subjecting the explants to various treatments such as mechanical wounding using glass beads (Grayburn and Vick 1995; Alibert et al. 1999), sonication (50 MHz) (Weber et al. 2003), and vacuum infiltration (Hewezi et al. 2003). Enhanced transformability with *Agrobacterium* was recorded with use of macrowounds by shaking with glass beads and microwounds generated by particle bombardment (Bidney et al. 1992; Grayburn and Vick 1995). Mechanical wounding procedures often lead to tissue damage and disruption affecting regeneration and reduced recovery of transformed shoots. Digestion of target tissues prior to transformation with macerating enzymes has been tried as these enzymes not only increase the area where the bacterium attaches to the cells but also results in release of compounds for inducing the *Agrobacterium vir* genes (Alibert et al. 1999; Weber et al. 2003; Ikeda et al. 2005). Weber et al. (2003) evaluated the expression of the reporter genes (*gus, gfp*) in shoot apices transformed through *A. tumefaciens* following wounding with macerating enzymes (cellulose, pectinase, macerozyme) and sonication (50 MHz) individually and in combination. While the effect of sonication was not found to be positive, treatment with enzymes (0.1% cellulose, 0.05% pectinase) singly enhanced the transformation efficiency. Incubation with acetosyringone (Laparra et al. 1995), combination of different treatments (Alibert et al. 1999) or transformation protocols (Bidney et al. 1992; Knittel et al. 1994; Malone-Schoneberg et al. 1994; Lucas et al. 2000; Muller et al. 2001; Molinier et al. 2002; Sawahel and Hagran 2006), co-transformation with cytokinin synthesis (*ipt*) gene (Molinier et al. 2002), and dehydration and rehydration of target tissues (Hewezi et al. 2002) were also tried to enhance the transformation efficiency. Most of these studies used transient expression assays (Alibert et al. 1999) or were restricted to characterization of the primary transformants (Hewezi et al. 2002; Weber et al. 2003).

Despite the reports on different transformation methods in sunflower, the transformation protocol of choice is mostly through *Agrobacterium*-mediated transfer of genes. Transient expression of the introduced gene is observed in the tissues subjected to different transformation methods, but stable transformation is observed only in tissues transformed with *Agrobacterium* (Laparra et al. 1995). The use of low inoculum (approximately 6×10^2 bacteria cells/mL) with long co-culture (LI/LC) period (15 days) led to large increases in sunflower transformation efficiency (Zhang and Finer 2016). Bacterial strain influences the infectivity and transformation. Transformation experiments of Benzle et al. (2015) using *Agrobacterium* strains that were isolated from galls and rhizospheric soil along with known wild-type and disarmed strains showed that hypocotyl tissues are very responsive with the highest transformation rates obtained with the widely used EHA 105 strain. While most of the articles provide the key steps followed for *Agrobacterium*-mediated

transformation in sunflower, the reader intending to start transformation experiments can refer the stepwise protocols with relevant notes described by Lewi et al. (2006) and Radonic et al. (2015). Manavella and Chan (2009) described protocols for the transformation of leaf discs via *Agrobacterium*-mediated method for providing valuable insights of several biological processes through functional validation of genes. The procedure described is simple, rapid, and molecular information can be obtained within a week, and it also precludes the need for use of heterologous systems for functional validation where the molecular events are not well conserved.

For the selection of putative transformants, hygromycin (*hpt*), kanamycin (*npt*II), and basta (*bar*) were conveniently used depending on the construct, crop, and target tissue. However, in case of sunflower, kanamycin is used widely as the plant selection agent (Table 1). Although it is found to have an inhibitory effect on plant regeneration and recovery of putatively transformed shoots, the ease of identification of green shoots (putatively transformed) from bleached shoots (untransformed) made it a preferred choice for plant selection. False transformants is a common problem in shoots selected on kanamycin, necessitating stringent selection using high concentration of the antibiotic or more selection cycles (Sujatha et al. 2012). Use of *bar* gene for selection avoided false transformants in T_0 generation and also premature flowering (Neskorodov et al. 2010; Escandon and Hahne 1991).

Premature flowering is one of the major concerns associated with tissue culture and transformation in sunflower leading to small-sized capitula with few disc florets, poor pollen production, and non-recovery of functional seed. Several manipulations like nutrient composition of media, low temperatures, reduced day length, and exogenous growth regulators have been suggested for overcoming this difficulty in sunflower. The problem is worth addressing in genetic transformation experiments as the time in culture is prolonged due to the selection cycles. Selection of transformants should be stringent, and the number of selection cycles should be kept to the minimum possible so as to avoid in vitro precocious flowering. Further, this bottleneck has been overcome to some extent by incorporating cytokinins like 2-isopentenyl adenine (2-iP) and kinetin and incubating the tissues at a temperature of 20 °C with 8/16 h light/dark photoperiod cycle (discussed in Sujatha et al. 2012) or grafting of in vitro recovered putative transformants on to healthy root stocks (Weber et al. 2003). Although T_0 plants were stunted with premature flowering, progeny obtained in subsequent generations either through selfing or backcrossing was identical in appearance to their non-transformed wild-type plants. Another solution is collection of pollen from transgenic plants for pollinating on healthy male-sterile flowers with normal seed set and progeny analysis of the first backcross generation (Everett et al. 1987).

Particle bombardment is the second preferred choice for genetic transformation of several crop plants. However, in case of sunflower, very few studies employed direct gene transfer method or a combination of particle bombardment with vector-mediated methods (Table 1). Preculture of explants enhanced the GUS expression (Hunold et al. 1995). Immature embryos were found to be more suitable for transformation through particle gun bombardment than cotyledons from mature seeds as the strong cuticle in cotyledons prevented particle penetration (Laparra et al. 1995).

Table 1 Genetic transformation studies in sunflower

S. no	Method of transformation	Gene(s) deployed	Remarks	References
1	Puncture of the stem cut surface by syringe needle	*Phaseolin* from bean	Tumorous calli	Murai et al. (1983)
2	Puncture with sterile needle and adding bacteria to the puncture sites	*Zein* from maize	Tumorous calli	Matzke et al. (1984)
3	Puncture with sterile needle and adding bacteria to the puncture sites	*β-Galactosidase* from *E. coli*	Tumorous calli	Helmer et al. (1984)
4	Puncture of the stem cut surface by syringe needle	*Zein* from maize	Tumorous calli	Goldsbrough et al. (1986)
5	Puncture with hypodermic syringe. Embryogenic callus induced from infected explants	*npt*II	Fertile transgenic plants	Everett et al. (1987)
6	Puncture of hypocotyls with hypodermic syringe	*npt*II	Tumorous calli	Nutter et al. (1987)
7	Puncture of hypocotyls with hypodermic syringe	*gus, npt*II	Transgenic plant	Hartman (1989)
8	*Agrobacterium*-mediated	*gus*, *npt*II	Integration of the transgenes confirmed	Schrammeijer et al. (1990)
9	*Agrobacterium*-mediated	*hpt*II, *npt*II	Transgenic neomycin-resistant plants	Hartman (1991)
10	*Agrobacterium*-mediated	*npt*II, *pat*, *gus*	Chimeric transgenic calli	Escandon and Hahne (1991)
11	*Agrobacterium*-mediated	*npt*II, *gus*	Chimeric transgenic shoots	Dek and Peerbolte (1991)
12	*Agrobacterium*-mediated	*npt*II, *gus*	Transgenic calli	Voronina (1991)
13	*Agrobacterium*-mediated	*npt*II, *cat*, *gus*	Chimeric transgenic shoots	Biasini et al. (1992)
14	Particle gun bombardment and *Agrobacterium*-mediated	*gus*, *npt*II	Microprojectile bombardment of plant tissues increased transformation frequency by *A. tumefaciens*	Bidney et al. (1992)
15	*Agrobacterium*-mediated	*npt*II, *gus*	Transgenic plants	Pugliesi et al. (1993)

(continued)

Table 1 (continued)

S. no	Method of transformation	Gene(s) deployed	Remarks	References
16	*Agrobacterium*-mediated	*npt*II	Stable integration of the transgene	Malone-Schoneberg et al. (1994)
17	Microprojectile bombardment and *Agrobacterium tumefaciens*	*gus* and *npt*II	Integration of the genes shown by Southern analysis	Knittel et al. (1994)
18	Particle bombardment using two particle delivery systems	*gus*	Transient expression of the *uidA* gene	Hunold et al. (1995)
19	*Agrobacterium*-mediated	*gus*	Fertile transgenic plants	Grayburn and Vick (1995)
20	*Agrobacterium*-mediated	*npt*II, *cat*, *gus*	Chimeric transgenic shoots	Laparra et al. (1995)
21	*Agrobacterium*-mediated	*gus*	Expression patterns of transgenes analyzed	Burrus et al. (1996)
22	*Agrobacterium*-mediated	*gus*	*Gus* expression up to 82.7% from transformed split shoot tip explants	Gurel and Kazan (1999)
23	*Agrobacterium*-mediated	*gus*	Stable integration of the transgene	Alibert et al. (1999)
24	*In planta*	*gus*	Stable integration of the transgene	Rao and Rohini (1999)
25	Particle gun bombardment and *Agrobacterium tumefaciens*	*npt*II, *gus*	Single-insertion events were observed in T_1 plants	Lucas et al. (2000)
26	*Agrobacterium*-mediated	*Oxalate oxidase*	*Sclerotinia* resistance	Scelonge et al. (2000)
27	*Agrobacterium*-mediated	*gfp*	Stable transformation efficiency was 0.1%	Muller et al. (2001)
28	*Agrobacterium tumefaciens* and particle gun bombardment	*gus*, *ipt*	Transient expression of *ipt* gene enhanced regeneration and transformation	Molinier et al. (2002)
29	*Agrobacterium*-mediated	*Oxalate oxidase*	*Sclerotinia* resistance	Hu et al. (2003)
30	*Agrobacterium*-mediated	*gus*, *gfp*	Stable integration of the transgene	Weber et al. (2003)
31	*Agrobacterium*-mediated	*gus*	*Gus* expression of 30–40% in the high oleic inbreds	Mohamed et al. (2004)

(continued)

Table 1 (continued)

S. no	Method of transformation	Gene(s) deployed	Remarks	References
32	*Agrobacterium*-mediated	*npt*II	Rooting on kanamycin is an effective selection method	Radonic et al. (2006)
33	*Agrobacterium*-mediated	*Human lysozyme*	*Sclerotinia* resistance	Sawahel and Hagran (2006)
34	Particle bombardment	*gus*	*Gus* expression of 3.1 and 4.5% in two high oleic inbreds	Mohamed et al. (2006a)
35	*Agrobacterium*-mediated	*gus*, *mgf*5	*Gus* expression of 4.1 and 4.8 % and *mgf5* expression of 3.3% in two high oleic genotypes	Mohamed et al. (2006b)
36	*Agrobacterium*-mediated	*gln2, ch5B*	Introduction of antifungal genes in sunflower	Radonic et al. (2008)
37	*Agrobacterium*-mediated	*npt*II	Stable integration of the transgene	Dagustu et al. (2008)
38	*Agrobacterium*-mediated	*bar*	Resistance to the herbicide Basta	Neskorodov et al. (2010)
39	*Agrobacterium*-mediated in planta	*β-1,3-Glucanase*	*Alternaria* blight resistance	Manoj Kumar et al. (2011)
40	*Agrobacterium*-mediated	*tvd-1*	Resistance against Alternaria leaf spot	Sirisha et al. (2011)
41	*Agrobacterium*-mediated	*LycB*, *npt*II	Somatic embryogenesis from IZEs with 0.2% transformation frequency	Liu et al. (2011)
42	*Agrobacterium*-mediated	*npt*II, *gus*	Average transformation frequency of 3.0% across genotypes	Sujatha et al. (2012)
43	*Agrobacterium*-mediated	TSV (*TSV-CP*)	Resistance to TSV infection	Pradeep et al. (2012)
44	*Agrobacterium*-mediated	*ProDH*1	Increased synthesis of L-proline and resistance to abiotic stress	Tishchenko et al. (2014)
45	Vacuum infiltration	*Xyn10A*	Co-expression of heterologous plant cell wall-degrading enzymes	Jung et al. (2014)
46	*Agrobacterium*-mediated	*HAM59 MADS-box*	Floral organogenesis	Shulga et al. (2015)
47	*Agrobacterium*-mediated RNAi	*PEPC*	Feasibility to increase the oil content in oilseed types	Zhao and Shi (2016)

(continued)

Table 1 (continued)

S. no	Method of transformation	Gene(s) deployed	Remarks	References
48	*Agrobacterium*-mediated	*hpt*II, *gfp*	Increased transformation efficiency with use of low inoculum with long co-culture period	Zhang and Finer (2016)
49	*Agrobacterium*-mediated *in planta*	TSV *(TSV-CP)*	Resistance to necrosis disease	Sunderesha (2017)
50	*Agrobacterium*-mediated	TSV(*TSV-CP*)	Resistance to necrosis disease	Singareddy et al. (2018)
51	*Agrobacterium*-mediated	*TaNHX2*	Improved tolerance to salinity	Mushke et al. (2019)

Comparison of transient expression in cotyledons and immature embryos showed particle penetration only in the epidermal layers in cotyledons while *gus* expression was intense and seen between epidermis and the fourth mesophyll layer in immature embryos (1.5–2 mm), indicating the importance of target tissues for bombardment (Hunold et al. 1995). Mohamed et al. (2006a) reported efficient and reproducible protocol for genetic engineering of high oleic sunflower genotypes (cv. Capella and SWSR2 inbred line). Bombardment parameters such as gold particle size, particle acceleration pressure, distance between macrocarrier assembly and target plate, preculture period of the explant, and number of bombardments per explant were optimized which resulted in transformation frequency varying between 3.1 and 4.5%. In all these studies of direct gene transfer, the conversion frequency of transient expression to stable transformation was low.

Rao and Rohini (1999) developed the in planta method which is genotype independent and technically easy and avoids the need for tissue culture-based regeneration and development of somaclonal variations, a frequent occurrence in shoots derived through tissue culture. However, this procedure leads to recovery of lot of chimeras from the T_0 plants and requires screening of large number of plants for obtaining the putative transgenics. Transformation efficiency in this procedure depends on the number and kind of cells that integrate the transgene and the development of germ cells from these transformed cells.

Table 1 presents the details of the genetic transformation studies undertaken in sunflower. Most of the studies were aimed at establishment of the transformation system. Transgenic development was mainly for incorporation of resistance to biotic stresses (*Sclerotinia sclerotiorum, Alternariaster helianthi,* necrosis disease), while few studies aimed at resistance to abiotic stresses (drought, salinity). Majority of the studies were confined to assess the effect of different variables in terms of transient expression of the introduced gene. Very few studies have determined the stability of the introduced gene at least till the T_4 generation (Lewi et al. 2006; Singareddy et al. 2018).

5 Genetic Transformation for Important Agronomic Genes

Despite the successes in genetic improvement of sunflower through conventional breeding, interspecific hybridization, and mutagenesis, several traits like improved mineral nutrition, tolerance to biotic and abiotic stresses, weed control, and seed quality traits need to be improved through biotechnological means. The review of Horn and Hamrit (2010) provides a comprehensive account of genes cloned and characterized in sunflower which include those related to developmental processes (embryogenesis, non-dormancy, and pollen- and pistil-specific genes), abiotic stresses (heat, drought), disease resistance (downy mildew), and seed traits (fatty acid biosynthesis, tocopherol biosynthesis). This section presents the progress made in the development of transgenic events for agronomically important traits, field evaluation, and the intellectual property protection with regard to development of transformation protocols and transgenics.

5.1 Biotic Stresses

Biotic stresses take a heavy toll of crop productivity in agricultural ecosystems. It has been reported that biotic stresses caused by insect pest(s), fungal, bacterial, and viral pathogens and weeds collectively result on an average of 45% yield losses in sunflower. Diseases that cause severe economic yield losses in both temperate and tropical regions include *Sclerotinia* wilt and rot (*Sclerotinia sclerotiorum*), downy mildew (*Plasmopara halstedii*), rust (*Puccinia helianthi*), *Verticillium* wilt (*Verticillium dahliae*), *Alternaria* leaf spot (*Alternariaster helianthi*), powdery mildew (*Golovinomyces orontii*), sunflower necrosis disease (tobacco streak virus), charcoal rot (*Macrophomina phaseolina*), and *Phomopsis* black stem canker (*Phomopsis helianthi*). Diseases not only reduce yield but also affect the seed oil content and quality. Management of the important biotic stresses such as insect pests and fungal pathogens has been carried mainly through deployment of resistant varieties bred by conventional means and chemical pesticides For diseases like rust, downy mildew, and powdery mildew, reliable sources of resistance are available in the cultivar germplasm or sexually compatible wild *Helianthus* species leading to successful introgression of resistance to desirable agronomic background. However, for diseases like *S. sclerotiorum* and *A. helianthi*, sources of resistance are identified in perennial types and higher ploidy types, and attempts at transfer of resistance to elite lines met with limited success.

Sclerotinia sclerotiorum *S. sclerotiorum* is an economically important disease affecting the crop worldwide particularly in the temperate regions causing root rot, mid-stalk rot, and head rot depending on the crop stage at the time of infection. The fungal sclerotia remain viable in the soil for up to 5 years and begin to attack various plant parts under favorable environmental conditions. Incorporation of resistance through interspecific gene transfer has been one of the options, but progress is rather

limited. Genetic resistance is not complete, and chemical control is not very effective. In *Sclerotinia* infection, oxalic acid has been identified as the key component, and hence, transgenic development is aimed at strategies to degrade oxalic acid. Genetic engineering for enhancing resistance to *S. sclerotiorum* was through introduction of the wheat germin oxalate oxidase (OXO) gene driven either by constitutive promoters or by a proprietary promoter (SCP1) and the 3′ region of the potato proteinase inhibitor (PINII) (Lu et al. 2000; Scelonge et al. 2000). Following the same strategy, resistance to white mold was significantly improved in the transgenic event TF28 (Hu et al. 2003). Radonic et al. (2008) engineered sunflower for resistance to *V. dahliae* and *S. sclerotiorum* through the introduction of antifungal genes like glucanase, chitinase, osmotin gene, and a ribosome inhibitor protein using two types of transformation vectors for exploiting the synergistic effect of different genes. Accumulation of coumarin phytoalexins (ayapin and scopoletin) in sunflower are also probable targets for enhanced resistance to *Sclerotinia* rot through genetic engineering (Urdangarin et al. 1999).

Alternariaster helianthi Among the candidate gene(s) deployed for enhancing plant defense against fungal diseases, pathogenesis-related proteins (PR-2) like β-1,3-glucanases and chitinases are widely used. Using in planta transformation method, transgenic lines harboring β-1,3-glucanase for conferring resistance to *A. helianthi* were developed (Manoj Kumar et al. 2011). The confirmed transgenic plants in T_1 generation were subjected to fungal bioassays using spore suspension of *A. helianthi* which showed various levels of enhanced resistance and seven best lines displayed delayed symptom development and less number of spots as compared to untransformed control. Sirisha et al. (2011) also attempted to incorporate resistance to this pathogen through the introduction of the *TVD1* gene.

Sunflower necrosis disease Sunflower necrosis disease (SND) incited by *Tobacco streak virus* of *Ilar virus* group has been of serious concern in the tropics and subtropical regions during the past decade and accounts for yield losses ranging from 10 to 80% depending on the severity and stage of attack (Jain et al. 2003, 2006). In India, SND has become a major problem since its first appearance in 2007 causing severe yield losses even up to 80%. Reliable sources of resistance in the cultivar germplasm for necrosis disease were not identified. Artificial screening of wild sunflowers through mechanical sap inoculation has resulted in the identification of few diploid perennial *Helianthus* species, but transfer of resistance to cultivated sunflower through interspecific hybridization and prebreeding was unsuccessful owing to crossability barriers and genomic constitution differences. Virus-resistant transgenics have been produced through incorporation of several genes/mechanisms such as coat protein, replicase protein gene, movement proteins, and satellite RNA strategy (Dasgupta et al. 2003). The success of coat protein-mediated resistance (*CP*-MR) has promoted the production of transgenic plants expressing multiple *CP* genes from more than one virus. Several important crops have been engineered for virus resistance using *CP*-MR approach and released for commercial cultivation.

Development of transgenics conferring resistance to the *Tobacco streak virus* through deployment of *TSV-CP* in sense and antisense directions was undertaken in India for conferring resistance to SND.

Pradeep et al. (2012) transformed sunflower (cv. CO-4) plants with *A. tumefaciens* strain LBA 4404 harboring the hpRNA cassette with the 421 bp *TSV-CP* sequence, and in vitro selection was performed with kanamycin. Infectivity assays with TSV by mechanical sap inoculation demonstrated that the sunflower transgenic lines exhibited resistance to TSV infection and accumulated lower levels of TSV when compared with non-transformed controls. Sunderesha (2017) developed SND-resistant transgenic lines through deployment of the *TSV-CP* gene using the *Agrobacterium*-mediated in planta transformation protocol. Resistant transgenic plants and infected wild-type plants subjected to ELISA showed enhanced accumulation of coat protein as compared to healthy (uninfected) sunflower plants. Genetic engineering of sunflower through deployment of the coat protein gene of TSV (*TSV-CP*) was attempted with constructs harboring the *npt*II gene for the selection of putative transformants on kanamycin (Singareddy et al. 2018). Expression analysis of the *TSV-CP* and *npt*II genes in different tissues at flowering and seed setting stages revealed constitutive expression of the transgene till seed maturation. Seed treatment procedures for controlling SND are effective till 35 days while the transgenic events displayed expression of the *TSV-CP* gene in reproductive parts and till seed maturity, thus conferring complete protection. One event (No. 481) out of five events characterized was selected for transfer of the *TSV-CP* gene into agronomically superior genotypes (Singareddy et al. 2018). Transgenic event No. 481 showed resistance to necrosis disease and plants grew to maturity in TSV-CP, while control plants (untransformed) showed mortality within 1–2 weeks following inoculation (Fig. 1).

5.1.1 Insect Pests

Globally, about 250 insect and acarine species have been recorded on sunflower (Rajamohan 1976). The crop is vulnerable to the attack of insect pests at the seedling stage with seedling and soil insect pests, vegetative stage due to foliage feeders and sucking pests, and the flowering stage with capitulum borers causing significant yield losses. The major polyphagous and generalist pests reported on sunflower are *Heliothis* spp., *Helicoverpa armigera*, and *Spodoptera litura*. A wide array of insect pests belonging to lepidoptera, coleoptera, and diptera are reported to attack this crop in North America which is the center of origin for sunflower including the wild *Helianthus* species. These include sunflower beetle (*Zygogramma exclamationis*), red sunflower seed weevil (*Smicronyx fluvus*), *Agrotis* spp., *Euoxa* spp., western corn rootworm (*Diabrotica virgifera*), *Hypurus* spp., *Cylindrocopturus adspersus* (Charlet et al. 1997; Cantamutto and Poverene 2007). Some of these insect pests can be effectively controlled through application of insecticides, exploiting genetic resistance available in wild *Helianthus* species, crop traps, natural controllers, etc. *Bacillus thuringiensis* (Bt) proteins can control insect damage due to lepidopteran foliar feeders, such as *Heliothis* spp., *Helicoverpa* spp., and *Diabrotica* spp.

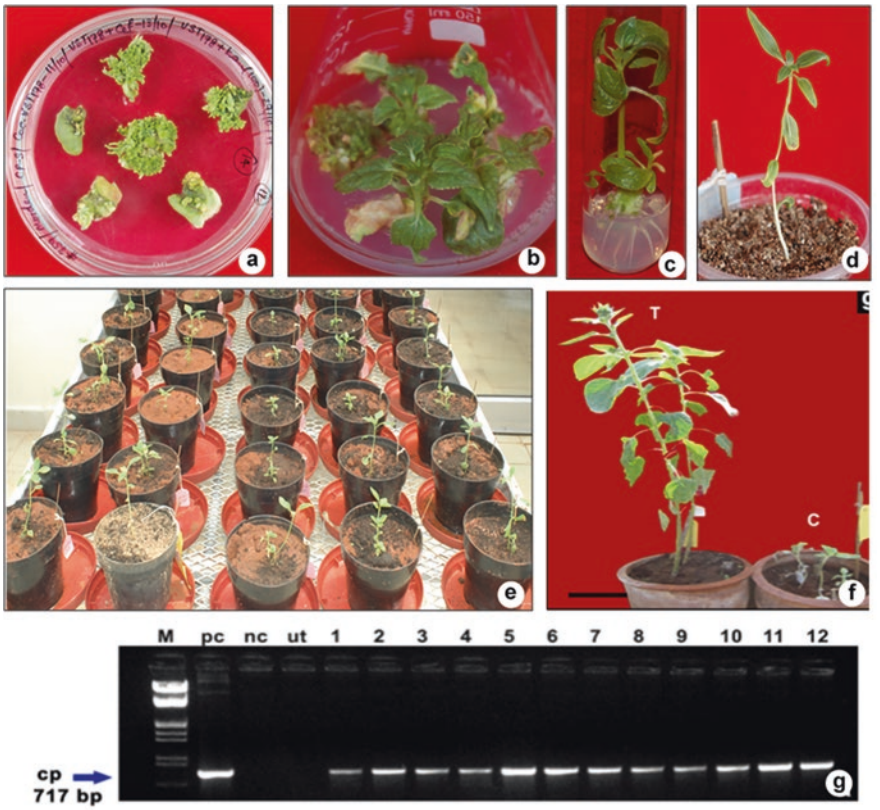

Fig. 1 Genetic transformation in sunflower through introduction of *TSV-CP* gene for virus resistance. (**a**) Selection of putative transformants on kanamycin; (**b**) Elongation of putative transformed shoots; (**c**) Rooting of elongated shoots; (**d**, **e**) Acclimatization of transformed shoots; (**f**) Differences in growth and reaction to SND between plants of homozygous transgenic line (481-10-12) (T) and control (C); (**g**) PCR analysis of the homozygous transgenic line (481-10-12) showing the 717 bp amplicon of the *TSV-CP* gene in all the plants (lanes marked pc—positive DNA control, nc—no DNA control, ut—DNA from untransformed sunflower, 1–12 represent DNA from plants of the transgenic event)

Experiments were carried out to assess the environmental risk and escape of the transgene into the wild using transgenic sunflower harboring the Bt *Cry 1Ac* gene (Snow et al. 2003). The study demonstrated that Bt transgenics increase the fitness of wild populations through reduced herbivory and enhanced fecundity. Sunflower ecosystem provides a favorable habitat for a multitude of beneficial insect species like honey bees as pollinators, parasitoids, predators, entomopathogenic microbes as biological control agents of insect pests which play a vital role in keeping the pest population below threshold level (Basappa and Santhalakshmi Prasad 2005). Hence, any transgenic event for insect resistance should be evaluated for their safety to the beneficial insects.

5.2 Abiotic Stresses

Sunflower is vulnerable to abiotic stresses requiring tolerance to physiological traits like water, salt, and heat. Abiotic stresses have their effects not only on seed yield but also on the oil content as well which is drastically reduced under stress conditions. Horn and Hamrit (2010) in their review enumerated the genes isolated by a candidate gene approach which include the abiotic stress responsive genes like heat shock proteins, desiccation tolerance, drought-related genes, herbicide-resistant against sulfonylurea and imidazoline, embryogenesis, and plant development. Tishchenko et al. (2014) demonstrated the efficiency of proline dehydrogenase (*ProDH1*) gene suppression aimed at increasing sunflower tolerance level to water deficiency and salinity, deploying the LBA 4404 strain harboring pBi2E with double-stranded RNA-suppressor from *ProDH1* gene of *Arabidopsis* through in planta method. The application of lethal doses of stressors (0.4 M mannitol and 2.0% sea salts) increased the L-proline level in transgenic regenerants. Its decline during the recovery period indicated the effectiveness of suppression of the sunflower *ProDH1* gene for increased osmotic stress. Tolerance to salinity is mainly mediated by the vacuolar transporters like Na^+/H^+ (NHX) which help Na^+ sequesteration from the cytosol into the vacuoles. Overexpression of NHX2 antiporter from wheat (TaNHX2) in sunflower enhanced stress tolerance, higher accumulation of Na^+ and K^+ in leaves and roots besides better performance under salt stress (200 mM NaCl) (Mushke et al. 2019). Physiological and biochemical characterization of transgenic plants showed increased accumulation of proline, high chlorophyll content, and higher activities of antioxidant enzymes. On the contrary, reduction in the levels of reactive oxygen species (ROS) and reduced levels of hydrogen peroxide, free oxygen radicals, and malondialdehyde (MOA) were recorded under salt stress.

5.3 Seed Quality Traits

The main objectives with regard to seed traits include enhanced oil content and desired oil quality. Fatty acid modification has been greatly achieved through traditional plant breeding and mutagenesis. Seed oil content and quality in terms of fatty acid composition and protein content are also important that need the attention. Level of oleic acid in seed oil has been obtained by altering the desaturation step from oleic. Oilseed sunflowers have seed oil content of 40–46%, particularly in the temperate regions while in the tropics, a range of 38–42% is recorded. The oil is chiefly composed of oleic acid, linoleic acid, vitamin E, polyphenols, etc. and is considered to be healthier due to its wide ranging benefits. However, high unsaturated fatty acid levels and lack of ω-3 fatty acids are the major reasons for its low oxidative stability and poor nutritional quality—a cause of cardiovascular and inflammatory/autoimmune diseases. Recent advances in understanding the biochemistry and genes encoding enzymes involved in fatty acid modification have

paved the way for metabolic engineering of oil stability and nutritional quality. Attempts are being made to develop high-oleic sunflower by knocking out delta 12-desaturase gene which encodes linoleic acid. Increased linoleic acid using PTGS technology was achieved (Lacombe et al. 2009; Chen et al. 2010). Rauf et al. (2017) reviewed the potential to induce long chain fatty acids like decosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) to enhance the medicinal and industrial value of sunflower oil.

Value of sunflower deoiled cake is lower as compared to other oilseed meals due to the unbalanced amino acid composition. The protein lacks lysine and has the presence of phenolic compounds like chlorogenic acid and caffeic acid, resulting in low intestinal digestibility. Although not much headway has been made toward enhancing the seed oil or modification of protein content, attempts were made to isolate and characterize promoters for seed-specific proteins such as Helianthinin (Jordano et al. 1989; Nunberg et al. 1994), Hads 10G1 (Prieto-Dapena et al. 1999), lipid transfer protein *Ha AP10* (Regente and de la Canal 2003), and oleate desaturase *HaFAD2-1* (Martınez-Rivas et al. 2001; Zavallo et al. 2010) for the modification of the seed phenotypes through biotechnological tools.

5.4 Expression of Sunflower Genes in Heterologous Systems

Transformation protocols are essential for functional validation of candidate genes, promoters, gene regulation, and protein–protein interactions. Owing to the problems associated with tissue culture and genetic transformation, heterologous systems were used to analyze the functions of genes isolated from sunflower. Nizampatnam et al. (2009) demonstrated the induction of male sterility by introducing *orfH522* gene isolated from sunflower in tobacco that could be useful for genetic engineering of male sterility in any other crops. *HaHB4*, a transcription factor belonging to the homeodomain-leucine zipper 1 (HD-Zip I) family for drought tolerance in *Arabidopsis*, showed more tolerance to water stress conditions than the wild-type plants (Dezar et al. 2005). Expression of mutated version of HaHB4 in wheat showed 9.4% larger water use efficiency than its control when evaluated in 37 field experiments (Gonzalez et al. 2019). Another transcription factor of the same family Hahb-10 which is regulated by light promoted early flowering when expressed in *Arabidopsis* (Rueda et al. 2005). *Arabidopsis* transgenic plants expressing HaHB11, a transcription factor of the sunflower HD-Zip I under the control of the CaMV 35S promoter and its own promoter, exhibited improved crop yield, biomass, and flooding tolerance to both submergence and water logging (Cabello et al. 2016). With regard to protein modification for enriching the sulfur amino acids (cysteine and methionine) and enhancing the nutritive value, sunflower seed albumin was stably transformed into narrow-leafed lupin (*Lupinus angustifolius* L.) (Molvig et al. 1997), alfalfa (Tabe et al. 1995), and *Trifolium repens* (Christiansen et al. 2000).

6 Genome Editing

For genome editing, it is essential to have precise information about the genes to be manipulated. A look at the 27 databases indicated the availability of information with regard to 82,992 genes, 141 bioprojects (oleic acid, orobanche-sunflower interactions, flooding tolerance, leaf senescence, fertility restoration, intraspecific divergence, etc.), 9749 sequence read archives (SRA), 1 genome assembly, proteins (conserved domains, proteins, identical proteins, protein structures), etc. in sunflower including promising *Helianthus* species (https://www.ncbi.nlm.nih.gov/search/all/?term=helianthus as on December 1, 2019). Genome editing through CRISPR/*cas9* technology has not been initiated and is still in its formulative stage in case of sunflower. Innovative Genomics Institute, California, is establishing tools for genome editing in sunflower. The main purpose is to understand the fundamental mechanisms in which sunflower responds to environmental stimuli, cope up with the various kinds of stresses, and produce seeds with high oil content. These tools are intended to have potential applications for further genetic improvement through the removal of deleterious mutations and enrichment of cultivated sunflower with alleles from wild *Helianthus* species that confer resistance to disease, drought, salt, or nutrient stress (https://innovativegenomics.org/projects/establishing-tools-sunflower-genome-editing/). Several sunflower genes were functionally validated in model crops like *Arabidopsis* and tobacco. Once the genome editing becomes a routine technique in this crop, it will certainly accelerate the breeding programmes aimed at genetic improvement for key yield contributing agronomic and seed traits.

7 Patents

Some of the procedures related to sunflower transformation are patented, and in most cases, regeneration and transformation target tissues are cotyledons and embryonic axes. These patents are owned customarily by private firms like Pioneer Hi-Bred International (92-09802, 92-09803, 98-51806) for transformation using microprojectile bombardment and *Agrobacterium* sp. and recovery of transformed plants from nodal culture without selectable markers; Biocem, France (95-05874) for the production of entirely transformed transgenic plants; South Dakota State University (94-03332) for direct injection of exogenous DNA into fertilized ovules (http://www.derwent.com); Du Pont (US8901377B2) for sunflower regeneration and transformation using radicle free embryonic axes. In addition, few modifications are being made, and patents were obtained. Monsanto Technology LLC (US6998516B2) claims use of high osmotic medium (200–750 mOsm) with 5–30% (w/v) of any of the carbohydrates like glucose, sucrose, mannitol, fructose, maltose, mannose, and xylose. The patent of Avesthagen Gengraine Technologies Pvt Ltd (EP1979482A1) is based on the selection of putative transformants and ability of the explants to utilize xylose as the sole carbohydrate source. The patent of Agrigenetics Inc. (US8324486B2) relates to a new distinctive sunflower cultivar designated as CI1151R containing one or more transgenes and selected from

progeny segregating for male sterility, herbicide resistance, insect resistance, downy mildew resistance, and oil content. The gene encoding *cis*-prenyltransferase was deployed for the production of latex in sunflower for which a patent (11/734,501) was granted (Hallahan and Keiper-Hrynko 2007).

8 Field Testing of Sunflower Transgenic Events

Despite development of transgenic events for several traits, there are no commercialized transgenic events in sunflower for field release till date. Cantamutto and Poverene (2007) summarized the transgenic events that were subjected to field experimentation. The intended effects of the field tested events include herbicide tolerance, increased nitrogen accumulation, resistance to lepidopteran and coleopteran pests (Snow et al. 2003), resistance to *S. sclerotiorum* (Hu et al. 2003), broomrape control, increased rubber yield (McMahan et al. 2006), enhanced protein quality, and modified stearate content (Rousselin et al. 2002). These events are mostly developed by private firms, and trials were undertaken in Argentina, the USA, France, the Netherlands, and Spain depending on the trait of interest. This clearly indicates the interest of private firms for genetic manipulation of sunflower and also the possibility of genetic improvement of sunflower through transgenic approaches for a wide range of traits related to nutrient-use efficiency, seed quality, and enhanced resistance to diseases and weeds. GM sunflower release permits in the USA and Argentina starting from 1991 steadily increased during 1998–2001, witnessed a steep decline thereafter due to imposed restrictions of the regulatory authorities in the wake of ecological concerns. GM technology for conferring resistance to herbicides (glyphosate and glufosinate-ammonium) has been developed. However, discovery of genes conferring resistance to imidazolinone and sulfonylurea group herbicides in wild sunflower populations paved way for the of non-GM herbicide-tolerant sunflowers (Baumgartner et al. 1999; Kolkman et al. 2004).

9 Conclusions

Sunflower is one of the most important oilseed crops grown worldwide. Regardless of the seed type, breeding for lines with high seed yield, oil content (oilseed types), nutritive-rich protein (confectionery types) coupled with resistance to diseases, pests, abiotic stresses, tolerance to herbicides is the main aim in sunflower improvement programmes. Genes governing yield contributing traits and those imparting resistance to a range of insect pests, diseases, and seed quality traits are either not available in the germplasm or extremely difficult to transfer through sexual hybridization warranting genetic improvement through gene manipulation techniques. Reproducible protocols of shoot regeneration through callus (indirect organogenesis or somatic embryogenesis) are yet to be established in this crop. Genetic transformation of sunflower has been very challenging to date, and transgenic lines were obtained using cotyledons from mature seeds, immature zygotic embryos, shoot tips

which invariably possess pre-existing meristems. Most of the efforts were at establishment of the transformation system while only few attempts were made at improving the input traits. Despite field testing of transgenic events in the USA, Argentina, and European countries, commercial releases failed to receive approvals due to ecological concerns and risk to wild sunflower populations which are native of the USA. With the available genomic resources and advent of genome editing tools, vast scope exists for genetic upgradation of sunflower through genetic modification for key traits to enhance productivity, oil quantity and quality, and also the value as medicinal and industrial crop.

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Genetic Engineering in Safflower (*Carthamus tinctorius* L.): Retrospect and Prospect

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Abstract

Safflower (*Carthamus tinctorius* L., Asteraceae) is an important edible oilseed crop. Because of the distinct seed oil profile, high α-tocopherol content, utilization as a leafy vegetable and useful petal pigments, it has special value among oilseed crops and is of much scientific interest. Recently, safflower has been improved for agronomical, nutritional and other traits with the introduction of specific genes from safflower and also other sources. The prerequisite for successful transformation is development of an in vitro propagation protocol, transformation method and gene of interest. Variation exists in regeneration frequency via organogenesis or somatic embryogenesis in different genotypes of safflower. Therefore, standardization of regeneration protocol is necessary for each genotype before gene transformation. Among different explants, cotyledons and api-

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cal shoot tips were found suitable for transformation and shoot regeneration. *Agrobacterium*-mediated transformation is the successful method for gene transfers in safflower. So far using this method, transformation has been achieved for the enhancement of γ-linolenic acid (GLA), α-linolenic acid (ALA), oleic acid, bioactive peptide, bioactive flavonoid and resistance to fungal pathogen *Alternaria carthami*. The commercial cultivation of genetically modified (GM) safflower is in progress in Australia, Canada and the USA. However, there is scope for improving the frequency of plant regeneration and genetic transformation. The present chapter describes the recent developments in genetic transformation and improvement of safflower.

Keywords

Safflower · In vitro propagation · Genetic transformation · Fatty acids · Edible oil · Pigments · α-Tocopherol

1 Introduction

Global population projections indicate that food production must continue to increase and double in the next few decades. Despite significant achievements, doubling food production is a big task as agriculture faces considerable challenges such as decline in arable land, constraints in the availability of clean water and energy and changes in climate (Smilovic et al. 2019). In such situations, an efficient strategy must be developed to improve crop plants for their nutritional quality and biotic and abiotic stress tolerance. This will maximize the use of cultivated land and increase the utilization of marginal soils (Kishor et al. 2005; Pingali et al. 2019). Biological and genetic diversity exists among different plant species with respect to biomass production and adaptation to stressful environments. Therefore, it is crucial to select suitable cultivars which can grow under harsh climatic conditions. One strategy that can also help us to improve plant productivity is by gene transfer using *Agrobacterium* which can make the plants to tolerate abiotic stresses like salinity, drought and temperature. Plants can also be made resistant to viral, fungal, bacterial and insect attacks besides improvement in the quality of oil (Govindaraj et al. 2015). Thus, with the development of plant genetic engineering technology, crop improvement becomes possible with the desired trait or character. This method accelerated the crop improvement as per the requirement for food, industry or even as per the changes in the environment (Lemmon et al. 2018).

At global level, it has been noted that sufficient edible oil is not available for human consumptions, and there is always a rise in the price of edible oils. This situation is due to the uncertain progress in the production of nine major oilseeds like groundnut, mustard, sesame, sunflower, safflower, soybean, niger, castor and linseeds. Progress in the introduction of high-yielding hybrids is poor compared with the cereal and cotton crops. Owing to these factors, the yield per hectare is low among oilseed crops. However, the per capita consumption of the vegetable oils is

increasing very rapidly due to an increase in population and improved economic status of the population. To meet this demand, along with other edible oil yielding crops, *Carthamus tinctorius* could play a pivotal role and has the potential of becoming the most attractive edible oil crop of the world (Patial et al. 2016; Bella et al. 2019; Anonymous 2019).

Safflower (*Carthamus tinctorius* L. $2n = 24$) is one of the unique edible oilseed crops and belongs to the family Asteraceae (Rudolphi et al. 2012). Some of the common names for the crop are safflower (English), hinghua (China), kusumba (India and Pakistan), golrang (Iran) and alazor (Spain). It originated in the Middle East and presently distributed worldwide (Mundel et al. 2004; El-Lattief 2012; Bella et al. 2019). It is an annual crop; the life cycle is usually completed within 130–140 days. The crop is a predominantly self-pollinating, but out-crossing has been reported up to 60% in some genotypes (Claassen 1950; Anonymous 2019). The safflower grain production can reach up to 1000–3000 kg/ha. The crop is mainly cultivated for its edible oilseeds and flowers. The young plants are also consumed as a leafy vegetable (Anonymous 1950). Among the genotypes, the colour of the flower may vary as yellow, orange, red and white. The petals are used for the production of a natural dye which has significance in textile, food and cosmetic industries. The yellow and red pigments obtained from petals have medicinal importance (Omidi and Sharifmoghaddasi 2010).

Although safflower crop is an underexploited crop, it exhibits worldwide adaptability. Presently, it is cultivated in more than 60 countries and can be grown almost in all cultivating areas around the world (Patial et al. 2016). The statistical analysis of data from 1961 to 2017 indicates that the area of cultivation ranges between 3.27 and 14.79 lakh hectares, and the maximum productivity reaches 11.10 lakh tons (FAO 2019). The analysis highlights the expansion of area under safflower cultivation and suggests the scope for further expansion and production. Canada, India, China, the Russian federation, the USA, Kazakhstan, Mexico, Australia, Spain, Pakistan, Argentina, Uzbekistan, Turkey and Iran are the major producers of safflower (FAO 2016; Janmohammadi et al. 2017; Shahrokhnia and Sepaskhah 2017). Safflower grows well in semiarid areas with low rainfall. At global level, it is the most important oilseed crop having eighth position after soybean, peanut, rapeseed, sunflower, sesame, linseed and castor (Bella et al. 2019).

Safflower oil has unique properties. It is rich in α-tocopherol (95% of the total tocopherols, i.e. α, β, γ and δ) (Furuya et al. 1987; Bella et al. 2019) and helps to lower blood cholesterol, also rich in omega-3 fatty acids. Because of high antioxidant property, it is the best remedy for chronic disorders like spondylosis, hypertension, abdominal pains, wounds stomach tumours and menstruation related problems (Zhou et al. 2014; Patial et al. 2016). It is considered to be the best frying oil due to high-oleic acid content and low level of polyunsaturated fatty acids (Anjani and Yadav 2017). In recent times, it has emerged as one of the most valuable agronomic crops in the world, due to its oil composition and nutraceutical and bioactive metabolites (Bella et al. 2019). The development of hybrids with larger seed, high oil content and improved quality of oil required to the industry increases the demand of the crop (Patial et al. 2016; Bella et al. 2019). The oil has the best antioxidant

property due to the presence of various phenolic components, pigments and tocopherols. There have been reports on extensive use of flower petal yellow (water soluble) and red (water insoluble) pigments (hydroxyl safflower A and B, anhydro-safflower yellow B and carthamin red) in food, cosmetic and textile dye (Anonymous 1950, 2019). The seed extract is a preventive for diseases like cancer and atherosclerosis. During the last two decades, safflower has attracted the attention as a health promoting oil because of high content of γ-linolenic acid and even as a biodiesel feed stock (Patial et al. 2016; Bella et al. 2019).

Attempts were made on improving the frequency of in vitro plant propagation and development of methods for genetic transformation in safflower (Orlikowska and Dyer 1993). Experiments were carried out to describe the transformation of desirable traits like improved nutritional qualities and resistant to abiotic and biotic stress (Patial et al. 2016; Bella et al. 2019). The current chapter gives an overview of genetic transformation and development of transgenics in safflower.

2 In Vitro Propagation Protocol: A Prerequisite for Genetic Transformation

Developments in DNA recombinant technology allows genetic transformation in several crop plants. The technology offers some benefits over traditional breeding methods for the improvement of crops for various traits like growth, nutritional quality, biotic and abiotic stresses (Chen et al. 2019). However, plant regeneration in vitro is a prerequisite for improvement of plants through genetic engineering. After incorporation of specific gene(s) for specific trait(s) into the cells, whole plants need to be regenerated. Accordingly, whole-plant regeneration via organogenesis or somatic embryogenesis is crucial from explants (Loyola-Vargas and Ochoa-Alejo 2018). Many attempts were made for the development of in vitro propagation protocols for many cultivars of safflower (Table 1).

2.1 Plant Regeneration Via Organogenesis

Attempts have been made for the establishment of in vitro propagation protocol from different explants of safflower (Table 1, Fig. 1). Most of the reported protocols (Table 1) for in vitro propagation used seedling explants like cotyledons, hypocotyls and leaves (Baker and Dyer 1996a; Nikam and Shitole 1999; Patial et al. 2016; Bella et al. 2019). Induction of capitula (inflorescences) directly from cotyledonary explants was achieved in the cultivar Mangira and A-1 of safflower (Tejovathi and Anwar 1984). In vitro capitulum formation at the shoot tip was also recorded in the cultivar A1 (Ying et al. 1992; Nikam and Shitole 1999). It appears that direct organogenesis protocol is the most suitable for the development of transgenics. However, the major drawback in most of the reported protocols for safflower is regeneration of shoots or roots and is associated with abundant callus formation, i.e. callus-mediated organogenesis (Table 1). However, it appears that direct shoot formation

Table 1 In vitro shoot regeneration and rooting of shoots in different cultivars of safflower (*Carthamus tinctorius* L.)

Cultivar	Explants and medium	Callus induction medium	Shoot induction medium	Medium for rooting of shoot	References
Indian CV: Th-black, NP-9 black, Partial hull black-10	One-week-old cotyledon, hypocotyl	MS + 0.5 mg/L NAA + 0.2–2.0 mg/L BA	Indirect induction of shoot, MS + 0.5 mg/L BA	MS + 6%, 7%, or 8% sucrose	George and Rao (1982)
Indian CV: Mangira, A-1	2- to 3-day-old seedlings: cotyledon		Direct induction of capitula, MS + 0.5 mg/L BAP + 0.1 mg/L NAA + 9.0% sucrose		Tejovathi and Anwar (1984)
Indian CV: Mangira, Tara, 18-66-18, 9-51, NS115-1, EC46984, A-1, S-4, JL-40, EC47006	Anther N6, B5, Chaleff's, LS, MS	Haploid callus production, MS + 0.5 mg/L BAP & KIN + 0.1 mg/L NAA + 3% sucrose	Indirect haploid shoot, MS + 2.0 mg/L BAP + 0.5 mg/L NAA + 2% sucrose	Rooting of haploid shoot, ½ MS + 0.1 mg/L NAA + 1% sucrose	Prasad et al. (1991)
American CV: Centennial	3- to 4-week-old seedlings: cotyledon, stem and leaf	MS + 1 mg/L BAP and 1 mg/L NAA	Indirect, shoot leaf-derived callus, 1 mg/L BAP, and 1 mg/L NAA	Attempts were made but not succeeded in root induction	Ying et al. (1992)
American var.: Centennial, Montola	3- to 20-day-old seedlings: cotyledons, leaves		Direct, shoots from 7-day-old explants, MS + 0.1 mg/L NAA + 0.5 mg/L BA or 0.1 mg/L TDZ + 2.5 mg/L $AgNO_3$	½ MS + 1.0 mg/L NAA + 1 mg/L riboflavin	Orlikowska and Dyer (1993)
Indian CV: Manjira, A-1	2- to 3-day-old seedlings: cotyledons, hypocotyls		Direct, capitula induction, MS + 0.5 mg/L BA + 0.1 mg/L NAA	MS + 2.0 mg/L 2,4,5 Clpop	Tejovathi and Anwar (1993)
Indian cultivar: Girna	10-day-old seedlings: cotyledon		Direct, MS + 1.0–2.0 mg/L NAA + 0.5 mg/L BA	MS + 0.2 mg/L NAA	Mandal et al. (1995)

(continued)

Table 1 (continued)

Cultivar	Explants and medium	Callus induction medium	Shoot induction medium	Medium for rooting of shoot	References
American var.: Centennial	3- to 6-day-old seedlings: cotyledons, leaf		Direct, MS + B5 vitamins + 0.1 mg/L NAA + 0.5 mg/L TDZ + 2.5 mg/L $AgNO_3$	½ MS salts + 1.0 mg/L NAA + 2.8 g/L phytagel + 2.5 mg/L $AgNO_3$	Orlikowska et al. (1995)
American var.: Centennial	3-day-old seedlings: cotyledons, hypocotyl		Direct, MS + B5 vitamins + 0.1 mg/L NAA + 0.5 mg/L TDZ + 2.5 mg/L $AgNO_3$	½ MS + 15 g/L sucrose + 2.5 mg/L $AgNO_3$ + 10 mg/L IBA + 1 g/L activated charcoal + 2.8 g/L phytagel	Baker and Dyer (1996a)
Indian, CV: Bhima	Root, hypocotyl, cotyledon, leaf	MS + 11.41 µM IAA + 4.43 µM BA	Indirect, MS + 8.04 µmol/L NAA + 2.21 µmol/L BA	MS + 7–8% sucrose + 2.8–5.7 µmol/L IAA	Nikam and Shitole (1999)
Indian, CV: A-1, A-300	2-day-old seedlings: embryo axis with one cotyledon	–	Direct, MS basal medium	Genetic transformation	Rao and Rohini (1999), Rohini and Rao (2000)
Indian cultivars: JSI-46, Bhima, A-300, S-144, Saradha, A-1, Tara, Girna	5- to 6-day-old seedlings: cotyledon		Direct, MS + 2.0mg/L NAA + 0.5 mg/L BA + 87.6 mM sucrose + 50 µmol/L $AgNO_3$	½ MS + 5.3 µmol/L NAA + 3% sucrose	Mandal and Gupta (2001)
Indian CV.: HUS-305, T-65, CO-1, N-7 A-1, and TARA	2- to 6-day-old seedlings: cotyledon		Direct, MS + 1.0 mg /L BAP and 0.1 mg /L Kinetin	½ MS + 0.2 mg/L NAA	Walia et al. (2005)
Indian CV: A-1, Manjira, HUS-305	After appearance of first pair of leaf: root, hypocotyl, cotyledon, primary leaf		Direct, MS + 0.5 mg/L TDZ + 0.5 mg/L NAA		Radhika et al. (2006)

(continued)

Table 1 (continued)

Cultivar	Explants and medium	Callus induction medium	Shoot induction medium	Medium for rooting of shoot	References
Indian CV: HUS-305	Stem segments		Direct, MS + 0.2 mg/L TDZ + 0.2 mg/L NAA		Sujatha and Kumar (2007)
Indian CV.: NARI-6	10- to 17-day-old seedlings: cotyledon, leaf		Direct, 1/4 MS + 1.5% sucrose + 250 mg/L glutamine + 100 mg/L arginine + 10 mg/L proline + 0.3 mg/L GA_3		Kumar et al. (2008a)
Indian CV.: NARI-6	10- to 17-day-old seedlings: cotyledon		Direct, MS + 10 mg/L myoinositol + 50 mg/L Thiamine hydrochloride + 5.0 mg/L TDZ	¼ MS + 0.2 mg/L NAA + 1.0 mg/L $AgNO_3$	Kumar et al. (2008b)
Turkish CV.: Dincer	10-day-old seedlings: cotyledon		Direct, MS + 0.5 mg/L TDZ + 0.25 mg/L IBA	½ MS	Basalma et al. (2008)
Chinese CV.: Tacheng	Cotyledon		Direct, MS + 3% sucrose + 0.7% agar + 0.2 mg/L NAA + 1.0 mg/L BA	¼ MS + 2.0 mg/L NAA + 0.5 mg/L IAA	Yang et al. (2009)
Indian CV.: Bhima CO-1, A-1, JSI-7	Mature embryo	MS + 2.0 mg/L Kin + 0.1 mg/L 2,4 D	Direct, MS + 2.0 mg/L BA + 0.5 mg/L NAA	MS + 0.1 mg/L BA + 2.0 mg/L NAA	Badri et al. (2009)
Indian CV.: A-1, Bhima, CO-1, HUS-305, JSF-1, Manjira, Tara	8- to 20-day-old seedlings: root, hypocotyl, cotyledon		Direct, MS + 0.2 mg/L TDZ + 0.2 mg/L NAA		Shilpa et al. (2010)
Australian CV.: WT, S-317	6- to 8-day-old seedlings: cotyledon		Direct, MS + 1 mg/L TDZ + 0.1 mg/L NAA	MS + 1 mg/L BA + 0.1 mg/L NAA	Belide et al. (2011)

(continued)

Table 1 (continued)

Cultivar	Explants and medium	Callus induction medium	Shoot induction medium	Medium for rooting of shoot	References
Turkish CV.: Dincer Iranian CV.: Sina	10-day-old seedlings: hypocotyl, cotyledon		Direct, MS + 0.1 mg/L NAA + 2 mg/L BAP	MS + 2 mg/L IBA	Motamedi et al. (2011)
Indian CV.: AKS-207, PVK-Pink	10- to 12-day-old seedlings: cotyleodonary leaves	MS + 2.0 mg/L TDZ + 0.5 mg/L NAA	Direct, MS + 2.0 mg/L TDZ + 0.1 mg/L NAA	MS + 3 mg/L NAA	Mohite et al. (2014)
Indian CV.: NARI-H-15	7- to 9-day-old seedlings: shoot tip and node	MS + 1.5 mg/L NAA + 1.5 mg/L CPPU	Direct, MS + 5.0 mg/L TDZ + 1.5 mg/L CPPU	MS + 1.0% sucrose + 2.0 mg/L NAA + 1.5 mg/L CPPU	Jaychandran et al. (2017)
Indian CV.: PKV Pink	10-day-old seedlings: hypocotyl, cotyledonary leaves		Direct, MS + 5.0 mg/L BA + 1 mg/L $GA_{3,}$ 4.0 mg/L BA + 1 mg/L GA_3		Surbahiyya et al. (2018)

Fig. 1 Callus-mediated shoot regeneration in cotyledon explant of *Carthamus tinctorius* L., *cv*. Bhima on MS + 5.0 mM BAP + 1.2 mM NAA

is achieved in the cotyledonary node explants of the cultivars Sharda, Bhima and PBNS-12 (Tables 1 and 3). The most desirable explants for shoot regeneration are the cotyledonary nodes (Dhumale et al. 2015; Patial et al. 2016). The problem of occurrence of hyperhydricity was reduced on the addition of cytokinin TDZ in place of BAP (Orilkowaska and Dyer 1993). Many aspects that influence shoot regeneration in safflower are discussed below.

2.2 Effect of Genotype on Plant Regeneration Frequency

Regeneration of shoots in safflower is highly influenced by the genotype, type of explants and age of explants (Patial et al. 2016). Variation exists in shoot regeneration frequencies (direct and indirect) in Indian, American, Australian, Turkish, Kazakhstan, Egyptian and German genotypes with the use of cotyledonary, hypocotyl and leaf explants (Table 1) (Shilpa et al. 2010; Belide et al. 2011; Bella et al. 2019). Variations in regeneration frequencies depending upon the genotypes was observed, and it has been suggested that cotyledonary node explants derived from Australian types produce maximum shoots followed by American, Kazakhstan, Egyptian and German genotypes (Patial et al. 2016). The data recorded in Table 1 indicate that the genotype specificity and low regeneration frequency are the major barriers for in vitro culture of safflower.

2.3 Influence of the Age of Explants

In safflower cultivars, shoot regeneration depends on the type and also the age of explants. Root explants did not respond for shoot formation. The young

cotyledonary explants give better responses in comparison with leaves, hypocotyls and stem explants in most of the cultivars. However, shoot regeneration frequencies from the cotyledonary nodes were much higher than those obtained from cotyledonary and hypocotyl explants (Patial et al. 2016). Protocol for the differentiation of shoots has been reported in about 41 cultivars of safflower (Table 1). In most of the protocols, shoot regeneration has been achieved from 3- to 11-day-old seedling explants. But, the response declined in other explants. Explants from 8-day-old seedlings displayed higher regeneration frequencies as compared with the explants from 20-day-old seedlings (Shilpa et al. 2010; Belide et al. 2011). Similarly, 6-day-old seedlings exhibited better frequency of regeneration in comparison with 8-, 10- and 15-day-old seedlings (Patial et al. 2016). These results indicate that the frequency of plant regeneration depends on the age of the explants in safflower.

2.4 Effect of Media Constituents on Plant Differentiation

The most common media used for plant tissue culture are Murashige and Skoog's (Murashige and Skoog 1962) medium (MS), Linsmaier and Skoog's (Linsmaier and Skoog 1965) medium (LS), Gamorg's (Gamborg et al. 1968) medium (B_5) and Nitsch and Nitsch's (Nitsch and Nitsch 1969) medium (NN) and woody plant's (Lloyd and McCown 1981) medium (WPM). Among these, MS medium is the most frequently used with maximum response (Gamborg and Shyluk 2013). However, it is also vital to determine the type of medium and optimum concentrations of plant growth regulators (PGR) added to it (Shimizu-Sato et al. 2009). Media such as MS and B5 have been used for the differentiation of shoots in safflower. Among these two, it is mostly the MS medium that gave optimum response. Different auxins and cytokinins were incorporated in the medium, but the best response for shoot regeneration was noticed on BAP alone followed by thidiazuron (TDZ) and zeatin. Kinetin was found less effective for shoot induction. Addition of silver nitrate improves the frequency of shoot formation in safflower (Baker and Dyer 1996a, b; Patial et al. 2016). The regeneration frequencies reported for some Indian and Australian genotypes of safflower remain in the range of 85–93%. Shoot regeneration frequencies in the range of 25–28% were also recorded for cotyledon and hypocotyl explants (Shilpa et al. 2010; Belide et al. 2011). Improvement in the shoot regeneration frequencies (33.3–40.6%) was recorded with the addition of TDZ and indole-3-butyric acid (IBA) from cotyledonary explants of the genotypes Sharda, PBNS-12 and Bhima (Patial et al. 2016).

2.5 Plant Regeneration Via Somatic Embryogenesis

Efficient in vitro propagation protocol is one of the basic requirements for genetic modification of crop plants. Several attempts have been made to develop protocols through organogenesis and somatic embryogenesis for different cultivars of safflower (Tables 1 and 2). However, the protocols developed are applicable to specific

Table 2 Somatic embryogenesis in different cultivars of safflower (*Carthamus tinctorius* L.)

Cultivar	Explants	Callus induction medium	Somatic embryogenesis (SE) induction medium	Somatic germination medium	References
Indian cultivar: Girna	10-day-old seedlings: cotyledonary leaves		Direct SE, MS + 2.22 BA + 5.37–10.74 µM NAA	½ MS + 1.07 µm NAA	Mandal et al. (1995)
Indian cultivar: Girna	10-day-old seedlings: cotyledons		MS + 2.22 µM BA + 5.37 µM NAA		Mandal et al. (2001), Mandal and Gupta (2003)
Indian cultivar: HUS-305	Endosperm		MS + 2.22–8.87 µM BAP/2.32–9.29 µM KIN/0.91–2.27 µM TDZ		Walia et al. (2007)
Indian cultivar: NARI-6	10–17-day-old seedling: Cotyledon and leaf explant.	MS + 6.0 mg/L TDZ + 1.5 mg/L IBA	MS + 6.0 mg/L TDZ + 1.5 mg/L IBA	MS + 0.2 mg/L GA_3 + 250 g/L glutamine + 100 mg/L arginine + 10 mg/L proline.	Kumar et al. (2008a)
Indian cultivar: NARI-6	5–7-day-old seedlings: Immature leaf explants.		MS + 4.0 µM TDZ + 5.0 µM IBA	MS + 1.5 µM + 1.0 µM spermidine	Kumar and Kumari (2017)

Table 3 Genetic transformation protocol for different cultivars of safflower (*Carthamus tinctorius* L.)

Cultivar	Explant	*Agrobacterium strain*	Trait	Gene	Selectable marker (concentration (mg/L))	Transformation efficiency (%)	References
Centennial	Leaf-derived callus	LBA4404	–	*nptII* GUS assay	Kanamycin 50 mg/L	15	Ying et al. (1992)
AKS 207, PKV Pink	Cotyledonary leaf	EHA-105	Lentil lectin gene (insect resistance)	*nptII* GUS assay	Kanamycin 50 mg/L	54, 47.6	Dhumale et al. (2016)
A-1, A-300	Embryo axis with one cotyledon	LBA4404 binary vector pKIWI105	–	*uidA* and *nptII*	Kanamycin 50 mg/L	5.3, 1.3	Rao and Rohini (1999), Rohini and Rao (2000)
S-317	Cotyledons	Binary vector pORE3	High oleic acid content	*nptII*	Secreted GFP and hygromycin	4.8	Belide et al. (2011)
WT	Cotyledons	Binary vector pORE3	High linoleic acid content	*nptII*	Secreted GFP and hygromycin	3.1	Belide et al. (2011)
Sharda, Bhima, and PBNS-12	Cotyledonary nodes	LBA4404	–	Bialaphos resistance (*bar*) gene	β-Glucuronidase (gus)	90	Patial et al. (2016)

cultivars of safflower (Table 3). Therefore, genetic manipulation was possible only in few genotypes of safflower (Table 4). The type of PGRs and their concentrations, media composition, relative humidity that exists in culture vessels and variable response of shoots to PGRs for rhizogenesis are key factors for successful regeneration of plants via somatic embryogenesis (Nikam and Shitole 1999; Kumar and Kumari 2011, 2017). Though there are good claims for efficient somatic embryogenesis protocols (Kumar and Kumari 2011), yet they did not help to improve genetic transformation of safflower (Tables 2 and 4).

2.6 Rooting of Shoots and Whole-Plant Regeneration

After successful transformation and shoot regeneration from transformed cells or tissues, there is a need for rooting of microshoots. Roots must connect with the vascular system of the shoots for proper establishment in the soil. Existing information reveals that the percentage of rooting of shoots in safflower is low (Table 1) and needs improvement (Nikam and Shitole 1993; Belide et al. 2011; Patial et al. 2016). This is irrespective of transfer of shoots for rooting on hormone-free as well as on hormone-added media (indole-3-acetic acid (IAA), α-naphthalene acetic acid (NAA), indole-3-butyric acid (IBA) and 2,4,5-trichlorophenoxy propionic acid (2,4,5-Cl_3, POP)). Additional components such as low and high concentrations of sucrose (0–9%), other carbohydrates, different chemicals like ascorbic acid and diverse methods were tried but the percent frequency of rooting remains low (Table 1) (Belide et al. 2011). In hormone-containing medium, formation of callus is very common at the basal parts of the shoots. In spite of the constraints, rooting was achieved in 95% of the shoots in a two-stage method. First, shoots regenerated from cotyledonary explants were treated for 7 days with 10 mg/L of IBA in half-strength MS medium, and then the shoots were transferred to hormone-free MS medium for 3 weeks (Baker and Dyer 1996a, b). To overcome the problem of rooting and successful establishment of transformed plantlets, in vivo and in vitro micro-grafting method was also applied. In ex vitro method, the stocks were developed from the seeds and in vitro genetically transformed shoot scions grafted on them and transformed shoots established successfully (Belide et al. 2011). Improvement in grafting method and increase in percent survival was recorded with the help of in vitro grafting method; the stock was developed from seeds in in vitro conditions, and then transformed shoot scions were grafted on them. Using these methods, in vitro grafted transformed plantlets were transferred to soil and grown in natural conditions. Such transgenic plants developed normal capitula and produced viable seeds (Patial et al. 2016).

3 Selection of True Transformants

Identification and selection of transformed cells from that of non-transformed cells is an important step in genetic transformation procedure. For this, the selection system has been developed with highly efficient, selectable marker genes and resistance to

Table 4 Genetically transformed cultivars of safflower (*Carthamus tinctorius* L.): method and outcome of transformation

Cultivar	Explant	Gene	Gene source	Trait	Transformation method	Strain	Outcome	References
Alteration and improvement in fatty acid profile								
Safflower *cv.* HUS-305	Hypocotyl	*δ-6-Desaturase*	*Borago officinalis*	GLA biosynthesis	*Agrobacterium*-mediated	LBA4404	GLA synthesis	Devi et al. (2009)
Safflower *cv.* A1	Hypocotyl	*FAD3*	*Arabidopsis*	ALA production	*Agrobacterium*-mediated	EHA105	ALA accumulation	Rani et al. (2018)
Safflower	–	*GLA* biosynthesis gene	*Mortierella alpina; Saprolegnia diclina*	GLA production	–	–	Increased in GLA levels of up to 50% and 70%, respectively	ISAAA (2017)
Safflower	–	Downregulation of *CtFATB* and *CtFAD2.2*	Safflower	High oleic acid	*Agrobacterium*-mediated	–	Accumulation of approximately 92% of oleic acid (C18:1) and very low (less than 2%) linoleic acid (C18:2) in the seed	www.Ogtr.gov.au
Production of pharmaceutically important compound								
Safflower		*apoAI*	Human	Apolipoprotein AI Milano	*Agrobacterium*-mediated	EHA101	Transgenic line found 7 g of $ApoAI_{Milano}$ per kilogram of seed	Nykiforuk et al. (2011)
Safflower ZHH0119 (Y line) and XHH007 (W line)	–	Chalcone synthase (*CHS1*)	Safflower	Quinochalcone glucosides	*Agrobacterium*-mediated	–	~20–30% increase in quinochalcone glucosides	Guo et al. (2017)
Safflower *cv.* Jihong No. 1	–	Chalcone isomerase (*CtCHI*)	Safflower	Flavonoid	*Agrobacterium*-mediated	–	Increased in flavonoid level	Liu et al. (2019)
Development of disease-resistant cultivar								
Safflower *cv.* A1	Apical meristem	*Chitinase*	Rice	Fungal pathogen *Alternaria carthami* resistance	*Agrobacterium*-mediated	LBA4404	Developed resistance against fungal diseases	Kumar et al. (2009)

antibiotics or herbicides (Miki and McHugh 2004) or ability to metabolize non-metabolizable component, viz. mannose (Joersbo et al. 1998) or xylose (Haldrup et al. 1998) or 2-deoxyglucose (Kunze et al. 2001). However, sensitivity to selective agents is widely variable between plant species and tissues under selection pressure. Therefore, it is necessary to standardize antibiotic sensitivity of the explants/callus cultures before genetic transformation is attempted. In safflower, the first attempt to use cotyledons, stems and leaf explants for *Agrobacterium*-mediated transformation has been reported in the American cultivar Centennial (Ying et al. 1992). Subsequently, embryo axis with one cotyledon and hypocotyl explants in Indian cultivar A-1, A-300 and HUS-305 (Rao and Rohini 1999; Rohini and Rao 2000, Shilpa et al. 2010) and use of cotyledons in Australian genotypes S-317 and WT were recorded (Belide et al. 2011). The selection of transformed shoots in safflower has been achieved using kanamycin (at 50 mg/L) and hygromycin (at 10 and 15 mg/L) (Shilpa et al. 2010; Belide et al. 2011; Motamedi et al. 2011).Transgenic shoot selection was also carried out by herbicide-based selection system and transformation vector containing the phosphinothricin resistance conferring bar gene (Patial et al. 2016).

4 Transgenics and Improvement of Fatty Acid Profile

Lipids are used as a food supplement, pharmaceutical and industrial raw materials. Different types of fatty acids have been reported in the lipid profiles of various oilseed crops (Murphy, 2016). The biosynthetic pathway of the lipids is well understood, and now it may be possible to satisfy the requirements, with the improvement of the quality of fatty acid production. Alteration and improvement of fatty acid profile has been attempted in the oilseed crops including safflower (Topfer et al. 1995; Shanklin and Cahoon 1998; Zhu et al. 2016; Villanueva-Mejia and Alvarez 2017; ISAAA 2017; FSANZ 2018b; Rani et al. 2018). The attempts carried out for the improvement of the fatty acid profile in safflower are as follows.

4.1 Enhancement of γ-Linolenic Acid (GLA)

The γ-linolenic acid (GLA) is an essential fatty acid which has pharmaceutical importance especially in curing the problems like eczema and mastalgia (Horrobin 1990). It is also used as a precursor for the biosynthesis of long-chain fatty acids. The source plants are limited, and the content in their oil is also very low. Therefore, having commercial importance, it attracts the attention of researchers for the production of high oil content enriched with GLA. It has been demonstrated that the enzyme δ-6-desturase plays a significant role in GLA biosynthetic pathway. The gene for δ-6-desturase was characterized and isolated from *Borago officinalis*. Using *Agrobacterium*-mediated gene transfer method, the gene for δ-6-desturase from *Borago officinalis* was transferred into the safflower cultivar HUS-305 (Devi et al. 2009). The seed-specific napin promoter consisting cassette was used to express the δ-6-desturase biosynthesis gene in the seeds of safflower. A group of researchers from Cory Nykiforuk of SemBioSys Genetics Inc., Canada, succeeded

in transferring and overexpressing a gene (name of the gene was not disclosed) isolated from the fungus *Mortierella alpina* to safflower. They reported 50% increase in GLA content of safflower, wheras the transgenic safflower obtained on transfer of the gene (name of the gene not disclosed) from fungus *Saprolegnia diclina* showed an enhanced synthesis of GLA in oilseed up to 70%. However, it has been mentioned that this variation in GLA levels is not linked to the gene dosage or the absence of triacyl glycerol backbone. The difference in the content of GLA is linked with the activity of enzymes obtained from two different fungal sources. At present, the content of GLA is the highest in the transgenic cultivars and has been commercialized as Sonova TM 400 (ISAAA 2017).

4.2 Overproduction of α-Linolenic Acid (ALA)

The α-linolenic acid (ALA) and n6:n3 fatty acid ratio and omega-3 long-chain polyunsaturated fatty acids (PUFAs) in the diet help to minimize the inflammatory, autoimmune, neuro and cardiovascular disorders (Rani et al. 2018). Omega-3 fatty acid content is ample in the fish but has several limitations in the availability and utilization. Safflower is the best source of omega-3 PUFAs, but scope exists for enhanced production of these fatty acids in the safflower with the help of recombinant DNA technology. The gene that encodes the enzyme delta-15 desaturase (FAD3) isolated from *Arabidopsis* was successfully incorporated with truncated seed-specific promoter via *Agrobacterium* into the cultivar A1 of safflower (Rani et al. 2018). The confirmation of transgenic nature of regenerants was carried out by PCR and southern blot. The detection and quantification of fatty acids in putative transformed plantlet seeds by GCMS analysis revealed the enhanced content of ALA (1.34, 2.69, 4.45, 2.58, 4.65, 18.2, 4.14, 5.91, 4.65, 2.77, 8.01, 1.62 and 2.63 mg ALA g^{-1} dwt of the total fatty acid contents of the seeds). Further it has been elaborated that this transgenic safflower becomes a source of nutritionally superior novel oil enriched with ALA. It not only has reduced ratio of LA to ALA but also adds importance for its use for good health (Rani et al. 2018).

4.3 Super High Oleic Acid in Safflower Oil (SHOSO)

The monounsaturated fatty acid such as oleic acid of safflower is highly heat stable and biodegradable. This property gives the commercial value to oleic acid and is suitable for utilization in oleo-chemical industry, which involves the production of bio-based plastics, foams, fluids, etc. Oleic acid could be the best alternative to petroleum-based resources in the manufacture of several industrial products such as lubricants and hydraulic fluids (GRDC 2010; Yelchuri et al. 2019). The safflower oil has a role to play in other industrial products such as infant formula (baby milk), cosmetics and soaps. Accordingly, the oil of GM safflower has been approved by the US-FDA for utilization as dietary supplement and also in industrial applications (Nykiforuk et al. 2012). This has become possible due to the efforts of the GO

Resources Pvt Ltd., which has genetically modified (GM) two cultivars of safflower (OECD Unique IDs: GOR-73226-6 and GOR-7324Ø-2) that are also referred as Event 26 and Event 40. The Commonwealth Scientific Industrial Research Organisation (CSIRO) carried out the molecular characterization of these GM cultivars (Wood et al. 2018). The events consist of a construct designed to downregulate two safflower fatty acid biosynthesis genes *CtFATB* and *CtFAD2.2*. The *CtFATB* gene encodes palmitoyl-ACP thioesterase and *CtFAD2.2* gene encodes Δ12 desaturase enzymes. The downregulation was achieved by targeting the seed using a promoter from flax (*Linum usitatissimum*) and RNAi technology. The DNA sequences from the vector pCW732 containing a cassette were introduced with the help of *Agrobacterium*-mediated transformation. This allows downregulation of the activity of Δ12 desaturase (CtFAD2.2) and palmitoyl-ACP thioesterase (CtFATB) within the seed through the mechanism of RNAi. In addition, the transformation event is also confirmed with the help of the hygromycin resistance gene (*hph*). The *hph* expresses the enzyme hygromycin B phosphotransferase (HPH) also known as aminoglycoside phosphotransferase (APH4). The *hph* confers resistance to the antibiotic hygromycin and expresses under the control of a constitutive promoter sequence which is introduced for in vitro selection. Downregulation of Δ12 desaturase (*CtFAD2.2*) and palmitoyl-ACP thioesterase (*CtFATB*) genes in seed altered the synthesis of fatty acids. This has allowed accumulation of approximately 92% of oleic acid (C18:1) and very low (less than 2%) linoleic acid (C18:2) in the seeds. The oil from genetically modified safflower cultivar has been called super high oleic acid safflower oil (SHOSO). These genetically modified cultivars are non-toxic to livestock, which was confirmed by grazing at late stages of plant growth (FSANZ 2018a). The licence was issued to the Australian company 'Go Resources' from CSIRO for commercialisation of SHO safflower (Singh et al. 2019). The SHOSO oil from genetically modified safflower has significantly higher stability than conventional oils. The oil also performs well or better than synthetic oils derived from fossil reserves. These properties may allow the SHOSO oil to attain a higher value than normal crop oils which should be reflected in the form of economic benefits to the growers (https://www.csiro.au/en/Research/AF/Areas/Crops/Oil-crops/SHO-safflower).

5 Pharmaceutical Compound Production in GM Safflower

5.1 Peptide Production in GM Safflower

Safflower is also a source of pharmaceutical and nutritional compounds. Therefore, attention has been paid for genetic transformation of safflower for production of pharmaceutical and nutritional compounds. The formation of fusion protein 'apolipoprotein AI Milano ($ApoAI_{Milano}$)' has been achieved in transgenic safflower seeds (Nykiforuk et al. 2011). The protein has pharmaceutical significance and is believed to act as an important mediator of reverse cholesterol transport (RCT). The ApoAI plays an important role in the prevention of coronary heart disease. These findings are supported by the infusion of HDL particles and ApoAI liposomes, and

overexpression of the human *apoAI* gene inhibits the disease process in animal models of atherosclerosis (Shah et al. 1998). The expression cassettes consist of $ApoAI_{Milano}$ protein gene with single-chain antibody oleosin (scFv-D9). For tightly controlled expression in a tissue- and temporal-specific manner during seed development, phaseolin promoter terminator was fused with them. Incorporation of cassette was achieved with the help of *Agrobacterium*-mediated gene transfer method. The transgenic plants contained 7 g of $ApoAI_{Milano}$ per kilogram of seed. The developed transgenic line was also selected for commercialization. Further, it has been demonstrated that the extracted $ApoAI_{Milano}$ fusion protein from seed is used for the production of Des-1,2-Apo-$AI_{Milano.}$ The nature of the product is confirmed by immunore activity against ApoAI antibodies, isoelectric point, N-terminal sequencing and electrospray mass spectrometry. The results of the study revealed that the plant-based expression system can be used as an efficient platform to produce high levels of biologically functional $ApoAI_{Milano}$ (Nykiforuk et al. 2011).

5.2 Production of Modified Protein of Interest

SemBioSys Genetics, Inc., a Calgary-based company, is planning to use genetically transformed safflower tissue to produce a modified protein of interest in the seeds. Due to the unavailability of wild relatives for crossing to produce fertile hybrids, the transgenic safflower has high chances to get permission for cultivation. The problems were minimized since safflower exhibits low seed dormancy and large degree of self-pollination. All these characteristics help to avoid food and feed contamination risks (Mundel et al. 2004). Transgenic safflower was produced with recombinant human insulin and has been submitted by SemBioSys Genetics Inc., to the United States Food and Drug Administration (FDA) for approval. The group confirmed and submitted the claim that transgenic safflower produced insulin is equivalent to pharmaceutical-grade human insulin. The company is planning to begin clinical trials (Baum 2008). The company also intends to submit a Clinical Trial Application (CTA) to the European authorities and assuming approval of the CTA and trials in the UK (ISAAA 2017).

5.3 *CtCHI* Gene of Safflower and Bioactive Flavonoid Production

Flavonoids of safflower have therapeutic importance. The dried petal powder (carthami flos) of safflower is used in traditional Chinese medicine for the treatment of cardiovascular and cerebrovascular disorders (Guo et al. 2017). Later, the phytochemical study of dried petal powder of safflower suggested that the disease-curing property is due to the presence of bioactive metabolites hydrosafflower yellow A (HSYA) and carthamin a quinochalcone glucosides. It has been demonstrated that out of 23, 4 uni genes (5 PALs, 1 C4Hs, 5 4CLs, 6 CHSs, 2 CHIs, 2 DFRs, 2 FLSs) have been found involved in flavonoid pathway which are responsible for

quinochalcone glucoside accumulation at the floret developmental stage (Guo et al. 2017). One of the flavonoid biosynthetic pathway genes, chalcone synthase (*CtCHS1*) has been over expressed in safflower cultivar ZHH0119 (Y line) and XHH007 (W line) with the help of *Agrobacterium*-mediated pollen-tube pathway method. In the florets of transgenic plants, increased expression of PAL2, PAL3, CHS1, CHS4 and CHS6 was found with a concurrent decline in the expression of CHI1 and CHI2. This has resulted in ~20–30% increase in quinochalcone glucoside content but 48 and 63% decline in quercetin-3-β-D-glucoside and quercetin in the florets. The study demonstrates that the *CtCHS1* gene plays an important role in quinochalcone glucoside biosynthesis rather than flavonol biosynthesis.

In several plants, chalcone isomerase enzyme regulates flavonoid metabolism. Expression of *CtCHI* gene appears very high at flower bud stage in the cultivar Jihong No. 1 of safflower (Liu et al. 2019). The correlation analysis between *CtCHI* expression and flavonoid accumulation at various flowering phases indicate that *CtCHI* might play a potential role during flavonoid biosynthesis in safflower. Therefore, with the help of *Agrobacterium*-mediated gene transfer method, attempts were made to express flavonoid biosynthetic pathway gene that encodes the enzyme chalcone isomerase (CtCHI) of safflower in *Arabidopsis.* Overexpression of *pBASTA-CtCHI* in transgenic *Arabidopsis* is associated with increased flavonoid levels as detected by HPLC. Subcellular localization of CtCHI in cell membrane and nucleus has been reported with transient expression in tobacco mesophyll cells using green fluorescent protein tagging (Liu et al. 2019). It is proved that the gene *CtCHI* plays a significant role in flavonoid metabolism of safflower. Thus, overexpression of *CtCHI* gene in safflower may be the future target.

6 Transgenic Safflower Seed Meal for Animal Feed

Seed meals are the ground residues left after extraction of oil from seeds. Year to year, seed meal is used as one of the common livestock feeds. The Arcadia Biosciences, Inc., Davis, CA, USA, proposed the petition for safe use of seed meal from a variety of bioengineered safflower as cattle and poultry feeds (FDA 2010). A safflower variety has been genetically modified for the improvement of fatty acid profile with the increase of certain fatty acids with concomitant decline in others. Transfer of gene that encodes the enzyme δ-6-desaturase isolated from *Saprolegnia diclina* Humphrey (water mould fungus) to safflower enhanced the production of γ-linolenic acid in the seed oil. After extraction of seed oil from transgenic cultivars like Centennial, the residue left can be safely used as cattle and poultry feeds if it meets the criteria laid down by FDA (2015).

7 Disease Resistance in Transgenic Safflower Cultivar

Crop yield is drastically hampered by insect pests, fungi, bacteria, viruses and nematodes. Preventing this loss is one of the major challenges in food production and sustainable agriculture. It has been demonstrated that the pathogen attacks are

usually tissue-specific and can cause damage to the roots, stems, leaves, fruits and seeds. But, application of chemical agents to control the pests has their own limitations. Further, use of chemicals to control the diseases is not a cost-effective method. At the same time, it is harmful to the animals if such leaves or stems or seed extract is used as feed. Safflower is susceptible for certain insects and microorganisms (fungi, bacteria, viruses) (Kumar et al. 2009). Hence, an attempt has been made for the development of genetically modified safflower for resistance to fungal pathogen *Alternaria carthami* (Matern and Kneusel 1993, Kumar et al. 2009). *Alternaria carthami* causes severe loss of yield in safflower. Resistance has been achieved against pathogenic fungi in several crop plants with the overexpression of pathogenesis-related proteins (PRP). Also, chitinase gene from rice has been successfully introduced into safflower cultivar A1 with the help of *Agrobacterium*-mediated gene transfer method. Transgenic safflower exhibited resistance to the pathogen *Alternaria carthami* (Kumar et al. 2009). This achievement opens the door for the development of transgenic safflower with resistance to biotic stresses.

8 Commercial Cultivation of Genetically Modified (GM) Safflower

The permission for commercial cultivation of GM safflower is issued to GO Resources Pvt. Ltd., by the office of the Gene Technology Regulator (OGTR) in Australia. The GM safflower has high mono-unsaturated fatty acid oleic acid (92%) and low content of linoleic acid (2%). Therefore, it is possible to produce high-purity oleic acid which has an application in the industry as a replacement to petroleum-based precursor to manufacture plastics, lubricants and cosmetics. At the same time, the meal left can be used as a feedstock. The GM safflower has been established as a commercial cultivar and can be grown by farmers in Australia, barring few states and territories. The responsibility of food safety is given to Food Standards Australia, New Zealand (FSANZ). However, there is no intension to use the GM safflower for human consumption but has intention to use it only for industrial oil applications. There are reports that high oleic GM soybean oil is safe for human consumption, and it is approved by FSANZ (FSANZ 2011). The risk assessment authorities concluded that GM safflower poses negligible risks to the health and safety of people or the environment. Therefore, licence was issued by the OGTR for an ongoing commercial release of GM safflower (OGTR, http://www.ogtr.gov.au/).

9 Future Prospects

Safflower is one of the oldest and best edible oilseed crops in human history. The crop is well adapted to semiarid regions of the world, does not need much irrigation or care and can withstand drought and low moisture stress. However, farmers find it difficult to grow due to non-availability of ready market, and poor economic

benefits. Further, due to spines, labour for harvesting the seeds demands higher wages compared to other crops. Also, the crop is susceptible to various biotic stresses such as aphids. Since it is heart-friendly oil, there is a need to create awareness among the public for the widespread use of safflower oil. A study report from NIN (National Institute of Nutrition), Hyderabad, suggested that among the 13 most used edible oils, safflower oil is the best cooking medium followed by sunflower oil, mustard oil and soybean oil.

Besides, safflower is also a major source of natural water soluble, yellow pigment (carthamidin, $C_{16}H_{20}O_{11}$) and water-insoluble red pigment (carthamin, $C_{12}H_{22}O_{11}$). Both red and yellow pigments are useful for colouring cloths and food stuff. These pigments have been used in traditional medicine for thousands of years. They are raw material of cosmetics and herbal medicine for feminine diseases. The active compounds red and yellow pigments which have been experimentally shown to enrich blood, to decrease fatigue, and to promote menstruation (Akihisa et al. 1994). Safflower pigments have been safe for use in processed foods and soft drinks (Meselhy et al. 1993). The safflower yellow and red pigments are emerging as a new source of pharmaceuticals and colouring agents due to their non-allergic and non-carcinogenic properties (Ghorbani et al. 2015). The production of these pigments has been achieved in in vitro cultures of safflower by various authors (Gao et al. 2000; Chavan et al. 2011; Ghorbani et al. 2015). However, the genetic modification was not attempted for improvement of production of these pigments. Efforts have been made for the improvement in fatty acid profile and pharmaceutical compounds in safflower using genetic transformation protocols (Fig. 2). But still there are

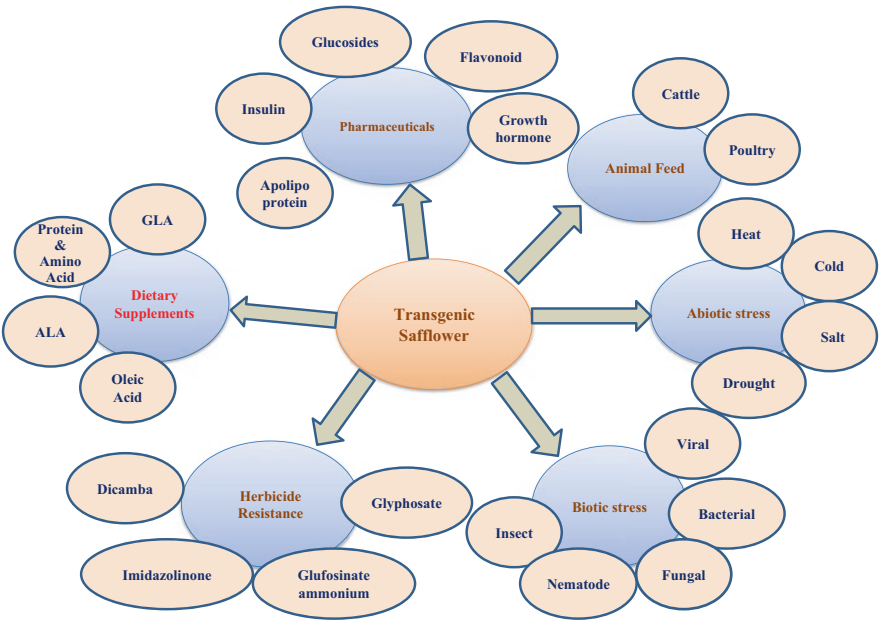

Fig. 2 Improvement of different traits in transgenic safflower

difficulties associated with high-frequency organogenesis/embryogenesis, hyperhydricity in cultures and low-frequency rooting of shoots. If we trounce these barriers, then the percent frequency of transformed plants can be increased. Application of biotechnological approaches and genetic engineering technologies showed drastic improvements in safflower. This will help farmers to give preferences for commercial cultivation of desirable safflower cultivars. Compared to other edible oils, safflower oil has special benefits due to its unique oil composition. Hence, bringing awareness among the public for its widespread utilization as a cooking oil is the key in our attempts to promote safflower cultivation programs. Also, in the larger interests of society, we need to accept genetically modified safflower while keeping the environmental risks to the minimum.

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Nutritional Value, In Vitro Regeneration and Development of Transgenic *Cucurbita pepo* and *C. maxima* for Stress Tolerance: An Overview

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Abstract

The *Cucurbita* genus, often called as cucurbits, include several economically important fruits and vegetable crops like cucumber, gourds, squash, watermelon, pumpkin, and melon. Several of the species including *Cucurbita pepo* and *C. maxima* have nutritional value and are utilized in folk medicine for treating gastrointestinal diseases and intestinal parasites. Such an activity is attributed to the presence of fatty acids, glycosides, resins, sterols, carotenoids, phenols, tocopherols, saponins, steroids, and terpenoids such as cucurbitacins. Squash and pumpkin are the major members of Cucurbitaceae family used as food and animal feed. The two major pumpkin varieties *C. pepo* and *C. maxima* are considered as highly polymorphic with manifold nutritional, including food preservative abilities and medicinal activities. Development of transgenic pepo for tolerance to various biotic and abiotic stresses coupled with nutritional value is the need of the hour. Very few reports are available on transgenic production of pepo. The present review summarizes the regeneration and transformation protocols for developing transgenic *C. pepo* and *C. maxima* and future prospects.

Keywords

Cucurbita pepo · *Cucurbita maxima* · Transgenics · Virus resistance · Yield improvement

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1 Introduction

The genus *Cucurbita* belongs to the family Cucurbitaceae (gourd family) and is native to the Americas. The family comprises nearly 130 genera and 800 species. The genus *Cucurbita* is cultivated in tropical and subtropical countries and comprises five major economically important crop species, viz., *C. argyrosperma* Huber, *C. ficifolia* Bouche, *C. maxima* Duchesne, *C. moschata* Duchesne, and *C. pepo* L. (Nee 1990; Smith 2001; Sanjur et al. 2002; Nanasato et al. 2011; Maynard and Paris 2018; Chomicki et al. 2019). The family Cucurbitaceae includes several herbaceous vines and diverse important species like pumpkins, squashes, gourds, and melons (Shah et al. 2010). Among them, cucumber (*Cucumis sativus* L.), watermelon [*Citrullus lanatus* (Thunb.) Matsum. & Nakai)], bitter gourd (*Momordica charantia*), bottle gourd (*Lagenaria siceraria*), and pumpkin/squash (*Cucurbita* spp.) are popular, economically important with a rootage of varied valuable products including drugs. Cucurbits are rich in carbohydrates, proteins, fatty acids, and antioxidants. *Cucurbita* products not only have nutritional value but also are used in culinary for biscuits, disserts, soup, and beverage production (Paris 1989; Dubey and Dubey 2012; Andolfo et al. 2017). *Cucurbita*, in Latin, means "gourd" and *pepo* means "large melon, pumpkin" (Mahoney 2002-2014). *Cucurbita pepo* is a phenotypically and genotypically polymorphic species of the *Cucurbita* genus, and its fruits are divided into eight groups which are equally distributed into two subspecies, *C. pepo* subsp. *pepo* and *C. pepo* subsp. *ovifera* (L.) D.S.Decker. Subspecies *pepo* has the cocozelle, pumpkin, vegetable marrow, and zucchini types, while subsp. *ovifera* has acorn, crookneck, scallop, and straightneck type fruits (Paris 2001; Xanthopoulou et al. 2019). *Cucurbita pepo* (squash) and pumpkin are highly polymorphic vegetables with a great consequence in food (Kathiravan et al. 2006) and pharmaceutical industries (Damiano et al. 2016). Three major pumpkin varieties, including *C. pepo*, *C. maxima*, and *C. moschata* are polymorphic in nature with antimicrobial activity (Adeel et al. 2014; Dinu et al. 2016). *Cucurbita* crops are consumed as food and also incorporated into processed foods. The pumpkin waste, a nutritional food produced in million tonnes is used as animal feed which reduces the dependency of livestock for cropland. The bioactive compounds present in the waste improve the nutritional value of egg, meat, and milk (Valdez-Arjona and Ramírez-Mella 2019). *Cucurbita* species serve as model plants for studying sex determination, vascular development, and fruit ripening (Lough and Lucas 2006; Pech et al. 2008; Bhowmick and Jha 2015; Sui et al. 2018). Cucurbit crops are also used in making medicines and musical instruments (McCreight 2017). Apart from this, antibacterial, antioxidant, and antiparasitic activities are reported in *Cucurbita* species (El-Aziz and El-Kalek 2011; Zhou et al. 2014).

2 Origin and Cultivation of *Cucurbita* spp

Members of Cucurbitaceae family are extensively cultivated in tropical and subtropical countries. *Cucurbita* species include pumpkins, squashes, gourds, and melons (Shah et al. 2010). They are mostly native to the American countries especially Mexico. Archeological studies observed that *Cucurbita* was domesticated to southern parts of Canada to Argentina, and Chile for more than 8000 years (Paris 1989; Dubey and Dubey 2012; Adnan et al. 2017; Andolfo et al. 2017). The current world production of cucurbits (including squash and gourds) in 2016 is more than 256 million tonnes (http://faostat.fao.org). Asia produces the highest yield (87.2%) of pepo followed by Europe (7.9%), America (2.9%), Africa (1.9%), and Oceania (Fig. 1). Within Asia, China dominates the market with 8.1 million tonnes from 4.4 lakh hectares followed by India with 5.5 million tonnes from 5.8 lakh hectares. *C. pepo* is cultivated across the globe as a dominant major crop, and the different types of cucurbits cultivated in all the geographical regions are represented in Table 1.

C. maxima is one among the four cultivated species of squash and the most diverse domesticated crops (Nee 1990; Ferriol et al. 2004). The species has originated in South America over 4000 years ago (Oris et al. 2002). There are several cultivars that have been developed in *C. maxima*. For example, Arikara squash is an heirloom variety with a fruit size weighing 2–6 kg. The shape of the fruit in this cultivar is usually tear-drop or round, and the color is mottled orange and green, and known for its eating qualities and seasonal decoration. Similarly, buttercup squash is a common cultivar with a flattish top and dark green skin weighing about 1.5–3 kg. On the other hand, Jarrahdale pumpkin has gray-colored skin. Kabocha is a Japanese cultivar with dark green skin and bright golden-orange flesh. Native American tribes have introduced different squash types into North America. Later, secondary centers of diversity were established with India, Bangladesh, Myanmar, and others (Nee 1990). Buttercup squash is roasted, baked, and mashed into soups. Likewise, it is popular as a vegetable and soup in several countries of South America and Africa.

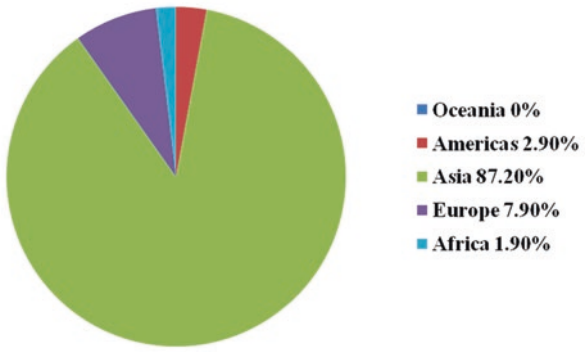

Fig. 1 Production of *Cucurbita pepo* across the globe

Table 1 List of cultivated *Cucurbita* and their geographical distribution

S. no.	Cultivated cucurbits	Common name	Geographical distribution
1.	*Cucurbita pepo*	Field pumpkin, summer squash, zucchini, vegetable marrow, courgette, acorn squash	Mexico, USA
2.	*C. argyrosperma*	Cushaw pumpkin	Panama, Mexico
3.	*C. kellyana*	Cushaw pumpkin	Pacific coast of western Mexico
4.	*C. palmeri*	Cushaw pumpkin	Pacific coast of western Mexico
5.	*C. digitata*	Finger leaf gourd	Southwestern United States (USA), Northwestern Mexico
6.	*C. ficifolia*	Finger leaf gourd, chilacayote	Mexico, Panama, Northern Chile, Argentina
7.	*C. foetidissima*	Stinking gourd, buffalo gourd	Mexico
8.	*C. maxima*	Winter squash, pumpkin	Argentina, Bolivia, Ecuador
9.	*C. moschata*	Butternut squash, "Dickinson" pumpkin, golden cushaw	Bolivia, Colombia, Ecuador, Mexico, Panama, Puerto Rico, Venezuela

Some of the pumpkins attain a size of 1190 kg, and the seeds of such pumpkins are used in treating parasites in animals (Obregon et al. 2004; World Record Achievements 2014). Pumpkin contains nearly 24.5 g of proteins besides 45.8 g of total lipids per kilogram of dry seeds (Salehi et al. 2019). Pumpkins are usually processed into flour which has a better shelf-life. The flour can be used for its flavor, sweetness, color, and dietary fiber (Salehi et al. 2019). The flour can be a supplement to cereal flours, bakery products, soups, sauces, and noodles (Noor-Aziah and Komathi 2009; Noor-Aziah et al. 2011). Pumpkin flours are rich in crude protein, crude fat, lutein, zeaxanthin, and fiber and hence suitable to enrich food with dietary fiber (Kulaitiene et al. 2014). Fruits of *C. maxima* are rich in vitamin C, vitamin E, minerals, pectins, and carotenoids (Kim et al. 2012; Kulaitiene et al. 2014).

3 Phytochemical Composition of *Cucurbita pepo*

C. pepo contains diverse types of carotenoids like α, β, and γ-carotene, neoxanthin, violaxanthin, lutein, zeaxanthin, taraxanthin, luteoxanthin, auroxanthin, neurosporene, flavoxanthin, 5,6,5′,6′-diepoxy-β-carotene, phytofluene, α-cryptoxanthin, and β-cryptoxanthin in fruit tissues. Table 2 shows different concentrations of these carotenoids in *C. pepo*. *Cucurbita* seeds are edible and rich in tocopherol (α-, β-, γ-, and δ-tocopherol), having concentrations ranging from 38.03 to 64.11 mg/100 g (Rabrenovic et al. 2014). *Cucurbita* species are also rich in carbohydrates, proteins, minerals, and fatty acids, and the compositions are shown in Tables 3 and 4.

Table 2 Major carotenoid contents present in different *Cucurbita* varieties

Cucurbita spp. varieties	α-Carotene (mg/100 g)[a]	β-Carotene (mg/100 g)[a]	Lutein + zeaxanthin (mg/100 g)[a]	References
Acorn Tay Bell	0.17	0.94	0.37	Murkovic et al. (2002), Kurz et al. (2008), Itle and Kabelka (2009)
Tonda Padana (Americano)	0.12	2.3	1.5	
Carneval di Venezia	0.03	0.06	ND	
Melonette Jaspée Vende	0.05	1.3	0.43	
Acorn Table	ND	0.36	0.09	
Table King Bush	ND	0.09	0.02	
Thelma Sander's Sweet Potato	ND	0.06	0.01	
Fordhook Acorn	ND	0.04	0.01	
PI 314806	ND	ND	ND	
Sweet Lightning	NQ	0.7	0.13	

[a]Values are expressed in dry base of edible flesh part; *ND* not detected

Table 3 Phytocomposition of *Cucurbita pepo*

	Cucurbita pepo			
Percentage (%)	Flesh	Peel	Seed	References
Carbohydrates	2.62–48.40	4.37–19.45	6.37–37.9	Murkovic et al. (1996), Amoo et al. (2004), Mitra et al. (2009), Younis et al. (2000), Badr et al. (2011), Noelia et al. (2011), Elinge et al. (2012), Mi et al. (2012), Jarret et al. (2013), Jacobo et al. (2015), Gutierrez (2016)
Protein	0.20–15.50	0.92–23.95	27.48–38.0	
Lipid/oil	0.055–0.18	0.47–6.57	21.9–54.9	
Fiber	0.37–11.25	1.23–29.62	1.00–14.84	
Ash	0.34–06.64	0.63–10.65	3.0–5.50	
Moisture	18.03–96.77	9.76–93.59	1.80–7.40	
Minerals (μg/g dry weight)				
K^+	160	2372.4		
P	11.38	476.8		
Ca^{2+}	3662	97.8–420.5	97.8–420.5	
Mg^{2+}	190	674.1		

4 Phytochemical Composition of *C. maxima*

C. maxima is a natural source of some bioactive components. The species contains carotenoids (mostly in the fruit peel) and tocopherols in the seeds (Mi et al. 2012). Different species vary in the quantity and quality of carbohydrates, proteins, minerals, fatty acids, and amino acids (Mi et al. 2012). Compared to that of flesh, seeds are rich in carbohydrates (12.90–24.45 μg/g), protein (14.31–27.48 μg/g), oil (30.70–52.43 μg/g), fiber (2.56–16.15 μg/g) (Salehi et al. 2019), K^+ (358.67 μg/g),

Table 4 Seed fatty acid composition of *Cucurbita pepo*

Fatty acid (%)	*Cucurbita pepo*	References
Myristic acid (C14:0)	0.1–0.23	Murkovic et al. (1996), Mitra et al. (2009), Elhardallou et al. (2014), Badr et al. (2011), Mi et al. (2012), Jarret et al. (2013), Gutierrez (2016)
Palmitic acid (C16:0)	9.5–14.5	
Palmitoleic (C16:1n7)	0.58	
Heptadecanoic acid (C17:0)	ND	
Stearic acid (C18:0)	03.1–8.67	
Vaccenic acid (C18:1n7)	01.8	
Oleic acid (C18:1n9)	21.0–46.9	
Linoleic acid (C18:2)	0.17–60.8	
Linolenic (C18:3)	ND	
Arachidic acid (C20:0)	0.39	
Gadoleic acid (C20:1n–9)	00.1–1.14	
Arachidonic acid (C20:4)	0.05	
Behenic acid (C22:00)	0.37	
Saturated	18.69–19.35	
Mono-unsaturated	32.40	
Poly-unsaturated	36.40	
Total unsaturated	7.6–80.65	

and Zn^{2+} (39.85 μg/g) (Elinge et al. 2012). Among the fatty acids, linoleic acid (34.77–56.60) and mono-unsaturated fatty acids (14.90–44.12) are very high in the seeds (Amoo et al. 2004; Kim et al. 2012).

5 Development of Protocols for In Vitro Plant Regeneration in *C. pepo* and *C. maxima*

Protocols were developed for *Cucurbita* plant regeneration via indirect somatic embryogenesis from cotyledons (Jelaska 1972). It was induced from shoot apex (Chee 1991) and cotyledons (Gonsalves et al. 1995). Direct organogenesis has been developed in 4 weeks with an efficiency of >50% using proximal regions of cotyledonary explants on Murashige and Skoog's medium (MS) (Murashige and Skoog 1962) supplemented with 1 mg/L 6-benzylaminopurine (BAP) (Ganapathi and Perl 2000; Ananthakrishnan et al. 2003; Lee et al. 2003; Vengadesan et al. 2005; Kathiravan et al. 2006; Zhang et al. 2008; Kim et al. 2010). The average regeneration efficiencies obtained were 62.5 ± 19.1% for *Cucurbita pepo* (Nanasato et al. 2013). Effective root system was induced in shootlets by incorporating 0.5 and 1.0 mg/L indole-3-acetic acid (IAA), or 0.5 and 1.0 mg/L naphthalene acetic acid (NAA) and also a combination of both (Shah et al. 2008). Plant regeneration via organogenesis was also achieved for two cultivars of *C. maxima* (Lee et al. 2003). Optimum plant regeneration was recorded from cotyledonary explants isolated from 4-day-old seedlings of *C. maxima*. They achieved 82 and 92% frequencies of regeneration on Murashige and Skoog's (MS) medium supplemented with 1 mg/L 6-benzyladenine. Shoots were rooted successfully on MS medium devoid of any growth regulators (Lee et al. 2003).

6 *Agrobacterium*-Mediated Genetic Transformations in *Cucurbita pepo*

Agrobacterium-mediated transformation was achieved for C-repeat binding factor 1 (CBF1) and *npt-II* genes in *Cucurbita* using shoot tip explants (Shah et al. 2008) on medium containing 150 mg/L kanamycin as a selectable agent. Tricoll et al. (1995) obtained transgenic *C. pepo* transfected with virus coat protein which conferred virus resistance with unknown transformation efficiency. Shah et al. (2008) recorded 0.7% efficiency via shoot organogenesis. Transformation efficiency increased by 2.7% with the addition of aluminum borate whiskers at 1% (w/v) (Nanasato et al. 2011). Vacuum infiltration method was employed to wounded explants which enhanced the transformation efficiency by threefolds (9.2 ± 2.9%) (Nanasato et al. 2013). Based on the results obtained, Ilina et al. (2012) suggested that composite *Cucurbita pepo* plants with transgenic roots can be used as a tool to study the development of roots.

7 *Agrobacterium*-Mediated Genetic Transformations in *Cucurbita maxima*

Cucurbita species are generally refractory to genetic transformation. Efficient and stable transformation protocols for *C. maxima* are meager (Ramirez-Ortega et al. 2015). Fu et al. (2010) studied the factors that influence *Agrobacterium*-mediated genetic transformation from cotyledonary explants of *C. maxima*. They optimized pre-culture time, infection time, and concentration of carbenicillin, cefotaxime, and kanamycin. They noticed high transformation frequency without any preculture treatment, *Agrobacterium* infection for 30 min, 100 mg/L acetosyringone, and coinfection for 5 days. The experiments conducted by Nanasato et al. (2013) suggested that cells with regeneration potential exist in the deeper layers of explants. Therefore, they applied vacuum infiltration to wounded explants in order to enhance *Agrobacterium*-mediated transformation. This technique has resulted in an increase of transformation efficiency from 3 to 9%. They noticed 54.2% regeneration efficiency and 0.2–0.3% transformation frequency of in *C. maxima*. Thus, it is suggested to use vacuum infiltration technique in order to achieve higher transformation efficiencies in *C. maxima* and other species.

8 Transgenic *C. pepo* for Biotic Stress Tolerance

Environmental stresses adversely affect the plants due to their sessile in nature. *C. pepo* is affected by many viral diseases which reduce the productivity and fruit quality. Cucumber mosaic virus (CMV), zucchini yellow mosaic potyvirus (ZYMV), watermelon mosaic 2 potyvirus (WMV 2), and papaya ringspot potyvirus type w (PRSVw) are the most important viruses that affect squash (*C. pepo*). Fuchs et al. (1998a) developed five transgenic squash lines expressing viral coat protein (CP) genes. They analyzed the transgenics in the field for their response to mixed

infections by CMV, ZYMV, and WMV 2. Among the five lines, CZW-3 expressing the CP genes from CMV, ZYMV, and WMV 2 exhibited higher resistance. Also, the transgenic line ZM-20 produced a 40-fold increase in marketable yield in comparison with wild-type plants. This study indicated that viable transgenic *C. pepo* lines can be developed that are virus resistant. However, if such lines are economically viable, they must be released to the farmers if they are safe both to animal and human health and to the environment. Fuchs et al. (1998b) also developed transgenic melon and squash containing the viral coat protein gene of the aphid transmissible strain WL of CMV. Transgenic squash line ZW-20 was grown under field conditions and found resistant to ZYMV and WMV 2 strains but susceptible to CMV. Such field experiments determined the potential use of transgenes for bringing out changes in virus-vector specificity. These transgenic lines have potential for commercial release and therefore of practical relevance. Transgenic summer squash *C. pepo* cultivars resistant to ZYMV, WMV, and/or CMV were developed by *Agrobacterium*-mediated transformation method with the appropriate viral coat protein (CP) gene sequences (Tricoll et al. 1995). While the squash resistant to ZYMV and WMV (Watermelon mosaic virus) were deregulated in 1996, squash resistant to CMV (Cucumber Mosaic Virus), ZYMV, and WMV in 1998 in the USA. Deregulated GEVR (Genetically Engineered Virus Resistance) summer squash has also been used as parents in conventional breeding to develop 11 virus-resistant summer squash cultivars. Qi (2011) developed *C. pepo* inbred line HSPMR7B1 with mutant allele Pm2 which exhibited resistance against powdery mildew. It has been predicted that gene flow from transgenic virus-resistant *C. pepo* plants to conventionally bred squash plants would increase *C. pepo* fecundity (Laughlin et al. 2009). Such an assessment would help us to assess the environmental risks of genetically engineered virus-resistant plants (Spencer and Snow 2001). They studied the fecundity of transgenic wild-crop hybrids of *C. pepo*, and the results infer that the F_1 generation does not represent a barrier to the introgression of beneficial crop genes into free-living populations of *C. pepo*.

In *Cucurbita*, pollen performance and their growth activities are severely affected by viral pathogens (Harth et al. 2016). Infection of zucchini yellow mosaic virus (ZYMV) limits the establishment and also severity of powdery mildew in wild populations (Harth et al. 2018). ZYMV is an economically important, seed-transmitted pathogen of cucurbits transmitted both horizontally and vertically. Fewer backcrossed transgenic *Cucurbita* plants displayed development of the symptoms (7%) in comparison with wild-type plants (26%) (Simmons et al. 2015). Ibaba et al. (2015) developed transgenic *C. pepo* plants resistant to three potyviruses that infect cucurbits frequently. The three viruses are ZYMV, *Moroccan watermelon mosaic virus* (MWMV), and *Zucchini shoestring virus* (ZSSV), common in South Africa. They used a viral coat protein gene for the purpose. Cotyledon explants were transformed using *Rhizobium*-mediated transformation with kanamycin selection pressure. All the 76 PCR-positive transgenic lines displayed resistance to viruses. The results suggest that genetic transformation is an effective

strategy to control potyvirus diseases in cucurbits. Harth et al. (2018) found that transgenic *C. pepo* plants are more resistant to powdery mildew because of the transgene. It conferred resistance to ZYMV with enhanced levels of salicylic acid. Protocols were developed to assess the risks of releasing genetically modified *Cucurbita* species. The risk of releasing transgenic *C. pepo* to the field conditions in Mexico was analyzed for 15 taxa of this genus. Concern was raised for growing transgenic *C. pepo* in those areas where sexually compatible relatives like *C. pepo*, *C. moschata*, *C. argyrosperma*, *C. fraterna*, and *C. ficifolia* are grown (Arriaga et al. 2006). Nevertheless, it appears that transgenic multiple virus-resistant cucurbit species are available, and multiple companies have applied for permission for field tests. It also implies that such crops can be marketed which will have an impact not only on plant productivity but also on the reduction of ecological damage caused due to chemicals used to control viruses.

9 Transgenic *C. pepo* for Hydrophobic Pollutants

Some cultivars of Cucurbitaceae such as melons, pumpkins, and zucchini are uniquely subjected to contamination of hydrophobic pollutants like organohalogen insecticides (DDT) and accumulate them. The molecular mechanisms for accumulation of these pollutants have not been detected so far. But, cDNA expression analysis of some *Cucurbita pepo* cultivars showed higher expression levels of zinc finger proteins (ZFPs) during accumulation of these pollutants. Cloned *CpZFP* genes were classified into two types: PBG (expressed in the cultivars like Patty Green, Black Beauty, and Gold Rush), and BG types (expressed in the cultivars like Black Beauty and Gold Rush) conferred the ability to accumulate hydrophobic contaminants (Inui et al. 2015).

10 Transgenic *C. maxima* for Stress Tolerance

Transgenic *C. maxima* with 16 kDa phloem protein CmPP16 exhibited tolerance to drought conditions via phloem transport with a higher photosynthetic activity compared to wild-type plants (Ramirez-Ortega et al. 2014). Ramirez-Ortega et al. (2015) also reported an efficient genetic transformation protocol from the cotyledonary explants. They achieved transformation via direct inoculation of *A. tumifaciens* and *A. rhizogenes*. *C. maxima* epicotyl explants were transfected with maize KNOTTED1 (*KN1*) gene fused with green fluorescent protein (GFP) under the control of vascular-specific promoter *rolC*. Efficiency of transformation ranged between 17.6 and 56%. Transgenics displayed leaf deformations such as leaf crumpling and lobed leaves (Ramirez-Ortega et al. 2015).

11 Conclusion and Future Perspectives

Cucurbits include diverse species with varied nutritional and medicinal values. Squash and pumpkin are the major members of Cucurbitaceae family, used as food, animal feed, and medicines. Development of transgenic pepo for tolerance to various biotic stresses especially for virus resistance coupled with improved nutritional value and enhanced shelf-life of fruit is the need of the hour. Very few reports are available on transgenic production of *C. pepo* and *C. maxima*. Transgenics were developed for tolerance to biotic stresses like powdery mildew, zucchini yellow mosaic virus, and cucumber mosaic virus. Transgenics also displayed higher expression levels of zinc finger proteins alongside the accumulation of pollutants. In such cases where commercial transgenics for viral resistance were developed, such GM plants must be released for the benefit of farmers, after evaluating their ecological safety. Thus, only a handful of genes were transferred till date, so huge potential exists for the improvement of fruit yield, viral resistance, and nutritional quality in both *C. pepo* and *C. maxima* using genetic engineering technologies. There is also a need to enhance the content of cucurbitacins using the methods of genetic engineering, since they exhibit anti-tumor, anti-inflammatory, anti-atherosclerotic, and anti-diabetic effects (Kaushik et al. 2015). This makes a great matrix to be exploited further for preventive as well as therapeutic purposes.

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Sugarcane Transgenics: Developments and Opportunities

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Abstract

Sugarcane is an important, commercial crop with potential industrial and bioenergy perspective. Despite considerable advancements in varietal improvement made using conventional breeding programmes, further genetic improvement of elite sugarcane genotypes is necessary. However, genetic improvement is restricted due to the genetic complexity, low fertility, non-flowering nature, and long duration of the crop. In this context, sugarcane biotechnology offers a valuable tool to introduce commercially important traits into elite genotypes. Redesigning of sugarcane varieties with tolerance to biotic (pathogen and insect pests), abiotic stresses (herbicide, drought, salinity, salt, etc.) and quality parameters (sucrose accumulation) by using genetic engineering offers a better way for tailoring the genetic architecture of plants. Advanced methodologies are now available to generate transgenic sugarcane with novel genes for desirable agronomic attributes. Genetically modified sugarcane has been approved for commercial cultivation in Indonesia and Brazil, and in other countries, transgenic products are in different stages of field trials and/or commercialization. These include transgenics with genes conferring resistance to diseases and pests, salt and drought tolerance, and high sucrose or herbicide tolerance. Sugarcane is also considered as a "biofactory" for the production of high-value bioactive com-

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pounds due to high biomass production potential. Many studies have shown the generation of products of industrial relevance in sugarcane. Transgenic technology offers potential opportunities for achieving increased productivity and resistance/tolerance to biotic/abiotic stresses and production of novel products in sugarcane for industrial and nonindustrial purposes.

Keywords

Sugarcane · Genetic transformation · Genetically modified crops · Diseases · Abiotic stress · Biofactory

1 Introduction

Agriculture production in India has increased considerably during the last five decades due to the development and large-scale cultivation of new higher-yielding varieties (Pingali 2012). This has resulted in self-sufficiency in food production and contributed tremendously to food security. However, it is projected that food security of this region might be at risk shortly due to increasing population and pressure for alternate land uses. Agricultural biotechnology is a tool for increasing food production and also making agriculture more sustainable from an environmental point of view (Hansson and Joelsson 2013).

Among the important agricultural crops, sugarcane is the second most important commercial crop in India. Sugarcane, botanically known as *Saccharum* sp., is a hybrid, tropical crop and is grown under diverse climatic conditions from sea level to 1500 m between 36.0N and 31.10S of the equator. It prefers a long warm growing season and a dry summer, cool but frost-free, ripening, and harvest season. In addition to sugar, sugarcane produces useful raw materials for many industries which utilize its different components to produce jaggery, khandsari, and a range of agro-industrial co-products, viz. alcohol, chemicals, paper, cattle feed, etc. In India, sugarcane is grown largely in the Indo-Gangetic plains of the subtropical region that faces extreme climatic conditions resulting in a low cane yield. *Saccharum officinarum* is cultivated in the tropical region, and *S. barberi* Jeswiet and *S. sinense* Roxb. are cultivated in the subtropical region.

Early attempts in the twentieth century were made to breed superior cultivars through interspecific hybridizations between *S. officinarum* (noble cane), the high sugar-containing species grown in the tropics and *S. barberi* Jeswiet, the north Indian species. For further improvement, the wild species *S. spontaneum*, known for its hardiness, was utilized in interspecific breeding programs to evolve cultivars for the subtropical region. *S. officinarum* contributed to high sucrose content, whereas *S. spontaneum* clones were used for the introgression of traits like ratoonability and increased adaptability. The hybrids were backcrossed to the *S. officinarum* parents to obtain high-sugared clones with improved stress tolerance. The hybrids thus obtained were intercrossed to evolve the existing commercial varieties. Thus,

conventional breeding approaches have been successful in producing high-yielding sugarcane varieties with resistance to disease and pests, which has sustained sugarcane productivity in the country (Mirajkar et al. 2019). Though tremendous achievement was made through conventional sugarcane improvement programmes, further genetic improvement of elite sugarcane genotypes by conventional breeding is difficult due to the complex polyploidy genome, poor fertility, narrow genetic variability, slow breeding gain, and the long time period (12–14 years) required for developing new cultivars. Traditional backcrossing to recover elite genotypes with desired agronomic traits is difficult due to low fertility, non-flowering, and complex environmental interactions and the process is very time-consuming. In this context, sugarcane biotechnology through genetic engineering is a very valuable tool to introduce commercially important traits into elite genotypes (Ming et al. 2010; Mirajkar et al. 2019). Some of the breeding objectives and traits for improvement are given in Fig. 1.

Recent advances in biotechnology of sugarcane highlight that the crop is on the threshold of genetic revolution as potential applications and benefits of molecular technology are being realized (Lakshmi et al. 2016). Among the new technologies, genetic transformation is the most important with considerable scope for trait-based improvement. High-yielding sugarcane varieties are confronted by various biotic stresses (fungi, bacteria, viruses, insects, nematodes) which cause huge loss in production and productivity. In this regard, genomic interventions and omics approaches can have a role in contributing to development of resistant germplasm (Devarumath et al. 2013, 2019). Since the first report of plant genetic transformation of tobacco (Herrera-Estrella et al. 1983), plant genetic manipulation has become the choicest

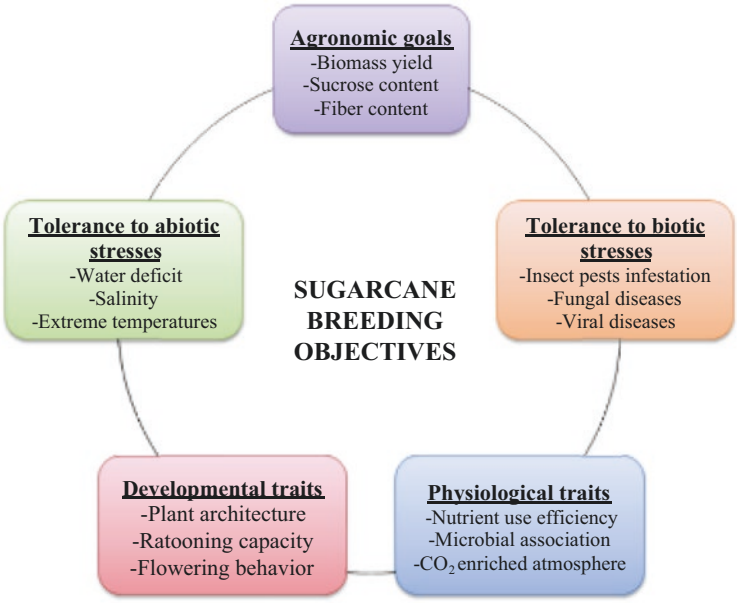

Fig. 1 Sugarcane breeding objectives and traits for improvement (Mirajkar et al. 2019)

tool for plant biotechnologists. Genetically modified (GM) crops have been developed for resistance to certain diseases, pests, herbicide tolerance, environmental conditions besides increase of nutritional value, bioremediation, production of biofuels, and pharmaceutical proteins. More than 191 million hectares of the world's croplands is planted with GM crops (ISAAA 2018, http://www.isaaa.org/resources/publications/briefs/54/default.asp).

2 Importance of Sugarcane Production and Constraints

Sugarcane production is considered a livelihood for millions of sugarcane producers and workers. However, its production is affected by both biotic stresses (pathogen and insect pests) and abiotic stresses like herbicide, drought, salinity, salt, and heavy metals (Shrivastava and Srivastava 2012). These stresses not only widen the gap between the mean yield and the potential yield but also cause yield instability in agriculturally important crops. Of the many factors affecting sugarcane production, drought is one of the main factors that restrict sustained sugarcane production. It is a rainfed crop, which depends heavily on the amount and duration of precipitation, humidity, moisture content, and temperature and soil conditions (Gawander 2007). During its 270–365 days of growth, the demand for water is high (1000–1500 mm). Development of sugarcane plant is divided into four distinct phases, i.e., germination, tillering, grand growth, and maturity. Tillering and grand growth phases are most susceptible to water stress. Ironically, these phases are also critical for sugarcane productivity. During these critical phases, water stress directly affects final yield through the reduction of growth, dry matter accumulation, cane yield, and juice quality (Naidu and Sreenivasan 1987). Due to this, the shortage of water adversely affects the crop yield. Genetic modification could reduce the water requirement of crops since traits that are used for genetic manipulation increase the rate of photosynthesis and depth of root structure and decrease the rate at which water is lost through transpiration. There is thus a greater need to understand the mechanism of the stress responses and means to reduce the impact through invigorating the plant for improving productivity.

Typically, sugarcane responds to water stress in the form of reduced length of stalk, decrease in shoot branching, and reduction in leaf senescence. Interestingly, a mild drought stress can have positive impact on sugarcane yield (Gentile et al. 2015). Withholding the irrigation before the harvest is an important strategy to enhance sucrose content in stalk. But if the water deficit becomes too severe, photosynthesis is inhibited resulting in lowering of cane and sucrose yields (Robertson et al. 1998). Sugarcane is a C_4 plant with high water use efficiency and has the capacity to maintain the leaf photosynthesis even with closed stomata. Studies undertaken with C_4 plants have shown that enzymes that comprise the metabolic CO_2 pump are more resistant to water deficit than the enzymes of C_3 photosynthesis (Ghannoum 2009). Despite the above facts, sugarcane is prone to drought-induced inhibition. Also, Andrade et al. (2015) suggested that patterns of gene expression in

sugarcane vary in different genotypes classified as drought tolerant indicating that there is high degree of complexity in response of sugarcane to water stress.

Large area under sugarcane production is susceptible to severe water stress, thus affecting sugarcane at one or the other stage of growth, suggesting that sugar yield and productivity are severely affected under limited moisture. In this case, biotechnology can provide a sustainable solution by enabling us to make efficient use of limited water resources. In India during 2001–2002 to 2015–2016, the area under sugarcane cultivation rose from 4.411 to 4.927 million hectares, cane production from 297.208 to 348.448 million tons, and sugar production from 18.528 to 25.125 million tons, respectively. In spite of the increase in area under cane cultivation and production, there is not much increase in sugar production (Co-operative Sugar, May 2017). This is expected to increase by at least 15–20% by 2020 to meet the need of increasing population dynamics. Sustainable cane production under abiotic and biotic stress conditions, nutrient management practice and improved sugar recovery are some of the concerns for the breeder. Until now, all existing commercial sugarcane varieties are developed by conventional breeding methods. This has resulted in the development of new varieties with certain characters like yield, high sucrose content, tolerance to abiotic stresses, and resistance to biotic stresses.

2.1 General Aspects of Genetic Transformation in Sugarcane

Sugarcane tissue culture is a prerequisite for developing efficient genetic transformation methods. Heinz and Mee (1969) first established the tissue culture system in sugarcane. Chen et al. (1987) first reported sugarcane transformation by introducing chloramphenicol acetyltransferase (*CAT*) gene into sugarcane protoplasts by electroporation method, but only callus was developed expressing *CAT* gene. Success in sugarcane transformation followed the development of microprojectile system by Bower and Birch in 1992. In this study, *npt*II gene was incorporated into sugarcane callus and regenerated transformed plants after killing the non-transformed cells by using geneticin. Chawdhary and Vasil (1992) reported successful transformation of pBarGUS genes into sugarcane suspension culture cells by particle bombardment and electroporation methods. The authors also demonstrated the stable integration of transgene by Southern blot hybridization technique but could not obtain regeneration of plants. Thereafter, significant progress has been made in sugarcane transformation and development of sugarcane transgenics for several traits. A general scheme of genetic transformation is presented in Figs. 2 and 3.

In sugarcane, different transformation techniques have been employed to introduce marker genes in sugarcane protoplasts, cells in suspension, and embryogenic calli. These include electroporation (Rathus and Birch 1992), polyethylene glycol (Chen et al. 1987), particle bombardment (Franks and Birch 1991; Babu and Nerkar 2012), Particle Inflow Gun (Snyman et al. 2006), and *Agrobacterium* (Arencibia et al. 1998; Elliott et al. 1998; Efendi 2003; Manickavasagam et al. 2004; Kalunke et al. 2009; Joyce et al. 2010; Mayavan et al. 2013; Dong et al. 2014) transformation. PEG-mediated transformation did not receive much responsiveness due to its

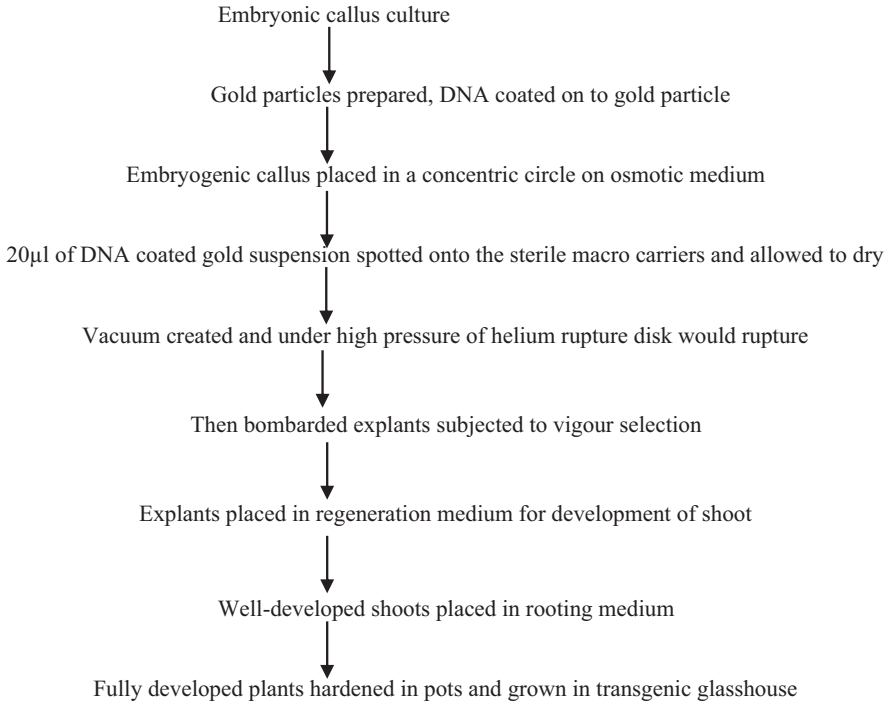

Fig. 2 Steps involved in the particle bombardment method of genetic transformation for sugarcane

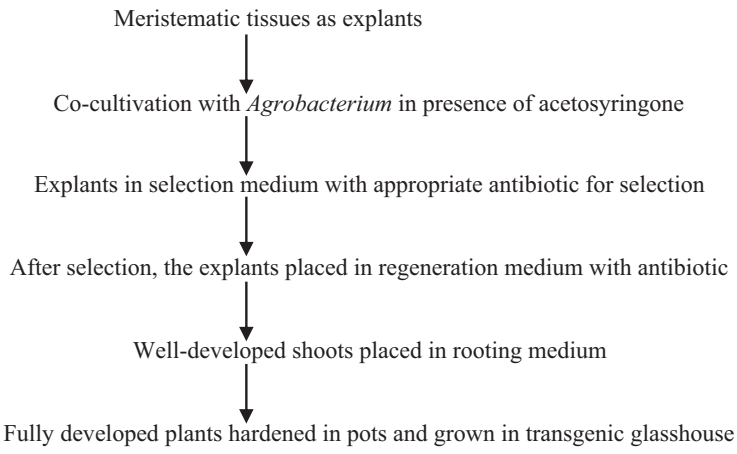

Fig. 3 Steps involved in *Agrobacterium*-mediated transformation of sugarcane

low efficiency and poor reproducibility. Transformation of sugarcane protoplasts of cultivar F164 was reported with low frequency of 1 in 106 treated protoplasts (Chen et al. 1987). Transformation by electroporation was found to be slightly better and reproducible over the PEG method. Rathus and Birch (1992) reported 1 out of 102–104 transformed cells by electroporated protoplasts in sugarcane cultivars Q63 and Q96.

3 Components of Genetic Transformation System

3.1 Promoters

Stable integration of the inserted gene into the genome of the target tissue is the key factor for success of the genetic transformation, selection of transformed cells, and expression of the transgene (Singh et al. 2010; Pillay 2013; Rashid and Lateef 2016). Expression of the transgenes requires a suitable promoter sequence (constitutive, inducible, or tissue-specific). Different promoters have been used in sugarcane transformation experiments including Emu, CaMV 35S (Franks and Birch 1991; Rathus and Birch 1992), Maize Adh1, Maize Ubiquitin promoter (Manickavasagam et al. 2004; Jain et al. 2007; Mulleegadoo and Dookum-Saumtally 2009; Joyce et al. 2010; Wang et al. 2017a), double CaMV 35S promoter (Babu and Nerkar 2012), TMV 35S, Emu and Act1 (Gallo-Meagher and Irvine 1993), Rab17 (Pillay 2013; Kumar et al. 2013; Reis et al. 2014), Prot ubi2.3 (Augustine et al. 2015a, b, c), CaMV 35S promoter with double enhancer (Raza et al. 2016), AIPC (Molinari et al. 2008), and Rubisco small subunit (scrbcs2) (Tang et al. 1996; Grof et al. 1996).

3.2 Type of Tissue

Type and age of initial tissue/material used for transformation determine the receptiveness of the transgene. Almost all culture systems such as protoplasts, cell suspension, embryogenic calli, leaf roll discs, regenerated young plantlets, axillary buds from sets, seed fluff, and inflorescence have been employed for raising culture systems for genetic transformation. Shoot tip explants (Khan et al. 2013), in planta genetic transformation using sugarcane seeds (Mayavan et al. 2013), axillary bud explants from 6-month-old plants (Manickavasagam et al. 2004; Mayavan et al. 2015), and young leaf whorl have been reported as the target tissue (Snyman et al. 2006) and open pollinated seed fluff transformation (Mayavan et al. 2013). Besides, production of sugarcane transgenic plants via in vitro culture of somatic embryogenic callus (Kalunke et al. 2009; Joyce et al. 2010; Kumar et al. 2014; de Alcantara et al. 2014) or cell aggregates of suspension culture (Efendi and Matsuoka 2011) is the method of choice. Sugarcane protoplasts (Arencibia et al. 1995), embryogenic calli, and apical meristems (Taparia et al. 2012) have also been used in sugarcane transformation studies.

Table 1 List of approved cases of genetically modified sugarcane[a]

Event	Characteristic	Approvals
CTB141175/01-A	Insect resistance	Brazil (food/feed; cultivation); Canada (food)
NXI-1T	Drought tolerance	Indonesia (food, cultivation)
NXI-4T	Drought tolerance	Indonesia (food, cultivation)
NXI-6T	Drought tolerance	Indonesia (food, cultivation)

[a]Source: International Service for the Acquisition of Agri-biotech Applications (ISAAA 2018)

Among these, embryogenic calli are the preferred explant system for transformation due to the high regeneration response (Taparia et al. 2012). The genetic transformation using polyethylene glycol (PEG) (Aftab and Iqbal 2001), microprojectile delivery system (Rani et al. 2012), and electroporation (Rakoczy-Trojanowska 2002) are best suited for protoplasts and cell suspension cultures. Bower and Birch (1992) reported the production of transgenic plants by means of bombardment of embryogenic callus with high-velocity DNA-coated micro-projectiles (Table 1).

3.3 Vectors

Reproducible methods of sugarcane transformation have been reported using different strains of *Agrobacterium tumefaciens*, which include AGL0, AGL1, EHA105, and LBA4404 and vectors like pGA492 (Manickavasagam et al. 2004), pKYLX80 (Gilbert et al. 2005), pAHC17 (Tang et al. 1996; Jain et al. 2007), Pu912 (McQualter and Dookun-Saumtally 2007), pHAC25 (Mullegadoo and Dookun-Saumtally 2009), pWBvec10a (Joyce et al. 2010), pMLH7133 (Efendi and Matsuoka 2011), pAHC27, pEmuKN, pR11F- (Pillay 2013), pGreen0029 (Kumar et al. 2013), pBract 302 (Reis et al. 2014), pGFP35S (Rasul et al. 2014), pTOK233, pMTCA31G (Arencibia et al. 1998), pCAMBIA1301, and pFFG19 (Babu and Nerkar 2012). Kumar et al. (2013) employed EHA105 strain of *Agrobacterium* harboring pGreen0029 vector containing *AVP1* (Arabidopsis Vacuolar Pyrophosphatase-1) gene driven under CaMV 35S promoter for genetic transformation against drought and salinity tolerance in sugarcane. Bax Inhibitor-1 gene from *Arabidopsis thaliana* (AtBI-1) into sugarcane offers suppression of ER (endoplasmic reticulum) stress in C4 grasses which can be an effective means for conferring improved tolerance to long-term water deficit (Ramiro et al. 2016).

3.4 Selectable Markers

The *Agrobacterium*-mediated transformation has the prospective advantage over biolistic method owing to its high efficiency and simple methodology of transgene integration. The selection system and co-cultivation medium were the most important features determining the success of genetic transformation and transgenic plant regeneration (Joyce et al. 2010). The most important and widely used selectable

marker in sugarcane is *npt*II gene conferring resistance to phytotoxic aminoglycoside antibiotics, kanamycin, and geneticin (Bower and Birch 1992; Fitch et al. 1995; Arencibia et al. 1998; Efendi 2003; Van Der Vyver 2010; Tang et al. 1996; Joyce et al. 2010). Another important selectable marker used is *hpt* (hygromycin phosphotransferase) gene conferring resistance to hygromycin (Christy et al. 2009; Arvinth et al. 2010; Philip et al. 2012; Augustine et al. 2015a, b, c). Inhibitory effect of selective agents is tissue- and species-specific (Cai et al. 1999). Therefore, it is necessary to know the minimal inhibitory concentration of selective agent for different sugarcane cultivars before attempting genetic transformation. Genetic transformation in sugarcane also involves use of reporter genes to establish the stability of transgene expression and any other effect of gene transfer process (Hansom et al. 1999). The *E. coli* PMI gene (*manA*) is used as the positive selectable marker gene for selecting transformed cells (Jain et al. 2007). List of selectable markers used for sugarcane transformation is given in Table 2.

4 Traits

There has been considerable success in the development of transgenic sugarcane plants. Sugarcane has been genetically modified for traits like sugar yield and quality (Botha and Groenewald 2001; Vickers et al. 2005), novel sugars with potential benefits to consumer (OGTR 2008), pharmaceuticals (Wang et al. 2005a, b), and abiotic and biotic stress tolerance (Devarumath et al. 2013, 2019). Table 2 has presented reports on the development of transgenic sugarcane for different traits. These include resistance to sugarcane mosaic virus (SCMV) (Gilbert et al. 2005; Jain et al. 2007), yellow leaf virus (Gilbert et al. 2009), sugarcane borer (Kalunke et al. 2009; Gao et al. 2016), and leaf scald resistance, antibiotic resistance, herbicide tolerance, and drought and salinity tolerance (Kumar et al. 2013; Reis et al. 2014). Production of naturally occurring compounds for use in bioplastics, enhanced nitrogen-use efficiency, altered plant growth, improved sucrose accumulation, altered plant architecture, improved cellulosic ethanol production from sugarcane biomass, incorporation of green fluorescent reporter gene, enhanced water use efficiency, and altered juice color (Manickavasagam et al. 2004; Mitchell 2011) are the results of transgenic technology. Further, GM sugarcane varieties that can produce high-value compounds, e.g., pharmaceutically important proteins, nutraceuticals, functional foods, biopolymers, enzymes, biopigments, and precursors, are paving ways to promote sugarcane as a biofactory (Grice et al. 2003; Suprasanna et al. 2011; Gómez-Merito et al. 2014).

4.1 Herbicide Tolerance

Sugarcane crop suffers from weed infestation and thereby yield losses. There have been few reports of development of herbicide tolerance using *PAT/bar* gene and selection using Basta (Falco et al. 2000; Mayavan et al. 2013). Genetically modified

Table 2 Genetic engineering of sugarcane for different traits (modified from Suprasanna et al. 2011 and references therein)

Trait	Gene	Transformation method	Reference
Reporter and selection system			
Neomycin phosphotransferase	*nptII*	Microprojectile	Bower and Birch (1992)
β-Glucuronidase	*uidA*	Microprojectile	Bower and Birch (1992)
β-Glucuronidase	*uidA*	Electroporation	Arencibia et al. (1995)
Hygromycin phosphotransferase	*hpt*	*Agrobacterium*	Arencibia et al. (1998)
Green fluorescent protein	*gfp*	*Agrobacterium*	Elliott et al. (1998)
α-Glucuronidase	*uidA*	Microprojectile	Wei et al. (2011)
Phosphinothricin acetyl transferase	*bar*	*Agrobacterium*	Manickavasagam et al. (2004)
Phosphomannose isomerase	*manA*	Microprojectile	Jain et al. (2007)
Herbicide resistance			
Bialaphos	*bar*	Microprojectile	Gallo-Meagher and Irvin (1996)
Phosphinothricin acetyl transferase	*bar*	*Agrobacterium*	Elliott et al. (1998)
Phosphinothricin acetyl transferase	*bar*	*Agrobacterium*	Enriquez-Obregon et al. (1998)
Phosphinothricin acetyl transferase	*bar*	*Agrobacterium*	Manickavasagam et al. (2004)
Glufosinate ammonium	*pat*	Microprojectile	Leibbrandt and Snyman (2003)
Disease resistance			
SCMV	*SCMV-CP*	Microprojectile	Joyce et al. (1998)
Sugarcane leaf scald	*albD*	Microprojectile	Zhang et al. (1999)
SrMV	*SrMV-CP*	Microprojectile	Ingelbrecht et al. (1999)
SrMV	*SrMV-CP*	Microprojectile	Rangel et al. (2005)
SCYLV	*SCYLV-CP*	Microprojectile	Gilbert et al. (2005)
Puccinia melanocephala	*Glucanase*, *chitinase*, and *ap24*	*Agrobacterium*	Enriquez et al. (2000)
Fiji leaf gall	*FDVS9 ORF 1*	Microprojectile	McQualter et al. (2004)
Sugarcane leaf scald	*albD*	Microprojectile	Zhang et al. (1999)
Pest resistance			
Sugarcane stem borer	*cry1A(b)*	Microprojectile	Arencibia et al. (1997)
Sugarcane stem borer	*cry1A*	Electroporation	Arencibia et al. (1999)

(continued)

Table 2 (continued)

Trait	Gene	Transformation method	Reference
Sugarcane stem borer	*cry1Ab*	Microprojectile	Braga et al. (2003)
Sugarcane stem borer	*cry1Ab*	Microprojectile	Arvinth et al. (2010)
Sugarcane stem borer	*cry1Aa3*	*Agrobacterium*	Kalunke et al. (2009)
Proceras venosatus	Modified *cry1Ac*	Microprojectile	Weng et al. (2011)
Sugarcane canegrub	*gna*	Microprojectile	Legaspi and Mirkov (2000)
Sugarcane canegrub	*gna*	Electroporation	Nutt et al. (1999)
Mexican rice borer	*gna*	Microprojectile	Setamou et al. (2002)
Mexican rice borer	*gna*	Microprojectile	Tomov and Bernal (2003)
Sugarcane stem borer	*gna*	Microprojectile	Irvine and Mirkov (1997)
Mexican rice borer	*gna*	Microprojectile	Nutt et al. (1999)
Ceratova cunalanigera	*gna*	*Agrobacterium*	Zhangsun et al. (2007)
Scirpophaga excerptalis	*Aprotinin*	Microprojectile	Christy et al. (2009)
Sugarcane weevil	*CCPI1*	Microprojectile	Schneider et al. (2017)
Drought and salt tolerance			
Osmotic adjustment and Oxidative stress	*P5CS*	Heterologous	Molinari et al. (2008)
Trehalose synthase	*Tsase*	*Agrobacterium*	Zhang et al. (2006)
Ethylene responsive factor	*SodERF3*	Wounding	Trujillo et al. (2008)
Drought-responsive factor	*Scdr1*	*Agrobacterium*	Begcy et al. (2012)
Arabidopsis Vacuolar Pyrophosphatase	*AVP1*	*Agrobacterium*	Kumar et al. (2014)
Heat Shock Protein 70	*EaHSP70*	*Agrobacterium*	Augustine et al. (2015a)
Pea DNA Helicase 45	*PDH45*	*Agrobacterium*	Augustine et al. (2015b)
Sugarcane MYB	*SoMYB18*	*Agrobacterium*	Shingote et al. (2015)
Arabidopsis vacuolar H+-pyro-phosphatase	(*H+-PPase*)	Microprojectile	Raza et al. (2016)
Arabidopsis Bax Inhibitor-1	*AtBI-1*	Microprojectile	Ramiro et al. (2016)
Metabolic engineering/alternative products			
Sucrose accumulation	Antisense soluble acid invertase	Microprojectile	Ma et al. (2004)
Fructo oligosaccharide	*IsdA*	*Agrobacterium*	Enriquez et al. (2000)
Polyphenol oxidase	*ppo*	Microprojectile	Vickers et al. (2005)
Polyhydroxybutyrate	*phaA*, *phaB*, *phaC*	Microprojectile	Brumbley et al. (2007)
p-Hydroxybenzoic acid	*hch1* and *cp1*	Microprojectile	McQualter et al. (2004)
Sucrose-phosphate synthase	*sps*	Microprojectile	Vickers et al. (2005)

(continued)

Table 2 (continued)

Trait	Gene	Transformation method	Reference
Mannose	*manA*	Microprojectile	Jain et al. (2007)
Polysterpolyhydroxy butyrate	*phb*	Plastid transf.	Petrasovits et al. (2007)
Sorbitol-6-phosphate dehydrogenase	*mds6pdh*	Plastid transf.	Chong et al. (2007)
Isomaltulose	*SI*	Microprojectile	Wu and Birch (2007)
Proline overproduction	*P5CS*	Microprojectile	Molinari et al. (2008)
ACC oxidase suppression	ACO antisense	*Agrobacterium*	Wang et al. (2009)

sugarcane plants resistant to phosphinothricin (PPT), the active compound of commercial herbicide, BASTA were generated by Manickavasagam et al. (2004), Mulleegadoo and Dookum-Saumtally (2009), and transgenic plants were confirmed by Southern blot hybridization. Herbicide resistance gene (*bar*) was used, and when glyphosate tolerance was assessed by spraying plants with 0.1–0.5% roundup, transgenic plants showed good growth (Wang et al. 2017a).

4.2 Abiotic Stress Tolerance

Sugarcane crop is threatened by adverse environmental conditions (abiotic stresses), such as water stress, salinity, and extreme temperatures. It is thus important to develop new sugarcane germplasm tolerant to salt, drought, and other stresses. It is also equally important to understand how plants have evolved adaptive genetic machinery including metabolic, cellular, and physiological processes, to promote growth and survival under stress environment (Devarumath et al. 2019). Improvement in stress tolerance in sugarcane has been achieved either by transferring a single gene or by transferring multiple genes or gene pyramiding. Transgenic sugarcane plants harboring *Grifola frondosa synthase* gene for tolerance to osmotic stress were developed (Wang et al. 2005a, b). Expression of *G. frondosa TSase* gene under the control of CaMV 35S promoter improved drought tolerance in sugarcane (Zhang et al. 2006) compared with non-transgenic plants. Similarly, overexpression of heterologous *P5CS* gene under stress inducible promoter (AIPC) was also reported to enhance drought tolerance in sugarcane (Molinari et al. 2008). McQualter and Dookun-Saumtally (2007) reported that the Arabidopsis *CBF4* gene under the control of the maize ubiquitin promoter improved drought tolerance in sugarcane.

The betaine aldehyde dehydrogenase (BADH) is involved in glycine betaine (GB) production in response to abiotic stress and, there is great potential for its use to improve abiotic stress tolerance (Fitzgerald et al. 2008). The *betA* and *betB* (encoding choline dehydrogenase from *Escherichia coli*) and *TsVP* (encoding V-H+-PPase from *Thellungiella halophila*) are also responsible for enhancing the abiotic stress tolerance in plants. Glyoxalase I and glyoxalase II that

confer improved salinity tolerance in tobacco and rice were used in sugarcane transformation (Rani et al. 2012). Drought tolerance has been attempted in sugarcane by using Arabidopsis Vacuolar Pyrophosphatase (*AVP1*) gene (Kumar et al. 2014), *SodERF3* (a novel sugarcane ethylene responsive factor), and *Arabidopsis bax inhibitor-1* gene and induced overexpression of a transcriptional factor *AtDREB2A* CA (Reis et al. 2014).

Heat shock proteins (HSPs) have a key role in stress tolerance mechanism in plants. *HSP70* gene isolated from *Erianthus arundinaceus* and driven by Port Ubi2.3 promoter was introduced in sugarcane variety Co 86032 through *Agrobacterium*-mediated method. The results suggested that EaHSP70 played an important role in sugarcane acclimation to drought and salinity stress and has potential for genetic engineering of sugarcane for developing drought and salinity tolerance (Augustine et al. 2015a).

Abiotic stress tolerance is improved by enhancing stress-responsive gene expression and upregulation in the activity of several defense mechanisms and sustaining membrane thermo-stability. For example, Augustine et al. (2015b) introduced the Pea DNA Helicase45 (PDH45) into sugarcane variety Co 86032 through *Agrobacterium*-mediated method. They used Port Ubi2.3 promoter to express this gene and analyzed V_o and V_1 plants for tolerance to soil moisture and exhibited significantly higher cell membrane thermo-stability, transgene expression, chlorophyll content, relative water content, and photosynthetic efficiency. Further EaDREB2 was overexpressed, pyramiding with the Pea DNA helicase gene (*PDH45*) in sugarcane cultivar Co 86032 to enhance tolerance to drought and salinity. When co-transformed with plant DNA helicase gene, DREB2 showed greater level of salinity tolerance than in single gene transfer (Augustine et al. 2015c).

4.3 Biotic Stress Resistance

4.3.1 Diseases

Diseases and pests cause significant economic losses to the sugar industries throughout the world. Sugarcane is susceptible to a crowd of viral, phytoplasma, and bacterial and fungal diseases. Still there are at least seven recognized sugarcane diseases of unknown etiology (Rott et al. 2000). In most sugar industries, diseases are controlled by an integrated approach involving the use of disease-free planting material, disease-resistant cultivars, applicable farm management practices, and strict quarantine measures. Some of the commercially grown sugarcane varieties are susceptible to more than one pathogen. Further, a number of elite, high-yielding lines developed in the breeding and selection programs do not see the light of the day for commercial release due to susceptibility to pathogens. Thus, there is current requirement to retain, maintain, or introduce resistance to various pathogens in the presently cultivated genotypes as well as other valuable sugarcane germplasm with commercial or breeding potential (Lakshmanan et al. 2005). About 8 bacterial and 160 fungal pathogens of sugarcane have been reported to date (Rott et al. 2000).

However, many elite sugarcane cultivars are susceptible to different bacterial and/or fungal diseases, limiting their commercial exploitation.

Sugarcane pathogens like mosaic virus and Fiji disease viruses cause significant yield losses in susceptible line. The coat protein-coding region of sugarcane mosaic virus (ScMV) has been introduced in sugarcane. Transgenic sugarcane plants containing this gene were evaluated and exhibited robust resistance over non-transformed plants. Embryogenic calli of hybrid CC84-75 was bombarded with plasmid pFM395 and pFM396 containing a ScYLV coat protein DNA fragment. After selection, 69 plants were regenerated out of which 46 plants were PCR positive. Transformed plants were inoculated with ScYLV and 10 months after infection, 37 plants were negative for ScYLV (Rangel et al. 2005). Fiji disease-resistant variants of Q124 have been produced by microprojectile transformation of Q124 callus with transgene developed from a translatable version of the FDV segment 9 ORF1. Forty-seven transgenic lines have been tested, and some resistant lines showed no symptoms of Fiji disease (McQualter et al. 2001).

4.3.2 Insect Pests

Pests of sugarcane are another major source of economic damage in all the cane-growing countries. Presently, sugarcane pests such as cane-grubs, borers, mealy bugs, wooly aphids, and other insects are controlled by integrated pest management (IPM) practices comprising biological, cultural, and insecticidal controls (Allsopp and Manners 1997; Allsopp and Suasa-ard 2000). Although IPM approaches are complementing for the already existing tolerance in sugarcane, increasing pest resistance by means of introducing novel insecticidal genes by transgenic approach will be useful strategy to support in maximizing and sustaining crop productivity (Allsopp and Manners 1997; Legaspi and Mirkov 2000; Falco and Silva-Filho 2003).

Genetic transformation in sugarcane with the *Nicotiana alata* proteinase inhibitor gene (Atkinson et al. 1993) or snowdrop lectin gene (van Damme et al. 1987) exhibited marked antibiosis to cane grubs (Allsopp and Suasa-ard 2000; Nutt et al. 2001). In another investigation by Legaspi and Mirkov (2000), they observed considerable growth inhibition of sugarcane stalk borers when fed on GM sugarcane engineered with lectin genes. Remarkable resistance to the borer *Diatraea saccharalis* Fab. was also reported in transgenic sugarcane that expressed a Bt-*Cry IA*(*b*) gene. Transgenic sugarcane plants were analyzed for insect resistance (Arencibia et al. 1999) and some resistance reported with soybean proteinase inhibitors (Falco and Silva-Filho 2003).

Cry1Ac gene, one of the cry1 genes from *Bacillus thuringiensis* (Bt) has been successfully used in sugarcane (Wang et al. 2017b). Inheritance of resistance to *Bacillus thuringiensis* Cry1Ab protein in the sugarcane borer, *Diatraea saccharalis* (Lepidoptera Crambidae), was analyzed by various genetic crosses (Wu et al. 2009). *Different expression vectors harboring the cry1Ac gene and marker genes were constructed* for transformation in sugarcane (Xu et al. 2008; Weng et al. 2011) and genetically modified sugarcane lines carrying *Cry1Ac* were evaluated using molecular marker techniques (Ismail 2013). Kalunke et al. (2009) reported transformation of *Cry1Aa3* gene in sugarcane and on-step regeneration of transgenic plants.

A synthetic gene coding for aprotinin, designed and codon optimized for better expression in plant system, was transferred to two sugarcane varieties CoC 92061

and Co 86032 through particle bombardment and hygromycin-resistant lines were analyzed by in vivo bioassay (Christy et al. 2009) and in vitro bioassay of Cry1F (Thorat et al. 2017). Arvinth et al. (2010) worked on pyramiding of aprotinin expressing sugarcane with *Cry1Ab* for shoot borer resistance.

4.4 Genetic Transformation for Byproducts/Others

Besides the prime purpose of increased crop production and enhanced food quality, transgenic plants also contribute to the production of therapeutic proteins, monoclonal antibodies, and edible vaccines for the pharmaceutical industry and environment friendly outputs like biodegradable plastics. Currently, sugarcane is being considered as an "ideal plant biofactory" for the production of these new products like pharmaceutical-grade proteins in sugarcane (D & MD report 2002; Gómez-Merino et al. 2014), polyhydroxybutyrate (PHB), environmental friendly biodegradable plastic (Bohmest et al. 2000; Zhang et al. 2002; Petrasovits et al. 2007), and bacterial phytase gene *appA* to improve the nutritional quality of sugarcane as animal feed (Santosa et al. 2005). Expression of sense or anti-sense version of these genes showed a reduced content of lignin in transformed sugarcane (Selman-Housein et al. 1999), production of biologically active GM-CSF in sugarcane (Wang et al. 2005a, b), almost doubled sugar content in sugarcane plants modified to produce sucrose isomer (Wu and Birch 2007). A novel vacuolar targeting determinant was used in sugarcane for vacoular localization of recombinant proteins (*gus* and *aprotinin* genes) thus paving the way for using sugarcane as a platform for production of high-value biomolecules (Palaniswamy et al. 2016). This technology has been given to Biotech Consortium India Limited (BCIL) for commercialization. However, for commercial acceptance of this technology, this needs to be validated with high-value pharmaceutically important proteins.

5 Plastid Transformation

Transgene(s) expression into the nuclear genome has led to a growing public distress because of the possibility of gene escape to their wild relative species through pollen. In most of the crops plants, plastids are maternally inherited. Engineering of the plastid genome is gaining importance as an alternative target to nuclear transformation. Chloroplast transformation has a number of advantages over nuclear transformation like high level of transgene containment and transgene expression and absence of gene silencing (Bock 2001, 2007; Daniel et al. 2001; Zhang et al. 2002; Maliga 2004, Sharma et al. 2005). Ruf et al. (2007) studied the transgene containment level in chloroplast transformation. Sugar-related crops like sugar beet were transformed using visual screening of plastid transformation by *gfp* gene, and transcription and protein expression were shown in transplastomic plants (Marchis et al. 2009), expression of synthetic *cry1Ac* in transgenic rice (Kim et al. 2009), and

eggplant (Singh et al. 2010). Kale et al. (2017) reported the development of sugarcane plastid transformation system using particle bombardment. However, strategies are required to be refined to obtain homotransplastomic lines.

6 Field Trials of Genetically Modified Sugarcane

Transgenic lines expressing different agronomic traits have been field evaluated to assess superiority of transgenic sugarcane. PT Perkebunan Nusantara XI (Persero) has developed NXI-1T, NXI-4T, and NXI-6T, genetically modified drought-tolerant sugarcane variety from BL-19 for drought-resistant qualities with the help of CDAST, Jember University, Indonesia. *BetA* gene responsible for the accumulation of betaine aldehyde, an osmoprotectant that helps in developing the drought tolerance, was used to develop drought-tolerant variety. Around 50 ha of land is currently under cultivation of these transgenic events. The Sugarcane Technology Center (Centro de Technologia Canavierira (CTC)), Brazil, has developed the GM sugarcane with "Bt (*Bacillus thuringiensis*)" gene responsible for borer resistance which has been approved for commercial cultivation. From the Biosafety point of view, vegetative mode of propagation of sugarcane provides good transgene containment and precludes any genetic segregation of transgenes (Altpeter and Oraby 2010). It is also suggested that GM sugarcane is one of the low-risk and safe plant species while food and environmental safety is considered, more so due to the flowering mechanisms and vegetative propagation characteristics (Altpeter and Oraby 2010; Zhou et al. 2016).

6.1 Status of GM Sugarcane in India

In India, the joint initiative (ICAR-SBI) of Sugarcane Breeding Institute, Indian Council of Agricultural Research, and Vasantdada Sugar Institute (VSI), Pune, is working towards developing drought-tolerant GM sugarcane that will need less water for cultivation. Developing drought-tolerant (less water use) GM sugarcane is not an end in itself as there are steps need to be taken to go for commercial release. The permission for field trials of drought-tolerant GM sugarcane variety, developed by the ICAR-SBI Coimbatore by overexpressing pea DNA helicase (PDH45), heat shock protein from *Erianthus* spp. (EaHSP70) and EaDREB2 genes, is under consideration with the Indian Biosafety regulatory authorities (Babu, personal communication).

6.2 Status of GM Sugarcane in Indonesia

PT Perkebunan Nusantara (a state-owned sugar milling conglomerate) of Indonesian Sugarcane Plantation Research Center (P3GI) and State University of Jember in

East Java have jointly developed transgenic sugarcane through the collaboration with Ajinomoto Company, Japan, and further approved for planting world's first GM drought-tolerant sugarcane (ISAAA 2013). This sugarcane variety can produce 10–30% more cane yield under drought condition than its conventional parental lines. According to the watchdog GMO Compass, field trials for GM sugarcane have been held in Brazil and other countries like Australia, Cuba, India, and the USA. As it is widely taken up in Indonesia, this "success" is likely to be cited as an important ground for regulators in other countries to license the commercial cultivation of GM sugarcane. As early as 2013, three different, drought-tolerant GM sugarcane events were approved for cultivation in Indonesia, but commercial cultivation has not yet taken place in Indonesia. Brazil is the second country after Indonesia to approve GM sugarcane for commercial cultivation. Raw sugar and refined sugar produced from Bt-sugarcane (CTS175-A) were approved for sale in Canada in April 2018.

6.3 Status of GM Sugarcane in Brazil

The world's largest sugarcane producer, Brazil, recently approved the commercial cultivation of GM sugarcane. Brazil has about 10 million hectares under sugarcane cultivation, the potential to plant GM cane is up to 15% of this area, and around 100 mills are now cultivating the GM cane. GM sugarcane variety "CTC 20 BT" is resistant to the country's main sugarcane borer (*Diatraea saccharalis*). This pest can cause losses of up to $5 billion annually. This new GM variety's pest resistance comes from the "*Bacillus thuringiensis*" gene, which has already been used in maize, soybean, and cotton in the country. The CTC 20 BT did not negatively affect soil composition, insect populations, or sugarcane biodegradability. It would take at least 3 years for first shipments of sugar produced from GM sugarcane to reach export markets. CTC has made applications in the USA and Canada to clear sale of sugar made from GM cane.

7 Transgenic Research in Sugarcane: Current and Future Perspective

Sugarcane (*Saccharum* spp. hybrid) is a complex polyploid crop of interspecific origin with a large and complex genome. Progress in traditional breeding of sugarcane is impeded by its narrow gene pool, complex genome, and long breeding/selection cycle (Mirajkar et al. 2019). With climate change imminent across the globe, development of varieties tolerant to multiple stresses is an immediate requirement. Sugarcane is an ideal candidate for transgenic improvement, and the crop is successfully genetically engineered to improve various traits of interest. Though sugarcane transgenic lines are in advanced stages of evaluation in different countries, commercial transgenic crop development is lagging behind compared to other major agriculturally important crops like soybean, maize, cotton, and canola. Water-deficit stress-tolerant sugarcane was the first transgenic trait approved for

commercial cultivation in Indonesia. More recently, Bt sugarcane resistant to damages caused by sugarcane borer (*Diatraea saccharalis*), the main pest of sugarcane in Brazil, has been approved for commercial cultivation. In Brazil, India, China, Thailand, the USA, and other sugarcane-producing countries, research is underway on developing transgenic sugarcane for different agronomic traits.

Genomics advances like next-generation sequencing (NGS) techniques and availability of sugarcane draft genome have led to the discovery genes for biotic and abiotic stresses and are expanding our knowledge about crop's response to stresses, and this is expected to accelerate sugarcane-based product development. On the other hand, progress in understanding molecular mechanisms of host–pathogen interactions-induced resistance based on plant's natural defense mechanism is a promising approach to successfully manage crop diseases through genetic engineering. Identification of novel pathogen signatures like pathogen-associated molecular pattern (PAMPs) and effectors, which are potential in inducing PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI), respectively, through their interaction with host targets like R genes, susceptibility genes, regulatory proteins, etc., would give an impetus in understanding and developing disease resistance in this complex polyploidy crop. Deploying available novel *cry* genes specific against particular sugarcane pests or pyramiding of genes more than one gene would enhance the development of durable insect-resistant sugarcane. Sugarcane root feeding insects are more of concern because biological and chemical controls have been attempted for their management, but successes are limited due to the subterranean habitat and the hidden nature of pests making its occurrence in the field undetectable until complete devastation. Hence, transgenic crops expressing *cry* toxin through transgenic approach could be an ideal management option considering the practical difficulties.

Sugarcane is emerging as a biofactory, and using this as platform, many of the commercial value biomolecules can be engineered for production. Transgenic sugarcane has shown tremendous potential and is expected to play an important role in the growing bioeconomy through biopharming. Considerable research is required to make GM sugarcane to reach farmland and industrial sector through improved biotic and abiotic stress tolerance, enhanced biomass and quality, and production of value-added products.

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