Genetic Engineering to Improve Biotic and Abiotic Stress Tolerance in Maize (*Zea mays* L.)

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1 Introduction

Maize is considered as a most valuable and highly productive cereal crop together with wheat and rice, supplying almost 60% of global food energy (Ross-Ibarra et al. 2017; Jiao et al. 2017). To accomplish the world's food requirement, which is estimated to touch 10 billion figures in the middle of the century, is challenging due to the changing climatic conditions (Tigchelaar et al. 2018). The rising demand for diverse germplasm to compete with global hunger is further threatened by various biotic and abiotic stresses. The major biotic factors include pathogens and insect pests, while abiotic factors such as drought, waterlogging, salinity, temperature, etc. cause a huge impact on maize yield over the world (Restrepo-Diaz et al. 2021). The detrimental effect on crop losses is highest and uncontrollable in the case of association of both biotic and abiotic factors (Josine et al. 2011). It is alarming the scientific community rapidly introduce maize cultivars having the ability to withstand adverse climatic conditions (Masuka et al. 2012; Dresselhaus and Hückelhoven 2018). Extensive efforts had been made in the fabrication of maize genotype through conventional breeding methods to maintain the yield potential for the previous six decades. The achievement of sustainable productivity requires the art of utilizing

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the natural variability to isolate the desired genotypes and further their application in stress-tolerant breeding programs (Yadav et al. 2015). Nevertheless, traditional methods helped to exploit the variations and further their introgression in cultivated maize varieties, but alternative strategies are required to meet the criteria of specific growing conditions (Haroon et al. 2020). Moving forward with conventional techniques is not convincing due to limited genetic stocks and inefficient selection measures for the plant stresses, which is more redundant in the case of abiotic factors (Bedada et al. 2016). To overcome these continuously evolving problems, the development of alternative strategies is mandatory to be adopted in the changing environment.

Genetic engineering (GE) aims to create specific alterations in the plant genome to introduce a novel functionality for the concerning trait. The GE approaches rely on the foreign gene expression and correlation with regulatory and signaling pathways of target plant species to generate or encode stress-specific metabolites (Anwar and Kim 2020). In the previous two decades, GE displayed phenomenal developments in the modification of the maize genes for triggering the defense mechanism. After the first event of genetic transformation back in 1996, maize emerged as a prioritized crop among the field crops at a commercial scale (Yadava et al. 2017; Raman 2017). *Agrobacterium*-directed delivery of genetic information gained the attention of plant breeders to engineer the maize genome because of its stable integration mechanism and fertile plant production, which follows typical Mendelian inheritance (Bedada et al. 2018). It is very important to enhance our understanding of molecular, biochemical, and cellular changes in response to a diverse range of stresses. The remarkable opportunities have been given by GE to conventional plant breeders for a better understanding of target traits (Ilyas et al. 2021). GE helped to identify the candidate genes, microRNAs (miRNAs), and transcription factors in maize associated with the tolerance mechanism and subsequently utilized them in stress-resistant breeding (Wu et al. 2019; Muppala et al. 2021).

In recent years, the climatic variations disturbed the plant growth environment greatly due to drought conditions and rise in temperature, significantly reducing the plant yield potential even after effective agronomical and biotic stress management (Restrepo-Diaz et al. 2021). Abiotic stress causes osmolyte accumulation, stomatal closure, reduced photosynthesis, and activation of stress-responsive genes. The soil productivity is further negatively influenced by salinity which becomes devastating in hot temperate areas, ultimately reducing the cultivation land (Turan et al. 2012). The combination of both biotic and abiotic stresses disorganizes the physiological, molecular, and biochemical framework of plants that leads to low photosynthesis and reduced water uptake ability, which failing crop productivity. The introduction of genes related to hormonal and enzymatic balance, ROS stabilizers, and helper in ion transporters significantly aids in maintaining the source-sink relationship during the adverse condition in maize (Landi et al. 2017). GE is more preferred for resistance genotype development which is only focused on target gene integration. On the other hand, it is not possible through plant breeding methods due to cross incompatibility and undesirable consequences of genomic background (Turan et al. 2012). Several orthologous genes have been identified and transferred into maize and vice

versa using GE from related plant species against biotic and abiotic stresses (Yin et al. 2004; Shou et al. 2004a, b; Turan et al. 2012; Bo et al. 2020).

Currently, GE through genome editing techniques creates fundamental insight into the biology of crop plants ultimately revolutionizing the agricultural sector at a commercial scale (Chen et al. 2019). The future of stress resistance GE in maize looks promising with CRISPR/Cas9 technique, although substitution of every novel technique needs to be simultaneously addressed to avoid any delay for the betterment of agricultural sciences. Furthermore, a combined approach of conventional techniques along with recent advancements in GE can accelerate the introduction of stress resistance varieties at low costs in maize. This chapter highlights the current scenario of GE techniques and status in biotic and abiotic stress resistance breeding in maize along with their challenges and new approaches with future perspectives.

2 Present Status of Genetically Modified Maize

Since the introduction of GE technology, the maize crop has received attention in the agricultural sector. Initially, the Food and Drug Administration (FDA) approved the production of genetically modified (GM) *Bt* corn developed by Ciba-Geigy in 1995 (Bawa and Anilakumar 2013). Further glyphosate-resistant corn variety, Roundup Ready corn, has been commercialized by Monsanto in 1999 (Monsanto 2013). Subsequently, the crystalline proteins, namely, Cry1 and Cry2 to target lepidopteran pests and Cry3 proteins for coleopteran pests attacked on maize have been identified (Schnepf et al. 1998). Therefore, maize was engineered with these toxic Cry proteins. Initially, the GM maize conferred either herbicide or insecticidal resistance, and further single cultivar has been engineered for stacked traits. In 2009, Monsanto and Dow AgroSciences produced a stacked GM maize, Genuity® SmartStax containing genes (*cp4 epsps*, *cry1Fa2*, *cry2Ab2*, *cry34Ab1*, *cry35Ab1*, *cry3Bb1*, *cry1A.105*, *pat*) to provide glyphosate tolerance and resistance against *Helicoverpa zea* (corn earworm), *Diatraea grandiosella* (southwestern corn borer), *Ostrinia nubilalis* (European corn borer), *Diabrotica virgifera virgifera* (western corn rootworm), *Diabrotica virgifera zeae* (northern corn rootworm), and *Diabrotica barberi* (Mexican corn rootworm), which was approved for cultivation in the United States (Moglia and Portis 2016; ISAAA 2021).

The ISAAA (2019) report states that GM maize is the second most important crop after GM soybean which has been cultivated globally on 60.9 mha area representing 32% of the total area under GM crops globally (190.4 mha). The maize has a maximum number (146) of approved events among all GM crops in 35 countries. Among the top ten GM events, seven are maize events, namely, NK603, GA21 (herbicide-tolerant), MON810, MON89034 (insect-resistant), TC1507, Bt11, and MON88017 (insect-resistant and herbicide-tolerant). The herbicide-tolerant event NK603 of maize has maximum approvals in 28 countries followed by soybean event GTS 40-3-2 for herbicidal tolerance. The GM maize has been grown in 15 countries, namely, the United States, Brazil, Argentina, Canada, Paraguay, South

Africa, Uruguay, Philippines, Mexico, Spain, Colombia, Vietnam, Honduras, Chile, and Portugal. The highest GM maize growing countries are the United States (33.17 mha) followed by Brazil (16.3 mha), Argentina (5.9 mha), Philippines (0.9 mha), and Vietnam (0.1 mha). According to ISAAA database, there is a total of 240 approved GM events available in maize across various countries, and out of these, 60 events are commercialized with various trade names. These 240 events include 212 events for tolerance to herbicides, 208 events for resistance against various insect pests, 13 events for product quality modifications, and 6 events for pollination control (ISAAA 2021). GM maize has a great contribution in saving million-dollar money for farmers by reducing the usage of pesticides and herbicides (Raman 2017). In North America, GM corn seed covers >90% of the corn seed market (Morder intelligence blog 2021).

The soluble protein Vip3A from *Bacillus thuringiensis* gain popularity these days to control pests that are resistant to Cry proteins due to its different action mechanisms. The Agrisure®Viptera™ having Vip3Aa20 protein which is effective against lepidopteran pests has been developed. The 34 events across different countries containing Vip3A proteins encoding gene along with other genes responsible for insecticidal and herbicidal resistance have been developed. The Bayer company also developed Trecapta technology™ to incorporate three genes Cry1A.105, Cry2Ab2, and Vip3Aa20 having different modes of action to impart tolerance against corn borers, corn earworms, black cutworm, fall armyworm, and western bean cutworm (https://traits.bayer.com/). Various events having approved trade names are Agrisure® Viptera™ 2100, Agrisure® Viptera™ 3110, Agrisure® Viptera™ 3111, Agrisure® Viptera™ 4, Agrisure® Viptera™ 3220, Agrisure® Viptera™ 3100, Agrisure® Duracade™ 5222 by Syngenta, and Power Core™ x MIR162 x Enlist™ by Dow Agro Sciences LLC (ISAAA database; Gupta et al. 2021). In 2013, the first drought-tolerant GM maize Genuity® DroughtGard™ of Monsanto has been released and commercialized in the United States, which contained the gene encoding cold shock protein B (CSPB) isolated from *Bacillus subtilis* (Moglia and Portis 2016; ISAAA database 2021). Maize has also been engineered for other traits, namely, modified product quality (*phyA2* for conversion of phytase phosphorus into inorganic phosphorous for its consumption in animal feed; *cordapA* for high lysine content with trade names Mavera™ Maize and Mavera™ YieldGard™ Maize; amy797E to increase the thermostability of amylase for enhancement in bioethanol production with trade name Enogen™), pollination control (*ms45* to restore fertility with trade name 32,138 SPT maintainer; *zm-aa1* for pollen sterility; barnase for male sterility/ trade name InVigor™ Maize), and increased ear biomass by targeting the bHLH TF *athb17* (Kumar et al. 2020; ISAAA database 2021). In this series, the Bayer's SmartStax™ Pro x Enlist™ (*cp4 epsps*, *cry2Ab2*, *cry1A.105, cry1F, pat, cry34Ab1*, *cry35Ab1, dvsnf7, aad-1*) and Bayer's SmartStax® Rib Complete® Corn Blend for herbicidal and insecticidal tolerance will be available in 2022 (ISAAA database 2021, https://traits.bayer.com/).

3 Acceptance and Impact of Genetically Modified Maize

The first genetically modified (GM) crop was commercialized in 1996s (Snow and Palma 1997; Benbrook 2012), and since then, it has been rapidly adopted in several countries (FAO 2015). In the recent decades, popularization of transgenic crops has considerably increased the crop yields by 22%, which has led to an approximately 68% increase in farmer profits (Klümper and Qaim 2014). Globally, in the past 22 years, the area of transgenic crops has increased considerably from 1.7 mha in 1996 to 191.7 mha in 2018, that is, around 113-fold increases (ISAAA 2018a, b). Presently, the cultivation of GM crops is dominated by soybean (~50%), maize (~30%), cotton (~13%), and canola (~5%) crops (ISAAA 2020). The 190 mha of GM crops have been majorly grown by 26 countries of which 46% was contributed by five industrial countries only, that is, the United States, Canada, Australia, Spain, and Portugal (ISAAA 2018a, b, 2020), demonstrating their role in the agricultural economy (Cao et al. 2011). The traits like herbicide tolerance and insect resistance have been mainly targeted to introduce into major crops like soybean, maize, canola, and cotton comprising about 53% and 14% of total GM area, respectively, and about 33% of total GM area for both traits staked in a crop (ISAAA 2016). Extensive research has been conducted to develop GM crops and has been widely accepted in many countries. But still, nearly 38 countries across the world have prohibited their cultivation due to human and environmental safety concerns (ISAAA 2016). Among GM crops, the highest numbers of GE events have been undertaken in maize for single or staked traits. After soybean, GM maize is the second largest crop to be globally adopted (Aldemita et al. 2015). As of 2015, a total 53.6 mha of GM maize has been cultivated globally, representing almost 28% of the 190.4mha of total GM crop cultivation (Statista 2021). Furthermore, GM maize has the highest potential of expansion due to its comparatively lower rate of adoption (30% of the global maize in 2015), and a huge number are under cultivation (ISAAA 2016). The acceptance of transgenic crops has been an issue for many years in many countries due to several human health and environmental concerns. It has been a concern that transgenics can cause allergic and carcinogenic reactions in people, although no evidence has been found yet (Ferber 1999). Furthermore, it can develop resistance to antibiotics that lead to the generation of super bugs. In addition, the digestion of foreign DNA from other sources like a virus or bacteria is also a question to consider, but still, no evidence has been found in any digestion difference from conventional DNA. Another big concern on acceptance of transgenics is the damage to the environment. The pollen from transgenic crops having toxins can be harmful to many nontarget insects such as Monarch butterfly larvae killed due to bacterial toxins in transgenics pollen (Losey et al. 1999). The other biggest concern is the hybridization of transgenic crops with weeds, which can cause super weeds development that will be resistant to herbicides. Genes utilized to develop insect/pest and diseases resistance in plants can benefit weed populations also allowing them to survive under harsh conditions too. But to date, these are just theoretical predications with little evidence to support them (Crawley et al. 2001). Several studies have been

carried out to analyze the impact of GM crops on agronomic, economic, and environmental aspects (Ortego et al. 2009; Burachik 2010; Arthur 2011; Xu et al. 2013; Wang et al. 2014; Nicolia et al. 2014; The National Academies Press 2016). However, these studies were not much useful to draw unambiguous conclusions. To answer the major questions for GM maize adoption, few meta-analyses have also been attempted to address the concerns related to yield, cost-benefit ratio (Marvier et al. 2007; Areal et al. 2013; Klümper and Qaim 2014), pesticide use (Klümper and Qaim 2014), and effects on nontarget (NT) invertebrates (Marvier et al. 2007; Wolfenbarger et al. 2008; Naranjo 2009; Comas et al. 2014). However, there are still some key issues such as effect on grain quality and nutrition, toxin values (Ercoli et al. 2007, 2011) in GM maize production, and its effect on important agroecosystem services including soil organic matter decomposition. According to Pellegrino et al. (2018), a meta-analysis was carried out for the agronomic, environmental, and toxicological traits of GM maize such as yield, grain quality, NT organisms, target organisms, and soil biomass decomposition. The results depict that GM maize performed better than its near-isogenic line in terms of grain yield (5.6–24.5%) with lower concentrations of mycotoxins (−28.8%), fumonisin (−30.6%), and thricotecens (−36.5%). It was analyzed that NTOs were not affected by GM maize, except Braconidae, a parasitoid of European corn borer due to BT maize. Biogeochemical cycle parameters like lignin content in stalks and leaves also did not fluctuate, while the biomass decomposition was higher in GM maize. Many GM crops for pest control are engineered by using BT toxins, crystal protein from the bacterium *Bacillus thuringiensis*. The US Environmental security company has analyzed that these toxins don't pose any hazard to human well-being. The endotoxins are insecticidal and show low environmental persistence by degrading quickly. Although these endotoxins are harmful to bugs, a few studies supported that they are harmless to wild mammals, birds, pets, and people. The use of Bt corn has saved 1.7 billion dollars from the European corn borer damage in US states while 10% yield increment. It has been estimated that by growing 50% of GM crops likes maize, oil seed rape, sugar beet, and cotton would decrease 14.5 million kg of pesticide use in a year sparing 7.5 mha from spray, saving 20.5 million liters of diesel, and avoiding roughly 73,000 lots of carbon dioxide being launched into the atmosphere. From 1997 to 2009, a decrease in 13 million kg of pesticide has been recorded in corn and soybean fields, adopting the GM versions. In the United States, the decrease in pesticide use has been projected approximately 2.5 million pounds a year (Madhusudhan 2016). It is a very well-known fact that genetically engineered crops have accelerated yields, increased taste of meals, and decreased the application of pesticides. Alternatively, these crops also pose some serious concerns related to human wellness and threaten environmental safety by the creation of super weeds, novel pest, negative effects on nontarget species, and the disturbance of ecosystem services. The countries adopted for transgenic crops have gained economic development through increased production and saving chemical and labor costs, in addition to preventing gigantic ecological damage. Slowly, many more developing countries are also accepting transgenic crops as they are gaining profits compared to earlier. Despite the large-scale cultivation of GM maize and its impact

assessment studies on agro-environmental aspects, the benefits and risks related to GM maize are still being argued, and safety concerns persist.

4 Genetic Engineering Approaches to Develop Transgenic Maize

There are several biotechnological platforms used to develop transgenic maize (Fig. 1). Several elements are required for genetic engineering such as the development of gene constructs possessing genes of interest (GOI), promoter, terminator, enhancer, and intron sequences, selectable markers, reporter genes, and binary and alternative vectors. A variety of plant transformation methods are developed to introduce the gene construct to regulate gene expression via suitable approaches such as overexpression, gene stacking, RNA interference (RNAi), and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein-9 nuclease (Cas9)-mediated genome editing.

4.1 Development of Gene Construct

(a) Gene of interest: With the advancement in DNA sequencing tools, crop plant research utilizing genetic engineering approaches has been revolutionized in past few years (Shendure et al. 2017). Through whole-genome sequencing, huge information has been generated that is to be utilized in association with powerful bioinformatic tools and sophisticated molecular biology methods. It helps to detect the functions of every gene associated with different biotic, abiotic, and agronomic traits. By utilizing different transgenic approaches, specific genes can be targeted to improve/modify their functions through their overexpression or knockdown with desirable phenotypes to improve targeted traits in plants. A few criteria need to be followed to improve a GOI such as expression level, gene structure, presence of conserved domains, GC content, and codon usage optimization for improved translation efficiency (Barahimipour et al. 2015). To construct the expression cassette, only the protein-coding region, that is, exons, should be inserted for plant transformation except in some cases where endogenous *cis*-regulatory elements or enhancer sequences are present that are essential for their expression, translation, or stability (Gao et al. 2015a, b; Zhang et al. 2018).

(b) Transcriptional promoter sequence: Promoters are DNA sequences located upstream of the 5′-UTR of the gene containing several regulatory elements to regulate transcription initiation (Yamamoto et al. 2007). Several important transcription factors (TFs) (e.g., DREB and ABRE, MYC/MYB TFs for abiotic stresses) play important role in transcriptional regulation by interacting with

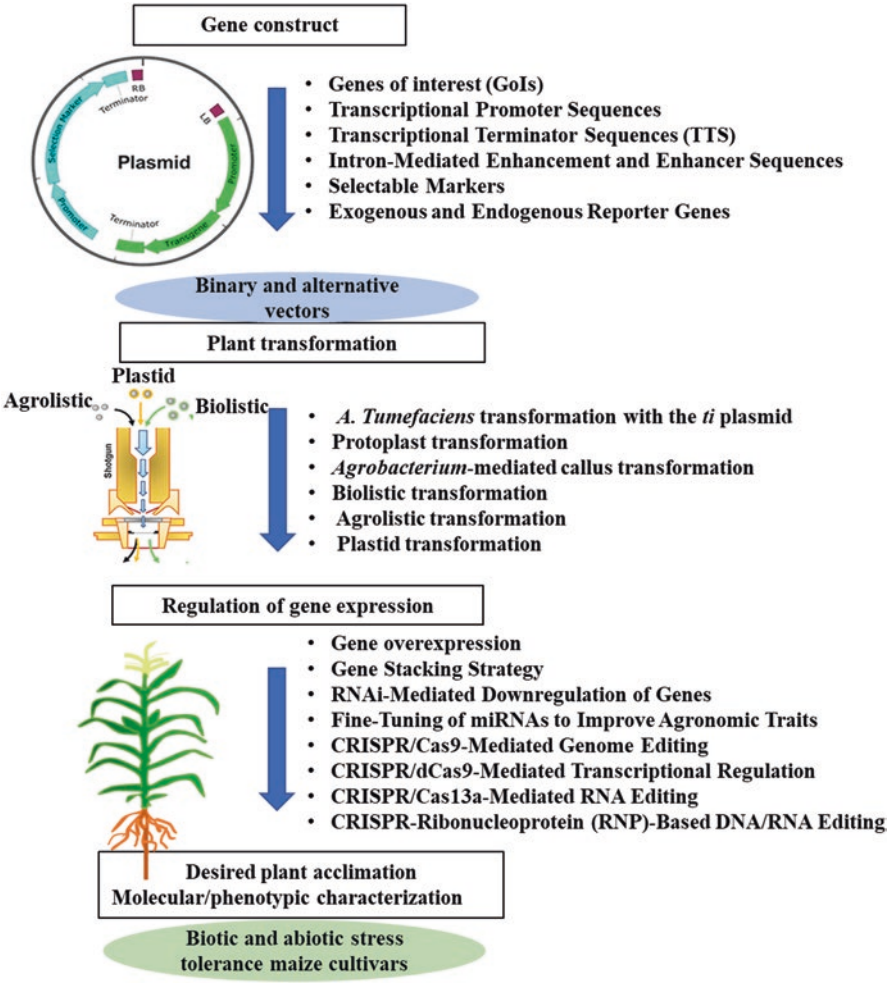

Fig. 1 Different genetic engineering elements and approaches to regulate maize gene expression for biotic and abiotic stress tolerance/resistance development

promoter sequences (Yamaguchi-Shinozaki and Shinozaki 2006; Ambawat et al. 2013). The selection of promoters directly affects the efficiency of new biotechnological tools (NBT) and the accessibility of powerful traits. There are several plants and viral and synthetic promoters that are available with constitutive, stress-induced (biotic and abiotic), tissue-specific, and developmental stage-specific features to regulate the overexpression of GOI in several crops (Basso et al. 2020).

(c) Transcriptional terminator sequence (TTS): TTS are conserved sequences present downstream of the protein-coding region and are recognized by the transcriptional machinery as transcription stop signals and consequently induce

decoupling of this machinery from the DNA (Loke et al. 2005). The most commonly utilized TTS in plants are as T-nos and T-ocs from the *nopaline synthase* and *octopine synthase* gene of *A. tumefaciens*, respectively, T-35S from the *Cauliflower mosaic virus* 35S terminator, rbcS1 or rbcS-E9 from the *ribulose-1,5-bisphosphate carboxylase* gene, small subunit, of *Pisum sativum*.

(d) Intron-mediated enhancement and enhancer sequences: Introns are the noncoding sequences present in primary transcripts that are removed before the translation of the exons, that is, coding sequence. In addition, some introns also act as intron-mediated transcription enhancers and improve the translation efficiency that are useful in genetic engineering (Laxa 2017)**.** The introns like *Adh1*, *Sh1*, *Bz1*, *Hsp82*, *Act1*, and *GapA1* from maize or rice genes are most commonly used introns to enhance the transcription levels in monocots, while the *rbcS*, *ST-LS1*, *Ubq3*, *Ubq10*, *PAT1*, and *atpk1* introns from petunia, potato, or *A. thaliana* genes are the most common in dicots (Gallegos and Rose 2015; Laxa 2017).

In contrast to introns, enhancers are (also noncoding DNA sequences) commonly present within the promoter sequence upstream of the TSS or in the 5′- or 3′-UTR. They bind many TFs to trigger the expression of genes sited upstream or downstream. In addition, they also regulate RNA expression, chromatin accessibility, and histone modifications and reduce DNA methylation levels (Weber et al. 2016); for example, in maize, an enhancer Hepta-repeat located 100 kb upstream of the booster1 gene improves its expression (Belele et al. 2013). Therefore, the introns and enhancers have huge potential in genetic engineering and need more validation studies to support the use of these sequences in specific crops.

(e) Selectable markers: The major challenge of genetic transformation is to insert the GOI into the genome of the cell and then to select this transformed cell with regeneration ability. It is feasible by the addition of selective agents, for example, hygromycin, kanamycin, geneticin, glyphosate, glufosinate-ammonium, and imazapyr and hormones used in the in vitro culture medium. There are two methods of selection, that is, via positive selection where non-transformed cells are unharmed without causing injury or death, while in negative selection, either the growth is inhibited or death of non-transformed cells. For positive selection, *uidA/gus* (β-*glucuronidase*), *manA* (*phosphomannose isomerase*), *xylA* (*xylose isomerase*), *PTXD* (*phosphite oxidoreductase*), and *DOG^{R}1* (*2-deoxyglucose-6-phosphate phosphatase*), genes isolated from microorganisms are mainly utilized in plant tissue culture (Izawati et al. 2015; Nahampun et al. 2016). For negative selection, the *nptII*, *hptII*, and *CmR* genes are used as selectable markers that confer resistance to antibiotics (geneticin/kanamycin, hygromycin, and chloramphenicol, respectively) blocking ribosome activity and finally inhibit protein synthesis.

(f) Exogenous and endogenous reporter genes: Reporter genes are efficient tools to monitor the efficacy of gene delivery vehicles and gene expression. It is cloned downstream of a regulatory region (e.g., promoter/enhancer), which generally controls the expression of a specific gene. Hence, introducing a reporter gene driven by a promoter of interest into the target cell can indirectly monitor the

expression of the gene. Using the reporter genes, various aspects of gene expression like promoter/regulatory elements, inducible promoters, and endogenous gene expression (Grandaliano et al. 1995; Ikenaka and Kagawa 1995) can be studied. It is an inexpensive, rapid, and sensitive assay to study gene delivery and gene expression, avoiding the development of a specific probe to assess the expression of every new GOI. The most commonly used exogenous reporter genes are chloramphenicol acetyltransferase, β-galactosidase (GAL), β-glucuronidase (uidA/GUS), β-lactamase, firefly luciferase, and Renilla luciferase; yellow fluorescent protein (YFP); green fluorescent protein (GFP); and red fluorescent protein (RFP). However, phytoene desaturase (PDS) is mostly used as an endogenous reporter gene to assess the RNAi assays in plants (Sundaresan and Gambhir 2002).

(g) Binary and alternative vectors: For decades, *Agrobacterium*-mediated genetic transformation has been widely used to generate transgenic plants. Initially, this technology involves complex microbial genetic methodologies to introduce GOI into the transfer DNA (T-DNA) of large tumor-inducing plasmids (Ti-plasmids, ~200 kb in length) making it complicated to delete or insert any DNA at specific sites. To make it easy, scientists developed more efficient binary vectors by splitting the T-DNA region and virulence (*vir*) genes into two replicons, which have enhanced the genetic transformation efficiency in crop plants. The superbinary vectors with supplementary *vir* genes, ternary vectors, and helper plasmid with an augmented number of *vir* genes have illustrated significant results (Che and Anand 2018; Anand et al. 2018). To enhance the transformation efficiency and stability of transgenes, it was crucial to optimize components with reduced T-DNA length. Therefore, vectors with multiple cloning sites or restriction enzyme sites adjoining the key transcription units are being engineered. For example, the pCAMBIA, pSITE, pGD, pMSP, pGPTV, and pRT100 vectors are amended binary vectors for plant transformation. The traditional vectors have some drawbacks of either non-optimization of their components, or they lack ideal components for a specific trait such as promoter, terminator, selection marker, or reporter gene. To resolve these limitations, new and optimized simple vectors have been synthesized for each explicit case.

4.2 Plant Transformation Methods

Plant transformation is the process to introduce the DNA segment into any species genome to alter its genetic constitution to achieve desired gene expression. In crop plants, the transformation was first described in tobacco in 1984, and since then, many plant transformation techniques have been developed (Paszkowski et al. 1984). Transformation methods to introduce diverse genes into plant cells include the indirect gene transfer through *Agrobacterium tumefaciens (Rhizobium radiobacter)*-mediated transformation (Sun et al. 2006), direct gene transfer into protoplasts (Karesch et al. 1991), and particle bombardment (Yao et al. 2006).

(i) *Agrobacterium*-mediated T-DNA transfer: It is the most common and widely used transformation protocol to introduce the GOI. A compatible interaction between host plant and *A. tumefaciens* consequences in T-DNA transfer facilitated by the T4SS into plant cells. The *Agrobacterium* harbor the tumor (*Ti*)- or root (*Ri*)-inducing plasmid from which the T-DNA is transferred into the plant genomic DNA through random integration by recombination. The T-DNA sequence has two borders, that is, left and right borders of 25 bp direct repeats, which are essentially required to perceive the T-DNA by the *vir*D and *vir*E proteins. TheT-DNA is introduced into the plant nucleus by single-stranded DNA (ssDNA)-associated virulence proteins encoded by *Agrobacterium* (Gelvin 2010). The T-DNA has been engineered into a binary vector by substituting the tumor-causing genes with promoters, GOI, and TTS. Due to the high rate of single transgenic events, this method has become most popular transformation tool among researchers. By using *A. tumefaciens* strains with varying degrees of virulence such as EHA105, LBA4404, GV3101, C58C1, and AGL1 in addition to better adaptation to a plant species with higher tolerance to recalcitrant tissues can further enhance the efficiency of this method.

(ii) Biolistic-mediated transformation: This method (particle bombardment or gene gun) was developed in 1987 as an alternative to the direct gene transfer through protoplast transformation. In this method, the DNA sequence is directly introduced into the plant genome, complexed with small gold or tungsten particles of 0.6–1 μM diameter. This method has shown better results to deliver foreign DNA into cell/tissue/organelle surpassing the barriers. These microcarriers with higher velocity were deposited on the membranes and bombarded against totipotent plant tissue. The major advantage of this method is that irrespective of plant species, it directly transforms tissues like embryo, pollen grain, meristems, and morphogenic cell cultures. In addition, a large number of transgenes can be attempted with this method, but very long DNA sequences cause a risk of DNA breakage during delivery, and insertion of many copies results in instability over successive generations.

(iii) Agrolistic-mediated plant transformation**:** This method combines the advantages of both *A. tumefaciens* with high-efficiency DNA delivery by biolistic. It has been mostly applied in recalcitrant plants, such as in cotton and soybean. The GOI is integrated into vector sequence as in T-DNA inserts to control the copy number. In addition, biolistic using microcarrier particles without DNA can also be utilized to cause superficial/minor injuries. The injured tissue can be co-cultivated with the suitable *A. tumefaciens* strain. However, biolistic methods being difficult, other alternative methods such as thermal shock before co-inoculation, needle injury, vacuum infiltration, co-cultivation in petri dishes containing co-culture medium or hydrated filter paper, or tissue sonication can be adopted (Dong et al. 2014).

(iv) Chloroplast genome transformation: The transformation of the chloroplast genome offers greater advantages over that of the nuclear genome in genetic engineering (Adem et al. 2017). This method has been enormously exploited to yield biopharmaceutical products such as vaccines, peptides, proteins,

human serum albumin, and antigens, in addition to resistance/tolerance against insect/pests, herbicide, drought, and pathogens in economically important crops. For chloroplast genome transformation, a typical vector contains the GOI, a selectable marker, an organelle-specific, and 5′- and 3′-UTRs that augment transcription and translation. The expression cassette must be skirted on the left and right borders by two genomic regions for site-specific insertion by homologous recombination (Verma and Daniell 2007). There is still a need to optimize this method for transformed and homoplasmic cell selection, and improved regeneration efficiency in many crop plants.

(v) Alternative plant transformation methods: To induce elite transgenic events, some of the desirable features are high transformation efficiency, inexpensive, and ease with reduced somaclonal variation to fulfill the current demand of agricultural production. Hence, alternative methods such as plant transformation methods free from tissue culture and mediated by *A. tumefaciens* using different explants like axillary buds, stem cuttings, or seeds have been standardized (Manickavasagam et al. 2004; Mayavan et al. 2015). Likewise, the plant transformation via pollen tubes has also shown the merits of being genotype-independent and tissue culture-free, with elevated efficiency and higher probability to generate selectable markers free events (de Oliveira et al. 2016). Similarly, other methods for *in planta* transformation have also demonstrated higher efficiency using carrier nanoparticles to efficiently deliver multiple cloning sites (Grossi-de-Sa). The *A. rhizogenes*-mediated root transformation and hairy root induction have been successfully used as a model for gene expression studies and function in several plant species (Daspute et al. 2019). However, due to the requirement of special handling, these methods are hardly utilized at present.

(vi) Clean-Gene technology: It is a safer way to develop genetically modified crops, that is, free from selectable marker genes, which may be undesirable from a biosafety point of view. It is a process to transform plants utilizing two different vectors, one carrying the transgene (GOI) and the other with the selectable marker or reporter gene (Kumar et al. 2010). Through *Agrobacterium tumefaciens*, these two vectors are integrated at different locations in the plant genomes, which can be segregated from each other at the next generation. So, it is an easy and efficient way to genetically modify plants safely. However, being easy and efficient to develop genetically modified plants, it has been rarely used due to low co-transformation efficiency, and different crop plants show different segregation patterns as in sugarcane and grapevine. Thus, to overcome these demerits, several strategies have been developed based on site-specific recombination systems or nucleases that mediate site-specific cleavage (Yau and Stewart 2013) to retain the GOIs. To generate marker-free plant, various site-specific recombination systems such as the Cre/Lox (Du et al. 2019), CINH/RS2 (Moon et al. 2011), FLP/FRT (Hu et al. 2008), and GIN/GIX (Onouchi et al. 1991) have been successfully used showing high efficiency in DNA excision. In addition, to generate transgene-free elite events has been also feasible by ribonucleoproteins (Cas9 nuclease plus a guide RNA)-based

genome editing, plant regeneration in nonselective medium, and screening of plant bulks using PCR (Liang et al. 2017).

4.3 Regulation of Gene Expression

There are a number of approaches to regulate gene expression from transcription initiation to RNA processing and to the posttranslational modification of a protein to modify the gene product (RNA or protein) (Fig. 1). Among those, gene overexpression is one of the widely utilized strategies to detect the gene function either inactivating (loss-of-function) or activating (gain-of-function) mechanisms. Several GOIs for economic agronomic traits have already been overexpressed in many crops. The overexpression of GOIs induced under different biotic and abiotic stresses have generated highly valuable phenotypes with reduced yield penalty (Wang et al. 2018a). Gene stacking strategy is another important technique to pyramid multiple abiotic and biotic stresses simultaneously, through which two or more GOIs in a single expression cassette have been utilized successfully to improve multiple economic traits in plants (Aznar et al. 2018). It is a powerful strategy to overcome the frequent breakdown of resistance by facilitating the long-term management of insect pests or pathogens. Another very important technique, that is, RNAi-mediated gene silencing has been extensively used to regulate the gene expression of agronomically important traits. Presently, a number of RNAi-based studied have been carried out to downregulate the essential genes related to economically important traits (Rosa et al. 2018). Another class of genes, that is, *MIR* genes, are the plant micro RNAs (miRNAs) which are typically 21–24 nucleotides in length and are transcribed in the nucleus from non-protein-coding genes. The differential expression of these genes upregulate or downregulate their target mRNAs associated with any phenotype (like growth, flowering, and senescence) or stress conditions (salinity, drought, and nutritional scarcity) (Hackenberg et al. 2015). Thus, the fine-tuning of these specific *MIR* genes by genetic engineering is a powerful genetic engineering strategy to improve key agronomic traits (Teotia et al. 2016).

Since the last decades, CRISPR/Cas9 or optimized nucleases such as CRISPR/Cpf1 or CRISPR/Csm1have been utilized successfully in plant genome editing (Wang et al. 2018a). It is a simple two-component system (guide RNA and Cas nuclease protein) that allows precise editing of target sequence(s) in the genome of an organism. In this process, a guide RNA (gRNA) recognizes the target sequence, which is complementary to it, and the CRISPR-associated endonuclease (Cas) cuts this targeted sequence (Liu et al. 2019). CRISPR-Cas9 induces the double-strand breaks (DSBs) at the targeted DNA site that are repaired either through nonhomologous end joining (NHEJ) or homology-directed repair (HDR) (Liu et al. 2019). Earlier techniques such as meganucleases, zinc finger nucleases (ZFNs), and transcription activator-like effector nucleases (TALENs) begin this new era. However, genome editing came into the limelight after the entry of clustered regularly

interspaced short palindromic repeats (CRISPR)/CRISPR-associated nuclease 9 (CRISPR/Cas9) (Chen et al. 2020). CRISPR/Cas9 technique is being mostly utilized in plant breeding programs for the sake of its high efficiency, easy performance, and high flexibility in comparison to earlier techniques.

The CRISPR-Cas system has been successfully practiced to engineer or edit several plant genomes. At present, CRISPR-Cas has multiplex editing capability as it can edit more than one gene at a time (Donohoue et al. 2018); in addition, it can target not only the open reading frame (ORF) (Liang et al. 2018) and untranslated region of a coding gene (Mao et al. 2018) but also noncoding RNAs (ncRNAs) (Li et al. 2018a) and microRNAs (Chang et al. 2016) as well as promoter regions (Seth 2016). The CRISPR/Cas9 and CRISPR/Cpf1 systems were engineered to control the expression of the GOIs (Tang et al. 2017) using a typical sgRNA into promoter sequence through deactivated Cas9 nuclease (dCas9) (lacks the HNH and RuvC domains) to produce DSB and is fused at the C-terminus to transcriptional activator or repressor domains (Lowder et al. 2017). There is another approach of CRISPR/Cas13a-mediated RNA editing in which Cas13a nuclease is utilized to target and cleave single-stranded RNA. It has been successfully developed in plant and mammalian cells to knock down any exogenous or endogenous RNA (Aman et al. 2018). Several advanced versions have been developed in CRISPR-based technology for DNA or RNA editing in plants. Among these, CRISPER-ribonucleoprotein (RNP)-based DNA/RNA editing technology has been considered the most important for the acquisition of novel traits in plants. The RNPs are accumulated in vitro and directly transferred into protoplasts or immature embryos followed by cell repair mechanisms that lead to mutations at the desired target site (Liang et al. 2018).

5 Genetic Engineering of Maize for Stress Tolerance

5.1 Genetic Engineering to Improve the Biotic Stress Tolerance in Maize

Biotic stresses are a significant threat to global food security. It comprises the damage brought about by living organisms like bacteria, viruses, fungi, insects, nematodes, and weeds to the plants. Due to the occurrences of climate variation, abiotic stresses appeared recently, whereas these biotic stresses were of historical significance. Previously, there are several incidences of biotic stresses that result in complete failure of the crops, causing famine, for example, potato blight in Ireland, maize leaf bight in the United States (Ullstrup 1972), the Great Bengal Famine in 1943 (Padmanabhan 1973), and coffee rust in Brazil (Rogers 2004). Globally, biotic stresses cause major yield losses resulting in around 800 million people being underfed and further intensifying the challenge of food security as 70% more food will be required by 2050 (Christou and Twyman 2004).

With the origin of new races of insects, pests, and pathogens with time, breeding for biotic stress-resistant crops is the principal challenge in front of plant breeders. Previously, traditional breeding strategies or classical breeding methods were undertaken and utilized the varietal germplasm, interspecific or intergeneric hybridization, induced mutations, and somaclonal variation of cell and tissue cultures for the creation of genetic variation but met with only bounded success. Although, as due to the complete breakdown of resistance, the speed of the evolution of the new races of pathogens could not get along with using these time-consuming tedious methods. In the same way, modern varieties are more susceptible to biotic and abiotic stresses in comparisons to their wild relatives and available land races, as during the time of development and selection for high yield loss of useful genes in terms of genetic erosion took place (Portis et al. 2004; Reif et al. 2005). Hence, for the development of effective and efficient resistance in a shorter duration, various transgenic approaches have been implemented by researchers to induce one or more useful characters, like herbicide tolerance, insect/pests, and disease resistance (Table 1).

Herbicide-Tolerant Transgenic Maize

Weed is an unwanted and undesirable plant that competes for nutrients, water, sunlight, and space with the crop plant, causing potential yield losses. Management of weeds by herbicides is one of the potent strategies, but almost all the weeds are herbaceous, and while protecting the crop plant, selective killing of the weeds is not always possible. Hence, the development of herbicide tolerance character in the main crop is a promising solution that can recommend the liberal use of robust nonselective and broad-spectrum herbicides. For weed control, based on selective and nonselective actions, two different types of herbicides are available. Among them, glyphosate and glufosinate, which are nonselective types of herbicides, are largely used. Hence, most of the herbicide-tolerant (HT) transgenic plants have been targeted to develop tolerance to these herbicides. The application of glyphosate inhibits 5-enolpyruvyl shikimate3-phosphate synthase (EPSPS) enzyme, which is involved in the shikimate pathway of aromatic amino acid biosynthesis. Glufosinate, which competitively inhibits glutamine synthetase enzyme (Lea et al. 1984), takes part in the conversion of glutamate and ammonia into glutamine. Inhibition of this enzyme by glufosinate leads to accumulation of ammonia, which constrains photosystem I and II reactions (Tachibana et al. 1986; Sauer et al. 1987). In maize, the development of herbicide tolerance transgenic constitutes the major portion of the GE field. Maize varieties having herbicide tolerance for glyphosate, chlorsulfuron, imazethapyr, phosphinothricin, etc. have been extensively adopted for cultivation, which ultimately benefits the environment as well as the farmers (Cao et al. 2011; Yadava et al. 2017). Few important genes, namely, *epsps, als, ahas, pat, bar,* etc., from bacteria and plants have been incorporated for the herbicide tolerance, which also play an important role in the selection of transgenic events (Yadava et al. 2017). Two different bacterial genes, namely, *pat* and *bar*, from *Streptomyces* spp. were

Table 1 Improvement of biotic stress tolerance in maize via genetic engineering

Target trait	Target gene	Donor organism	Genetic transformation technique	References
Sulfonylurea herbicidal tolerance	P450	Soil bacterium *Streptomyces griseolus*	Ectopic expression or a promoter specific for the tapetum	Werck-Reichhart et al. (2000)
Imidazolinone herbicidal tolerance	Acetohydroxy acid synthase gene (AHAS)		Targeted modification of endogenous genes using chimeric RNA/DNA introduced single point mutation	Zhu et al. (2000)
Sulfonylurea herbicidal tolerance	Aceto lactate synthase (ALS)		Cas9-gRNA causes single point mutation in the ALS gene from proline to serine	Chilcoat et al. (2017)
2,4-D herbicidal tolerance	Aryloxyalkanoatedioxygenase-1(AAD-1)	*Delftia acidovorans*	Transformation	Han and Kim (2019)
Glufosinate herbicidal tolerance	Phosphinothricin N acetyltransferase (PAT)	*Streptomyces viridochromogenes*	–	Green and Owen (2011)
Glufosinate herbicidal tolerance	Bialaphos resistance gene (BAR)	*Streptomyces hygroscopicus*	–	Han and Kim (2019)
Glyphosate herbicidal tolerance	GAT4601 Glyphosate N-acetyltransferase	*Bacillus licheniformis*	–	Han and Kim (2019)
Glyphosate herbicidal tolerance	CP4EPSPS 5-enolpyruvylshikimate-3-phosphate synthase	*Agrobacterium tumefaciens* strain CP4	–	Han and Kim (2019)
Glyphosate herbicidal tolerance	2MEPSPS	*Zea mays*	A double mutant version (T102I/P106S) of EPSPS mutation	Han and Kim (2019)
Glyphosate herbicidal tolerance	MEPSPS	*Zea mays*	A modified EPSPS (two amino acid substitutions)	Han and Kim (2019)

Chlorsulfuron herbicidal tolerance	ALS2 P165S	*Zea mays*	Targeted mutagenesis by CRISPR/Cas9	Svitashev et al. (2015)
Insect resistance	dsRNA-spray	Lepidopteran	RNA interference	Li et al. (2015)
Insect resistance	*dvvgr dvbol*	Corn root warm *D. virgifera*	RNA interference	Niu et al. (2017)
Rhopalosiphum padi insect resistance	ß-1-3glucanase	*Zea mays*	CRISPR/Cas9 lead to the reduction of callose deposition in maize sieve tubes	Kim et al. (2020)
Spodoptera exigua, Harmonia axyridis Insect resistance	cry1Ab/cry2Aj	*Bacillus thuringiensis* (Bt)	Agrobacterium-mediated transformation using cotyledonary node explants	Chang et al. (2017)
Corn borer (*Sesamia cretica, Ostrinia nubilalis, Chilo agamemnon*	*Spodoptera littoralis* chitinase gene	Insect chitinase cDNA from cotton leaf worm (*Spodoptera littoralis*)	Cloning and transformation	Osman et al. (2015)
Smut resistance	*Lipoxygenase 3(Lox 3)*	*Zea mays*	Site-directed mutagenesis by CRISPER/Cas9	Pathi et al. (2020)
Heliothis zea	cry1Ab	*Bacillus thuringiensis* (Bt)	Cloning and transformation	Koziel et al. (1993) Armstrong et al. (1995)
Pectinophora gossypiella	cry1Ac	*Bacillus thuringiensis* (Bt)	Cloning and transformation	Buschman et al. (1998)
Fusarium ear rot *Fusarium graminearum, Fusarium proliferatum, and Fusarium verticillioides*, insects and plant pathogenic fungi	chalcone isomerase 3-like (CI3)	Fusarium ear rot resistant inbred	Cloning and transformation	Dowd et al. (2018)
European corn borer	cry1Ab, cry1Ab	*Bacillus thuringiensis* (Bt)	Cloning and transformation	Sanchis (2011)

(continued)

Table 1 (continued)

Target trait	Target gene	Donor organism	Genetic transformation technique	References
Western bean cutworm, European corn borer, black cutworm, fall armyworm	cry1F	*Bacillus thuringiensis* (Bt)	Cloning and transformation	Sanchis (2011)
Western Corn Rootworm	cry3Bb1	*Bt subsp. kumamotoensis*	Cloning and transformation	Sanchis (2011)
Western, Northern, and Mexican Corn Rootworm	cry34/35Ab1		Cloning and transformation	Sanchis (2011)
European corn borer, corn rootworm	cry1Ab + cry3Bb1	*Bacillus thuringiensis* (Bt)	Cloning and transformation	Sanchis (2011)
Western bean cutworm, European corn borer, black cutworm, fall armyworm, Western/Northern/ Mexican corn rootworm	cry1F + cry34/35Ab1	*Bacillus thuringiensis* (Bt)	Transformation as electroporation, or particle bombardment	Ellis et al. (2002)
Diabrotica virgifera and other coleopteran insects	Genes encoding proteins	–	RNA interference	Baum et al. (2007)

used for creating the glufosinate-resistant crops. Both of these genes encode the phosphinothricin acetyl transferase (PAT) enzyme, which detoxifies this herbicide by acetylation. From1996 to 2018, a total of 351 HT events have been approved for cultivation (ISAAA 2019). Out of these, the maximum number of 210 HT events has been commercialized in maize, followed by Argentine canola (34), soybean (33), potato (4), carnation (4), rice (3), sugar beet (3), and wheat (1). Among the commercialized transgenic crops, HT transgenic crops inhabit the largest area. Next to the abovementioned herbicides, HT transgenic maize crop specific to other herbicides, such as 2,4-D (Han and Kim 2019), dicamba, isoxafutole, mesotrione, oxynil, and sulfonylurea (Chilcoat et al. 2017), has been commercialized recently as mentioned in Table 1.

Insect-Resistant Transgenic Maize

Among the biotic stresses, major crop loss is caused by insect pests and diseases. All around, about 67,000 insect species are causing severe losses to important crops. Previously, farmers mainly depend on the expensive chemically synthesized insecticides as a control measure of insect pests, but these chemicals increase the economic burden on the farmers as well as the environment unfriendly. Hence, to get the better of these pitfalls of insecticide use, genetic engineering of crops to develop insect resistance has gained popularity. Insect-resistant transgenic crops have the second largest area under cultivation, that is, 23.3 mha in 2017 (ISAAA 2017). Globally for cultivation, 304 transgenic events have been accepted, among which maximum 208 events comprising various insect resistance genes in maize have been accepted for cultivation. Generally, a distinct variant of *cry* gene as insecticidal genes and very few events of *vip* gene, which manage the harmful insects attacking the crops, has been transferred to most of the commercial crops (Kereša et al. 2008). The *cry* genes, which are isolated from *Bacillus thuringiensis* (Bt) (a soil bacterium), are among the widely utilized genes to develop insect-resistant transgenic crops. The *cry* genes from different isolates of *B. thuringiensis* offer resistance against a wide range of insect pests, that is, lepidopterans, coleopterans, and dipterans (McPherson et al. 1988). Several cry gene variants have been reported and utilized in gene stacking to develop stable insect resistance (Sanchis 2011; Chang et al. 2017). An additional advantage of the cry gene application is the nontoxicity of the cry protein to mammals. The insect-pest resistance in maize through GE demonstrated the potential of preventing environmental degradation, consumer acceptance, and cost-effectiveness to the farmers.

Disease-Resistant Transgenic Maize

Diseases caused by the pathogens, such as fungi, bacteria, viruses, and nematodes, result in substantial crop yield loss. Plant diseases are commonly managed by the application of agrochemicals, but hazardous effects caused by the use of

agrochemicals on the environment permit the investigation of other strategies to handle the plant diseases. Additionally, there might be the possibility of the emergence of chemical-resistant pests due to the undiscriminating use of chemicals. So, to get over the issues imposed by plant pathogens, there is a need for the development of inherent disease resistance in crop plants. For this, identification of genes responsible for disease resistance and transferring the same to plants through breeding or biotechnological approaches. As yet, globally 29 transgenic events possessing resistance against several diseases have been commercialized. Most of the virus-resistant transgenic crops have been developed via gene silencing techniques, such as co-suppression/RNAi and antisense RNA targeted against viral genes (Fei et al. 2007). In maize, various disease resistance such as smut resistance (Pathi et al. 2020) and Fusarium ear rot (Dowd et al. 2018) have been targeted through a transgenic approach as mentioned in Table 1. Parallel to herbicide and insect-pest resistance via GE, the development of disease-resistant varieties has been less focused on maize. GE promises enhanced disease resistance against various pathogens without affecting the beneficial microbes (Hilder and Boulter 1999; Wally and Punja 2010).

5.2 Genetic Engineering to Improve the Abiotic Stress Tolerance in Maize

Recently, climatic factors like temperature and rainfall are becoming arbitrary resulting in the transpose of temperature from the optimal state, alteration in precipitation pattern, perpetual drought, and heat negatively affecting crop production and productivity. In the last few decades, due to erratic climate changes, plants are becoming more vulnerable to abiotic stress, which threatens global food security issues (Bhusal et al. 2021). The maize production has been hampered by prolonged drought, heat, cold, variable precipitation, and increase salinity in the soil. Therefore, it is the need of the hour to develop a variety that can show tolerance to abiotic stress and able to sustain crop production. Conventional plant breeding strategy has not proved its success in addressing abiotic stress at a notable level. Genetic engineering provides innumerable applications in crop improvement via direct transfer of closely or distantly related genes of interest with desirable traits (Parmar et al. 2017). This resultant in the development of significant tolerance to abiotic stress in crops in a shorter period in comparison to conventional plant breeding techniques (Datta 2013; Marco et al. 2015). Here, we briefly describe abiotic stress tolerance in maize improved via genetic engineering, which was able to reduce losses due to climatic changes (Table 2).

Table 2 Improvement of abiotic stress tolerance in maize via genetic engineering

Target trait	Target gene	Donor organism	Genetic transformation method	Gene expression technique	References
Drought	*TsCBF1*	*T. halophila*	*A. tumefaciens* strain *LBA4404*	Overexpression	Zhang et al. (2010)
	TsVP and BetA	*T. halophile and E. coli*	*A. tumefaciens* strain *LBA4404*	Overexpression	Wei et al. (2011)
	ZmPLC1	*Z. maize*	*A. tumefaciens* strain *LBA4404*	Overexpression	Wang et al. (2008)
	rpk and nced	--	*A. tumefaciens* strain *EHA105*	Overexpression	Muppala et al. (2021)
	beta	*Escherichia coli*	*A. tumefaciens* strain *LBA4404*	Overexpression	Quan et al. (2004)
	ZmVPP1	*Zea maize*	*A.tumefaciens* (*GV3101 + pSoup*)	Overexpression	Wang et al. (2016a, b)
	LOS5	*Arabidopsis*	*A. tumefaciens* strain *EHA105*	Overexpression	Lu et al. (2013)
	Zm-Asr1 and C4–PEPC	*Z. maize*	*A. tumefaciens*	Overexpression	Jeanneau et al. (2002)
	ZmLEA14tv	*Z. maize*	*A. tumefaciens* strain *EHA105*	Overexpression	Minh et al. (2019)
	ZmVPP1	*Z. maize*	–	Overexpression	Jia et al. (2020)
	ZmNAC111	*Z. maize*	*A. tumefaciens* strain *LBA4404*	Overexpression	Mao et al. (2015)
	SbER1–1 and SbER2–1	*S. bicolor*	*A. tumefaciens strains EHA105 and GV3101*	Overexpression	Li et al. (2019)
	AnVP1	*A. nanus*	*A. tumefaciens* strain *LBA4404*	Overexpression	Yu et al. (2021)
	TPS1	*S. cerevisiae*	*A. tumefaciens* strain *LBA4404*	Overexpression	Liu et al. (2015)
	ZmTIP1	*Z. maize*	*A. tumefaciens* GV3101	Overexpression	Zhang et al. (2019)
	ARGOS8	*Z. maize*	Particle bombardment	CRISPR/Cas gene editing	Chilcoat et al. (2017)
Heat	*ZmHSFA2 and ZmHSBP2*	*Z. maize*	*A. tumefaciens*	Overexpression	Gu et al. (2019)

(continued)

Table 2 (continued)

Target trait	Target gene	Donor organism	Genetic transformation method	Gene expression technique	References
Salinity	*ZmWRKY114*	*Z. maize*	*A. tumefaciens* strain GV3101	Overexpression	Bo et al. (2020)
	ZmHAK4	*Z. maize*	*A. tumefaciens*	Overexpression	Zhang et al. (2019)
	ZmWRKY104	*Z. maize*	–	Overexpression	Yan et al. (2021)
	ZmHKT1;5	*Z. maize*	*A. tumefaciens* strain LBA4404	Overexpression	Jiang et al. (2018)
	SAG4 and SAG6	*Z. maize*	*A. tumefaciens*	Overexpression	Luo et al. (2019)
	ZmHKT1;1	*Z. maize*	*Floral dip*	Overexpression	Ren et al. (2015)
	ZmCPK11	*Z. maize*	*A. tumefaciens* strain EHA105	Overexpression	Borkiewicz et al. (2020)
	ZmEREB20	*Z. maize*	*A. tumefaciens* strain GV3101	Overexpression	Fu et al. (2021)
Cold	*AnAFP*	*A. nanus*	*A. tumefaciens*	Overexpression	Zhang et al. (2020b)
	ZmSEC14p	*Z. maize*	*A. tumefaciens* strain EHA105	Ectopic expression	Wang et al. (2016a, b)
	ZmLEA3	*Z. maize*	*A. tumefaciens* strain EHA105	Overexpression	Liu et al. (2016)
	ZmMYB48	*Z. maize*	*A. tumefaciens* strain LBA4404	Overexpression	Wang et al. (2017)
	ZmASR	*Z. maize*	*A. tumefaciens*	Overexpression	Li et al. (2018a, b)
Waterlogging	*HaOXR2*	*H. annuus*	*A. tumefaciens* strain LBA4404	Overexpression	Torti et al. (2020)
	6-BA	–	–	–	Hu et al. (2020)
	Spermidine	–	–	–	Liu et al. (2014)

(continued)

Table 2 (continued)

Target trait	Target gene	Donor organism	Genetic transformation method	Gene expression technique	References
Drought+salt	*SsNHX1*	*Suaeda salsa*	*A. tumefaciens* strain LBA4404	Overexpression	Huang et al. (2018)
	ZmmiR156	*Z. maize*	*A. tumefaciens* strain EHA105	Overexpression	Kang et al. (2020)
	ZmSCE1e	*Z. maize*	*A. tumefaciens* strain LBA4404	Overexpression	Wang et al. (2019)
	ZmBES1/BZR1-5	*Z. maize*	*A. tumefaciens* (Floral dip method)	Overexpression	Sun et al. (2006)
	ZmPIP1;1	*Z. maize*	*A. tumefaciens* strain EHA101	Overexpression	Zhou et al. (2018)
	ZmPIF3	*Z. maize*	*A. tumefaciens* strain EHA105	Overexpression	Gao et al. (2015a, b)
Drought+heat	*ZmWRKY106*	*Z. maize*	–		Wang et al. (2018a, b)
	ZmHsf06	*Z. maize*	*A. tumefaciens* strain GV3101	Overexpression	Li et al. (2015)
	ZmNF-YA3	*Z. maize*	–	–	Su et al. (2018)
Drought+salt+heat	*ZmERF1*	*Z. maize*	–	–	Shi et al. (2016)

Drought Tolerance Transgenic Maize

Maize being a drought-sensitive crop is highly affected at the seedling stage, which is the most critical stage of the growth period. At vegetative growth period particularly during V1 to V5, drought resulted in a reduction in crop growth, elongation of the vegetative growth period, and shrinkage of the reproductive growth period (Aslam et al. 2013; Aslam et al. 2015) consequently affecting the overall development of plant throughout their life cycle. Drought for a shorter duration resulted into reduction of 28–32% dry weight during the vegetative growth stage, while dry weight was reduced to 66–93% at the time of reproductive growth stages especially during tasseling and ear formation, respectively (Cakir 2004). Moreover, extended drought period hinders tassel and silk development, which leads to reduction of productivity by 15–25% (Nesmith and Ritchie 1992). Wang et al. (2019) observed the reduction in ear elongation and kernel size along with moderation in carbohydrate metabolism and plant growth hormone regulation under drought. Henceforth, there is an urgent need for the development and improvement of varieties with enhanced drought tolerance to achieve maximum maize production and productivity with less water requirement. There are reports on the development and

improvement of drought-tolerant maize varieties by conventional and molecular breeding approaches (Gedil and Menkir 2019). But the main drawback with these methods is lesser crop improvement in longer time duration coupled with dependent on the availability of gene-pool or germplasm (Anwar and Kim 2020). Therefore, genetic engineering as an alternative technique can be employed to improve drought tolerance more rapidly with reduced time (Zhang et al. 2000). There are a number of natural and synthetic genes and transcription factors whose incorporation can improve drought tolerance to maize via genetic engineering techniques (Parmar et al. 2017). In general, dehydration-responsive element binding (DREB), late embryogenesis abundant (LEA) proteins, proline accumulators, polyamines, and mitogen-activated proteins play a key role in improving drought tolerance to crops (Bidhan et al. 2011). Among all the factors, DREB proteins (a subfamily of APETALA 2/ethylene-responsive element binding factor (AP2/ERF) family) and mitogen-activated proteins are mostly targeted for maize drought tolerance. Shou et al. (2004a, b) reported the enhanced drought tolerance by incorporation of nicotiana protein kinase (NPK1) in transgenic maize via genetic transformation. The NPK1 is a tobacco mitogen-activated protein kinase kinase kinase (MAPKKK) enzyme that induces heat shock proteins (HSPs) and glutathione-S-transferases (GSTs) to protect photosynthetic machinery during drought stress. The function of LEA proteins has been briefly explained by Amara et al. (2014) in maize during dehydration state. Amara et al. (2013) improved drought tolerance in transgenic maize plants by overexpression of group 5 LEA Rab28 candidate gene during drought. This increases the accumulation and stability of Rab28 protein, which leads to enhance water stress tolerance. Likewise, Du et al. (2015) identified 59 trihelix TFs (GT factors) via in silico approach, which was distributed on maize chromosomes 1 to 10 (11, 8, 5, 9, 9, 2, 1, 4, 3, and 7 genes, respectively). These GT factors exhibit spatial-temporal expression toward drought tolerance in maize. Out of 59, 17 GTs were upregulated, while three were downregulated in response to drought. He et al. (2018) reported that overexpression of *ZmPYL3, ZmPYL9, ZmPYL10, and ZmPYL13* played a significant role in imparting drought resistance in transgenic plants by enhancing ABA signaling, proline, and other drought-related marker genes. Likewise, several drought-responsive genes and TFs have been identified such as *TsCBF1* from *T. halophila* (Zhang et al. 2010); *TsVP* and *BetA* from *T. halophile and E. coli* (Wei et al. 2011)*; ZmPLC1*, *ZmVPP1*, *ZmTIP1*, and *ZmNAC111* maize (Wang et al. 2008; Wang et al. 2016a; Zhang et al. 2020a; Mao et al. 2015); *LOS5* from Arabidopsis (Lu et al. 2013); *SbER1–1 and SbER2–1* from sorghum (Li et al. 2019); *AnVP1* from *AnVP1* (Yu et al. 2021); and many more, whose overexpression result in drought-tolerant in maize (Table 2). In a nutshell, the identified TFs and drought-responsive genes may serve as potential markers for drought improvement in maize via a transgenic approach.

Heat Tolerance Transgenic Maize

Being a tropical rainfed crop, maize is highly influenced by sporadic heat stress in the Asiatic region. Maize grown in subtropical areas are severely affected by higher temperature during early reproductive and grain filling stages (Prasanna 2011). By looking into the increasing demand for maize, another spring season has been added to take one more cropping season where it is exposed to extremely hot summer period of the year (February to May) especially during late vegetative and reproductive growth stages, which are unfavorable for crop growth resulted into yield loss (Lobell and Burke 2010). Siebers et al. (2017) observed a significant drop in production when heat waves warmed the canopy during silking stage for continuously 3 days. Maize encounters drastic physiological drought due to increased vapor pressure deficit connected to the higher temperature and less humidity under heat stress led to a relatively higher reduction in yield as compared to drought stress (Pavani et al. 2019). Therefore, the development of heat-tolerant varieties is required to sustain maize production and productivity under heat-stressed conditions. Since the heat sensitivity is tremendously fluctuating throughout the developmental growth stages of the plant, the development and improvement of heat tolerance is a challenging task by conventional plant breeding methods (Driedonks et al. 2016). To avoid environmental interaction and influence, genetic engineering can be the best option for the improvement of heat tolerance in maize. There are several reports that explain the role of HSPs, a kind of transcription factor that activates HSPs to interact with signal transduction via calcium and reactive oxygen species to provide thermo-tolerance to plants in response to cytoplasmic heat stress (Li and Howell 2021). Ribeiro et al. (2020) engineered the maize plants with *WPGD1* and *WPGD2* transgenes coupled with an endosperm-specific promoter, which increases the activity of *6PGDH* (6-phosphogluconate dehydrogenase) thereby being able to redeem defective *pgd3*-defective kernel phenotype consequently escalate heat tolerance. Overexpression of *OsMYB55* gene upregulates the HSPs in maize which reduces negative impacts of high temperature and improves tolerance to heat stress (Casaretto et al. 2016). According to Zhao et al. (2021), overexpression of abscisic acid-induced calcium-dependent protein kinase *ZmCDPK7* in transgenic maize lines resulted in the regulation of heat stress tolerance by upregulation and downregulation of the respiratory burst oxidase homolog *RBOHB* and phosphorylation of HSp *sHSP17.4*. This alteration by *ZmCDPK7* enhances thermostability, photosynthetic rates, and antioxidant enzyme activity, while it downregulates H_2O_2 and malondialdehyde (MDA) contents under heat stress. Transformation of *Arabidopsis thaliana* trehalose phosphate synthase gene (*AtTPS1*) enhances heat tolerance in maize by involving in trehalose biosynthesis (Almeida et al. 2003). Ko et al. (2007) identified that thermotolerance (*TTO6*) gene is responsible for heat shock stress tolerance in maize clones, which is 69% similar to *GASA4* gene from *Arabidopsis thaliana*. Similarly, *ZmHSFA2* and *ZmHSBP2* from maize have significantly contributed to heat tolerance (Gu et al. 2019). The above studies suggest that the identified domains can be employed to develop long-term heat-tolerant genotypes in maize through transgenic approaches.

Salinity Tolerance Transgenic Maize

Globally, around 50% of irrigated and 20% cultivated land is highly affected by salinity stress, which influences the production and productivity of crops (Wang et al. 2017). Maize is moderately sensitive to salinity, which adversely affects its growth and development. Salinity diminishes shoot growth by suppressing leaf growth rate, internode growth, number, and rate of elongation cells. At the molecular level, salinity stress results in membrane damage, protein denaturation, accumulation of oxidative substances, and reduction of relative water content in leaves. These all result in the reduction of photosynthetic rate consequently yield loss in maize (Szalai and Janda 2009). Maize undergoes several biochemical and physiological changes to adapt under salinity stress. The focal point of genetic engineering is to identify key genes/factors associated with the molecular, physiological, and biochemical pathway of salinity stress to improve salinity tolerance by overexpression of identified genes. Typical transcription factors (TF) like DREB, MAPK, and MYB (myeloblastosis) can enhance heat response by actively participating in signal transduction pathways in transgenic plants. MYB TFs were firstly identified in maize, and till today, around 200 MYB families are known to be responsible for abiotic stress response (Du et al. 2012). Wu et al. (2019) studied the effect of a transcriptional activator, that is, ZmMYB3R, whose overexpression results in enhancement of salinity tolerance in transgenic lines by modifying the root architectural system and hormone regulation. ZmMKK4 belongs to MAPKK gene family, which encodes for group C in maize and resides inside the nucleus. Overexpression of ZmMKK4 results in salt tolerance in transgenic *Arabidopsis*. These transgenic *Arabidopsis* lines exhibit higher germination rate, lateral root numbers, chlorophyll content, catalase, and peroxidase activity (Kong et al. 2011). The class of deubiquitinating enzymes (DUBs) and ubiquitin-specific proteases (UBPs) is engaged in the growth and development of the plant. The proteins UBP15, UBP16, and UBP19 are homologs of UBP16 of *Arabidopsis,* which actively participate in signal transduction during salt stress in maize. Overexpression of *ZmUBP15, ZmUBP16*, and *ZmUBP19* led to rescue ubp16-1, which impart salinity tolerance in transgenic *Arabidopsis* (Kong et al. 2019). Wang et al. (2007) performed cloning and functional characterization of *ZmCBL4*, a putative homolog of *Arabidopsis* calcineurin B-like protein/salt overly sensitive CBL4/SOS3 protein with unique features. Constitutive expression of *ZmCBL4* showed enhanced tolerance to salinity in transgenic lines of *Arabidopsis*, which is similar to SOS3 in the salt signaling pathway. These transgenic lines exhibit better shoot and root development under salt stress. Besides all these TFs, there are various other genes, and TFs have been diagnosed such as *ZmHKT15*, *ZmHAK4*, *ZmCPK11*, *ZmWRKY114*, *ZmEREB20*, and*ZmWRKY104* (Jiang et al. 2018; Zhang et al. 2019; Borkiewicz et al. 2020; Bo et al. 2020; Fu et al. 2021; Yan et al. 2021) whose transformation has resulted into better performance under the saline condition in maize. The detailed information concerning donor organism, transformation method, and genetic engineering approach has been given in Table 2. The identified functional genes can be useful for gene stacking against salinity tolerance by genetic engineering with high precision in a shorter period.

Cold Tolerance Transgenic Maize

Among abiotic stresses, low temperature (chilling and freezing temperature) is another major stress, which limits the growth and development of plants thereby reducing the production and productivity of maize (Meng and Sui 2019). Chilling and freezing stress is responsible for catastrophic events of the biological and physiological process like membrane destruction, ion leakage, stomata opening, the release of toxic substances, reduced photosynthesis and respiration, reduction in unsaturated fatty acids, increased level of reactive oxygen species (ROS) production, seed germination, and seedling establishment which adversely affect vegetative and reproductive growth stages (Einset et al. 2007). To improve cold tolerance, it is necessary to maintain structural integration of lipid membrane and their dynamic transitional ability from a liquid crystalline to the gel phase. Plants have developed some enzymatic or nonenzymatic adaptive mechanisms like antioxidants, superoxide dismutase (SOD), catalase, ascorbate peroxidase (APX), etc. Conventional plant breeding takes a long time to improve cold tolerance. Moreover, most of the time, the improved lines exhibit nonsignificant results in low temperature. To narrow down the pros of conventional breeding methods, genetic engineering can play a significant role in tolerance toward chilling and freezing. Genetic engineering alters transfer and expresses the targeted gene (antifreeze proteins and TFs) in transgenic lines to reduce the effects of cold stress (Goel and Madan 2014). There are several enzymes such as glycine betaine, antioxidants, catalase, peroxidase, etc. whose integration results in cold improvement under stress conditions. TFs involved in cold stress are MYB, MAPK, LEA, CBL (calcineurin B-like proteins), DREB, ZIP (basic leucine zipper), NAC {NAM (no apical meristem), ATAF (*Arabidopsis* transcription activation factor), and CUC Cup Shaped Cotyledon)}, CBFs (cold-inducible master transcription factors), etc. (Shou et al. 2004b). Meng and Sui (2019) investigated the effect of *ZmMYB-IF35* gene in response to cold stress in transgenic lines of *Arabidopsis*. The transgenic lines with the integration of *ZmMYB-IF35* gene result in chilling tolerance by increasing the activity of SOD and APX enzymes. These enzymes act as a scavenger and result into the protection of chloroplast membrane and maintain the integrity of lipid membrane. *ZmMKK1* belongs to *MAPK* group in maize for cold tolerance. Cai et al. (2014) performed isolation and functional characterization of *ZmMKK1*. Overexpression of *ZmMKK1*can show enhanced chilling tolerance in transgenic tobacco. The transgenic lines exhibited increased seed germination, early seedling establishment, longer root growth, lower production of malon-dialdehyde and relative electrolyte leakage, and increased level of soluble sugar, proline content, and inhibition of ROS production. Constitutive expression of tobacco *MAPKKK (NPK1)* at a low level increased the freezing tolerance in transgenic maize by mimicking the effect of H_2O_2 signaling, increasing soluble sugar and osmolytes, which act as cryoprotectants and stabilize the membrane integrity (Shou et al. 2004b). Similarly, *AnAFP* from *A. nanus (*Zhang et al. 2020b) and *ZmSEC14p*, *ZmLEA3*, and *ZmASR* from *Z. maize* (Wang et al. 2016b; Liu et al. 2016; Li et al. 2018b) have been identified and briefly explained in Table 2. These different studies suggest that overexpression

of identified *CBFs* and other TAFs in transgenic plants can induce cold and freezing tolerance in maize. Therefore, these detected genes/TFs can be used in the development of cultivars tolerant to cold and freezing stress.

Waterlogging Tolerance Transgenic Maize

Maize being a dry land crop is highly sensitive to waterlogging stress (Zaidi et al. 2010). Excessive water severely hampers the growth and development of plants and limits the quantity and quality of products (Liang et al. 2020). Approximately 10% of the world's arable land is highly affected by waterlogging stress. The annual maize yield was reduced to about 25–30% in India due to waterlogging (Zaidi et al. 2010). Since the solubility and diffusion rate are very low in waterlogging stress, it leads to a reduction in the amount of available oxygen to plants. To overcome the effects of flooding, plants exhibit some morphological, physiological, and biological adaptation. These adaptations include the development of adventitious root, lysigenous aerenchyma tissue, and hydrophobic surface formation to improve diffusion rate and decrease radial oxygen loss for reduction of oxygen leakage from the rhizosphere (Pan et al. 2020). But the adaptation to waterlogging stress is slightly different in maize from other cereals and marshland plants, as generally it does not develop aerenchyma cells under excessive water (Gong et al. 2019). The genomic regions associated with aerenchyma formation have been studied by Gong et al. (2019) after the introgression of maize with wild relative (*Zea nicaraguensis*) to improve tolerance of oxygen deficiency. But to develop improved lines with these conventional methods is not up to the mark and required an alternate strategy such as genetic engineering. This technology has the potential to enhance waterlogging tolerance in maize. Du et al. (2010) isolated and functionally characterized *zmzf* (zea maize zinc finger) promoter from Mo17 inbred line, which is waterlogging inducible promoter and highly specific to root traits. The transformation of *zmzf* can result into the development of lines with waterlogging tolerance in maize. A group of VII ethylene response factors (ERFVIIs) plays a significant role in waterlogging tolerance in plants. However, in the case of maize, *ZmERFVIIs* is a nonresponsive gene toward waterlogging. In light of this, Yu et al. (2019) identified tightly linked gene *ZmEREB180* (waterlogging-responsive gene) with *ZmERFVIIs*, which is upregulated by ethylene under excessive water. Du et al. (2015) reported 59 trihelix TFs, which were differentially expressed under excessive water stress. Out of 59, 14 GTs were upregulated during waterlogging. These GTs are associated with the primary and secondary structure of proteins, amino acid composition, solubility, and folding state of protein, which serves as valuable information during stress. Overexpression of *ZmEREB180* in transgenic lines of maize is able to enhance the survival rate via adventitious root formation, modulation of ROS, and antioxidant levels under submergence conditions. A gram-negative aerobic bacterium, *Vitreoscilla*, contains a type of hemoglobin Vitreoscilla hemoglobin (*VHb*), which contributes to waterlogging tolerance in plants. Overexpression of *VHb* gene in maize through particle bombardment exhibits waterlogging tolerance under submergence conditions. The transgenic lines resulted into an increment of the activity

of alcohol dehydrogenase, peroxidase enzyme, which ultimately improves primary root length, lateral root number, root dry weight, and shoot dry weight (Du et al. 2016). Likewise, other genes and TFs like *HaOXR2* from *H. annuus* (Torti et al. 2020), *6-BA* (Hu et al. 2020) and *Spermidine* (Liu et al. 2014) have significantly enhanced maize improvement through their overexpression under waterlogging (Table 2). Henceforth, the identified genomic regions or TFs responsible for waterlogging tolerance can be utilized by genetic engineering approaches to develop tolerant and resistant cultivars in maize.

6 Conclusion and Future Perspectives

Currently, with changing climatic conditions and food security challenge, employment of new biotechnological tools facilitates wider range of solutions. Hence, the development of genetically modified crops is the hottest topic ever and has been grown as the fastest agricultural technology in the world. In the international market, there is an issue for the acceptance of GM food crops due to its adverse effect on health, but it can be tackled by isolating harmless genes from specific sources or by inducing toxicity properly. To resolve the concerns regarding GM crops, there is the need to opt strict legislation in addition to rigorous technical assessments considering the societal values and demands. The development of transgenic crop conventionally through introduction of foreign gene raise the concerns of toxicity and risks to humans, environment, natural biodiversity, and other nontarget organisms. Hence, to bypass such concerns, the endorsement of alternative technologies like cis-genesis and intra-genesis has come in limelight where transformation belongs from sexually compatible gene pool. Different versions of novel genome editing techniques through using clustered regularly interspaced short palindromic repeats (CRISPR)/Cas system enable precise editing of endogenous gene and site-specific insertion of a GOI. The adoption of these genome editing techniques has the potential to discourse many regulatory issues associated with transgenes and resolve the uncertainty and inefficiency related with conventional random mutagenesis and transgenesis. Some of these methods can also develop crop plants free from any foreign gene, which might help it to fetch higher consumer acceptance in comparison to the transgenic crops and would get quicker regulatory approvals.

The regulation of genetically modified crops varies across nations. By identifying the shared facts, opinions, technical expertise, and experiences of various competing interests like researchers, bureaucrats, politicians, and societal interests should harmonize the regulation of transgenics development. Some countries have widely supported the cultivation of transgenic crops due to their increased yields, decreasing the use of pesticides that save the environment and the cost of pesticides and the production of crops with increased nutritional value. Hence, to fit in the climatic changing scenario, the concept of producing transgenic crops is a powerful tool in the current era. To spread the use of transgenic crops at large requires more research at field level considering each aspect of human and environmental safety. It will help to get clarity to fully take the potential advantage of this useful invention.

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