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OPINION PAPER

Engineering abiotic stress tolerance via CRISPR/Cas-mediated genome editing

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Abstract

Abiotic stresses, including drought, salinity, temperature, and heavy metals, pose a major challenge for crop production and cause substantial yield reduction worldwide. Breeding tolerant cultivars against these abiotic stresses is the most sustainable and eco-friendly approach to cope with this challenge. Advances in genome editing technologies provide new opportunities for crop improvement by employing precision genome engineering for targeted crop traits. However, the selection of the candidate genes is critical for the success of achieving the desired traits. Broadly speaking, these genes could fall into two major categories, structural and regulatory genes. Structural genes encode proteins that provide stress tolerance directly, whereas regulatory genes act indirectly by controlling the expression of other genes involved in different cellular processes. Additionally, *cis*-regulatory sequences are also vital for achieving stress tolerance. We propose targeting of these regulatory and/or structural genes along with the *cis*-regulatory sequences via the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) system as a robust, efficient, and practical approach for developing crop varieties resilient to climate change. We also discuss the possibility of creating novel quantitative trait loci for abiotic stress tolerance via the CRISPR/Cas-mediated targeting of promoters. It is hoped that these genome editing tools will not only make a significant contribution towards raising novel plant types having tolerance to multiple abiotic stresses but will also aid in public acceptance of these products in years to come. This article is an attempt to critically evaluate the suitability of available tools and the target genes for obtaining plants with improved tolerance to abiotic stresses.

Keywords: Abiotic stress, CRISPR/Cas9, climate-resilient crops, genome editing, sensitivity genes, tolerance genes, transgenic.

Introduction

Climate change threatens agriculture and food security (Piao *et al.*, 2010; Hasegawa *et al.*, 2018). Excessive emission of greenhouse gases is responsible for frequent episodes of high temperature and drought stress to crops (Asseng *et al.*, 2015). An increase of 1 °C in atmospheric

temperature is predicted to reduce the yield in wheat, rice, and maize by 6, 10–20, and 21–31%, respectively, thus threatening global food security (Asseng *et al.*, 2015; Yang *et al.*, 2017; Wang *et al.*, 2019). Importantly, the negative impact of such abiotic stresses is more severe in Africa and South Asia, which are already experiencing food insufficiency (Hasegawa *et al.*, 2018). Thus, breeding climate-smart crops which can tolerate abiotic stresses such as recurrent heat stress, drought, and/or salinity would be a sustainable approach to cope with such challenges. Although conventional breeding has contributed significantly towards crop improvement for abiotic stress tolerance, more efficient and modern technologies with immediate impacts are surely needed to address this challenge (Driedonks *et al.*, 2016). Further, it has been estimated that agricultural production will have to be increased by at least 70–85% by the year 2050 to feed the projected global population of 9.7 billion (Alexandratos and Bruinsma, 2012; Ray *et al.*, 2013). Owing to the impressive progress made in molecular biology techniques, the ability and the ease in development of genetically modified (GM) crops is being perceived as a 'gene revolution' in agriculture, thus potentially contributing to food security. Illustrative examples in this category are the nutritionally enhanced crops such as golden rice and the herbicide/insect-resistant crops such as Bt-cotton (Napier *et al.*, 2019). However, the full adoption of GM crops in many countries is still awaited (Shukla *et al.*, 2018). Thus, continuous efforts are needed towards the invention and adoption of new breeding techniques such as 'genome editing' to accelerate both the rate of crop improvement and public acceptance.

During the last decade, advances in genome editing techniques have revolutionized crop improvement programs, thus enabling highly efficient and precise gene editing down to the level of a single base (Lu and Zhu, 2017; Zong *et al.*, 2017). The availability of refined genome editing tools offers excellent opportunities for trait discovery and development. This would further widen the range of novel traits, thereby leading to more efficient and targeted improvement of crop traits, especially tolerance towards abiotic stress (Dalla Costa *et al.*, 2017; Klap *et al.*, 2017). In this article, we discuss the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) genome editing techniques which could potentially be utilized for rapid development of abiotic stress-tolerant crops. These tools may further aid in promoting and enabling the usage of the products concerning their societal acceptance primarily because of its precision and 'foreign DNA-free engineering' approach. Some countries have openly adopted genome-edited crops; whereas, many countries are still debating on this issue. However, it is expected that this technology will certainly go a long way towards enabling a relatively easy adoption of genome-edited crops in most countries. We also introduce two new terminologies for defining the genetic factors which negatively or positively regulate the response of plants towards abiotic stresses. These are referred to as 'sensitivity genes (or *S* genes)' and 'tolerance genes (or *T* genes)'. This is in agreement with the established terminologies prevalent in the area of biotic stresses where *S* genes and *R* genes are well accepted.

Targeting structural genes to achieve abiotic stress tolerance

Structural genes are an important class of targets which can enhance specific features of stress tolerance. Reactive oxygen species (ROS) play essential roles in plants by acting as signaling molecules for the regulation of gene expression (Ribeiro *et al.*, 2017), plant defense against viral pathogens (Wu *et al.*, 2017), and symbiotic nitrogen fixation between plants and the soil rhizobia (Sinharoy *et al.*, 2016). However, overproduction of ROS, which is a typical response of plants towards abiotic and oxidative stresses, can impart different types of growth abnormalities such as a reduction in photosynthesis rate, enhanced cell death, and even male sterility, leading to reduced crop yield (Hu *et al.*, 2011; Zafar *et al.*, 2019). Thus, keeping a check on ROS production and scavenging is vital to maintain redox balance in cells (Mittler, 2017). Dozens of genes encoding antioxidant enzymes such as catalases (CATs), superoxide dismutase (SOD), glutathione reductases (GRs), glutathione *S*-transferases (GSTs), and many peroxidases (PODs) participate in the scavenging of ROS molecules. These genes can be referred to as the *T* genes which contribute to abiotic stress tolerance (Hu *et al.*, 2011; Mittler, 2017). However, several *S* genes causing excessive production of ROS (which is usually referred to as oxidative stress), reduced antioxidant activity, and enhanced programmed cell death (PCD), and the genes that cause disturbance in hormonal homeostasis making plants sensitive to abiotic stresses have been reported (Fang *et al.*, 2015; Liu *et al.*, 2016; Zhao *et al.*, 2017). Molecular breeders and geneticists have identified several *T* genes associated with abiotic stress tolerance and incorporated them into plants to achieve tolerance. For example, papain-like cysteine proteases (PLCPs) are found to be enhanced under abiotic stress conditions in various plant species such as sweet potato (*SPCP2*) and wheat (*TaCP*). Arabidopsis lines overexpressing *SPCP2* and *TaCP* showed enhanced tolerance to drought stress (Chen *et al.*, 2010; Zang *et al.*, 2010; Liu *et al.*, 2018). Another example of this category are the melatonin biosynthetic genes. Melatonin is an antioxidative molecule which helps plants in scavenging ROS and reactive nitrogen species (RNS). Plants overexpressing melatonin biosynthesis genes were found to be tolerant to different abiotic stresses (Zuo *et al.*, 2014; Byeon and Back, 2016; Antoniou *et al.*, 2017). Further, most of the *T* gene alleles have been lost during crop domestication programs, but it is believed that the wild species may still possess them. This could be due to the fact that breeders mostly select 'yield-contributing genes' during the selection programs, thus overlooking the genes contributing to stress tolerance.

S genes which negatively regulate abiotic stresses have been underexplored so far. Thus, knocking out *S* genes may contribute towards stress tolerance by disrupting the pathways involved. For example, *Oryza sativa* stress-related RING finger protein 1 (OsSRFP1) is an E3 ubiquitin ligase and functions as a negative regulator for multiple abiotic stresses by enhancing the level of H_2O_2 (an important ROS species) and reducing the activities of antioxidant enzymes in plant tissues (Fang *et al.*, 2015). Knockdown of *OsSRFP1* increased plant tolerance to abiotic stresses via disrupting H_2O_2 biosynthesis and positively regulating antioxidant activities (Fang *et al.*, 2015). Several sensitivity genes such as *OsDIS1* (*O. sativa* drought-induced SINA protein 1) and *DST* (drought and salt tolerance) have been tested for such functional transformation via RNAi-mediated gene silencing (Huang *et al.*, 2009; Ning *et al.*, 2011). Although several *S* genes have been reported in different crop species (Table 1), studies regarding their engineering for improving the tolerance towards abiotic stress are

sparse. Nevertheless, several *S* genes causing susceptibility to biotic stresses (diseases and insect pests) have been targeted (mutated) to achieve resistance against biotic stresses in different crops (Wang *et al.*, 2014; Pyott *et al.*, 2016; Thomazella *et al.*, 2016; Zhang *et al.*, 2017).

Targeting the regulatory genes to enhance abiotic stress tolerance

Regulatory genes such as those encoding transcription factors (TFs), phosphatases, and kinases are another important class of targets for modulating the level of expression of several downstream genes and activating many stress signals. For example, in Arabidopsis, a NAM-ATAF1/2 and CUC2 (NAC) TF gene, *ANAC069*, functions as a negative regulator of abiotic stresses (*S* gene). *ANAC069* regulates the expression of multiple stress-responsive genes by binding specifically to the core motif sequence C[A/G]CG[T/G] in their promoter region. This results in decreased ROS-scavenging capability and a high level of proline biosynthesis, leading to increased sensitivity to salt and osmotic stress (He *et al.*, 2017). *ANAC069* knockdown mutants obtained via T-DNA insertion showed enhanced tolerance to salt and osmotic stress (He *et al.*, 2017). Similarly, overexpressing *T* genes such as *AtMYB44* confers drought and salt tolerance via enhancing abscisic acid (ABA)-induced stomatal closure (Jung *et al.*, 2008). Also, overexpression of *ZmWRKY106* enhances drought and heat tolerance in transgenic plants by regulating the expression of stress-related genes, reducing the ROS content, and increasing activities of antioxidant enzymes (Wang *et al.*, 2018).

Another class of important targets in this category are the miRNAs which are small non-coding RNAs regulating the expression of numerous genes at the post-transcriptional level by targeting specific mRNAs. miRNAs function by gene silencing via complementary base pairing with mRNA transcripts, and thus regulate various biological processes. Some miRNAs have been described to regulate the abiotic stress response, which targets the specific stress-responsive genes. For example, *osa-MIR393* negatively regulates salt and alkali stress tolerance by regulating the expression of three crucial stress-responsive genes (*LOC_Os02g06260*, *LOC_Os05g41010*, and *LOC_Os05g05800*). Overexpression of *osa-MIR393* caused enhanced sensitivity to salt and alkali stress (Gao *et al.*, 2011). Thus, silencing of this gene can lead to improved tolerance to these stresses. Similarly, another miRNA, miR399, regulates phosphorus homeostasis in Arabidopsis by targeting ubiquitin-conjugating E2 enzyme (AtUBC24). miR399 is up-regulated upon phosphorus starvation and suppresses its target E2 enzyme. Wild-type plants take up

Table 1. A list of representative sensitivity (S) genes proposed as the potential targets for improving tolerance towards abiotic stresses through genome editing

Name	Target gene/miRNA/TF	Species	Stress	Reference
RGLG2	*AtERF53*	Arabidopsis	Drought	Cheng *et al.* (2012)
OMTN2, *OMTN3*, *OMTN4*, *OMTN6*	N/A	Rice	Drought	Fang *et al.* (2014)
AtPUB19		Arabidopsis	Drought	Liu *et al.* (2011)
ARR1, ARR10, ARR12		Arabidopsis	Drought	Nguyen *et al.* (2016)
OsDIS1		Rice	Drought	Ning *et al.* (2011)
OsiSAP7	N/A	Rice	Drought	Sharma *et al.* (2015)
MODD	OsbZIP46	Rice	Drought	Tang *et al.* (2016)
FtMYB10	N/A	Arabidopsis	Drought and salt	Gao *et al.* (2016)
GhWRKY17		*Nicotiana benthamiana*	Drought and salt	Yan *et al.* (2014)
PagGla, *PagGlb*, and *PagGlc*	N/A	Arabidopsis and Poplar	Salt	Ke *et al.* (2017)
GmWRKY13		Arabidopsis and soybean	Salt	Zhou *et al.* (2008)
ZmWRKY17	*ZmCAM2*	Maize and Arabidopsis	Salt	Cai *et al.* (2017)
AtWRKY15		Arabidopsis	Salt	Vanderauwera *et al.* (2012)
CmWRKY17	N/A	Chrysanthemum and Arabidopsis	Salt	Li *et al.* (2015)
OsERF922	TF	Rice	Salt	Liu *et al.* (2012)
GhSARP1		Arabidopsis and cotton	Salt	Liu *et al.* (2016)
OsRMC	N/A	Rice	Salt	Serra *et al.* (2013)
PIF3	*CBF* genes	Arabidopsis	Cold	Jiang *et al.* (2017)
AtVDAC1	N/A	Arabidopsis	Cold	Li *et al.* (2013)
OsMATE1 and *OsMATE2*	N/A	Rice	Arsenic and disease	Tiwari *et al.* (2014)
CBF1/DREB1B, CBF3/DREB1A		Arabidopsis	Cold	Novillo *et al.* (2004)
OsGIRP1		Rice	Radiation stress	Park *et al.* (2015)
miRNA399	E2 enzyme	Arabidopsis	Pi toxicity	Chiou *et al.* (2006)
osa-MIR393	*LOC_Os02g06260, LOC_Os05g41010, LOC_Os05g05800*	Rice and Arabidopsis	Salt and alkali	Gao *et al.* (2011)

*TF; transcription factor.

inorganic phosphorus (Pi) via the roots and readily translocate it to shoots and other plant tissues. However, plants overexpressing miR399 showed impaired Pi remobilization leading to Pi toxicity due to its overaccumulation in shoots (Chiou *et al.*, 2006). Thus, the identification and targeting of particular miRNAs involved in abiotic stress regulation via precise genome editing tools could improve abiotic stress tolerance in crops.

Importance of *cis*-regulatory sequences in tackling abiotic stresses

Cis-regulatory sequences have fundamental importance in regulating the expression of genes as these sequences facilitate the recruitment of specific TFs. The role of *cis*-regulatory sequences in abiotic stress regulation has been well documented (Liu *et al.*, 2014). These sequences are mostly present in the promoter region of genes, and presence/absence/variation in the position/sequence of these sequences would influence the expression of the gene which could lead to the induction, reduction, or even no expression of the gene. Several *cis*-regulatory sequences such as the W-box (TTGACC) and GCC box (AGCCGCC) function as negative regulators of abiotic stress response/tolerance by providing binding sites for particular TFs such as GhWRKY17 and OsERF922, respectively (Table 2). Thus, these *cis*-regulatory sequences could serve as a suitable target for creating nucleotide-level mutations using recent genome editing tools that may improve tolerance to abiotic stress tolerance in crops. For example, *Arabidopsis thaliana* ANAC069 inhibits the expression of several stress-responsive genes (*T* genes, e.g. *SOD*, *POD*, *GST*, and pyrroline-5-carboxylate synthase, *P5CS*), which have ROS-scavenging activities, and thus negatively regulates salt and osmotic stress tolerance. ANAC069 regulates the expression of these genes by interacting with *cis*-elements and binds specifically to the DNA sequence C[A/G]CG[T/G] (He *et al.*, 2017). Mutation in this core sequence causes the failure of gene regulation by ANAC069 and thus would lead to stress tolerance. Novel promoter variants can also be created to produce useful novel phenotypic variation and new quantitative trait loci (QTLs) by 'gain-of-function' mutation for various traits including abiotic stress tolerance. Notably, CRISPR/Cas9-driven mutagenesis of *cis*-sequences in the promoters of several genes created a continuum of genetic and phenotypic variation which resulted in the creation of novel QTLs and improved tomato size and yield (Rodríguez-Leal *et al.*, 2017). This approach could be efficiently exploited for the improvement of complex traits such as abiotic stress tolerance via generating novel alleles and QTLs (Fig. 1).

CRISPR/Cas9-mediated targeting of abiotic stress tolerance genes

The discovery of programmable nucleases that generate double-strand breaks has revolutionized molecular biology by opening up the way for targeted genome editing. Zinc finger nucleases (ZFNs) take the credit as the first genome editing tool that caused a breakthrough in genome engineering by employing programmable nucleases (Chandrasegaran and Carroll, 2016). Soon after, transcription activator-like effector nucleases (TALENs) that are based on bacterial TALEs further expanded the capability of genome engineering. These tools were quickly adapted to ~40 different organisms for genome engineering (Chandrasegaran and Carroll, 2016). However, the discovery of CRISPR/Cas9 received a great deal of attention from scientists around the world due to its apparent benefits over ZFNs and TALENs (Mao *et al.*, 2013). Unlike ZFNs and TALENs, which use protein motifs for target identification, CRISPR/Cas9 depends on RNA–DNA recognition to create the double-strand break. Other advantages of CRISPR/Cas9 over ZFNs and TALENs are (i) simplicity of the target design; (ii) efficiency of introducing mutations by directly injecting the RNAs encoding Cas9 protein and guide RNA (gRNA); and (iii) the ease of multiplexing, causing targeted mutations in multiple genes in a single event (Ma *et al.*, 2015; Malzahn *et al.*, 2017). It is easy, flexible, and efficient because designing the CRISPR/Cas9 vector is relatively less tricky than previous techniques such as ZFNs or TALENs due to the availability and easy access to the improved bioinformatics tools, which could be used to identify the most appropriate sequences to design the gRNAs with no further need for screening libraries to fish out the most efficient target. In recent years, several modifications and improvements have been made to the CRISPR/Cas9 system that enabled researchers to make precise modifications in any organism of interest with 'nucleotide-level' precision, and that too in an exceptionally rapid manner. These advancements have contributed significantly to the wider adaptability of this technique among eukaryotes.

In addition to mutagenesis, CRISPR/Cas 9 can be used to induce (CRISPR activation or CRISPRa) or repress (CRISPR interference or CRISPRi) gene expression by fusing catalytically inactive Cas9 (dCas9) with a transcriptional activator or repressor

Table 2. A list of representative cis-regulatory sequences causing sensitivity towards abiotic stresses

cis-sequences	TFs	Response	Species	Reference
W-box (TTGACC)	GhWRKY17	Drought and salt sensitivity	Cotton	Yan *et al.* (2014)
C[A/G]CG[T/G]	ANAC069	Salt and osmotic sensitivity	Arabidopsis	He *et al.* (2017)
W-box (TTGAC)	GmWRKY13	Salt sensitivity	Soybean	Zhou *et al.* (2008)
W-box (TTGACC)	ZmWRKY17	Salt sensitivity	Maize	Cai *et al.* (2017)
GCC box (AGCCGCC)	OsERF922	Salt sensitivity	Rice	Liu *et al.* (2012)
DBS (TGCTANNATTG)	DST	Drought and salt sensitivity	Rice	Huang *et al.* (2009)
MYBR (A/TAACCA and C/TAACG/TG)	MYBC1	Freezing stress sensitivity	Arabidopsis	Zhai *et al.* (2010)
GCC like DNA Motif in *OsRMC promoter*	OsEREBP1 and OsEREBP2	Salt sensitivity	Rice	Serra *et al.* 2013)

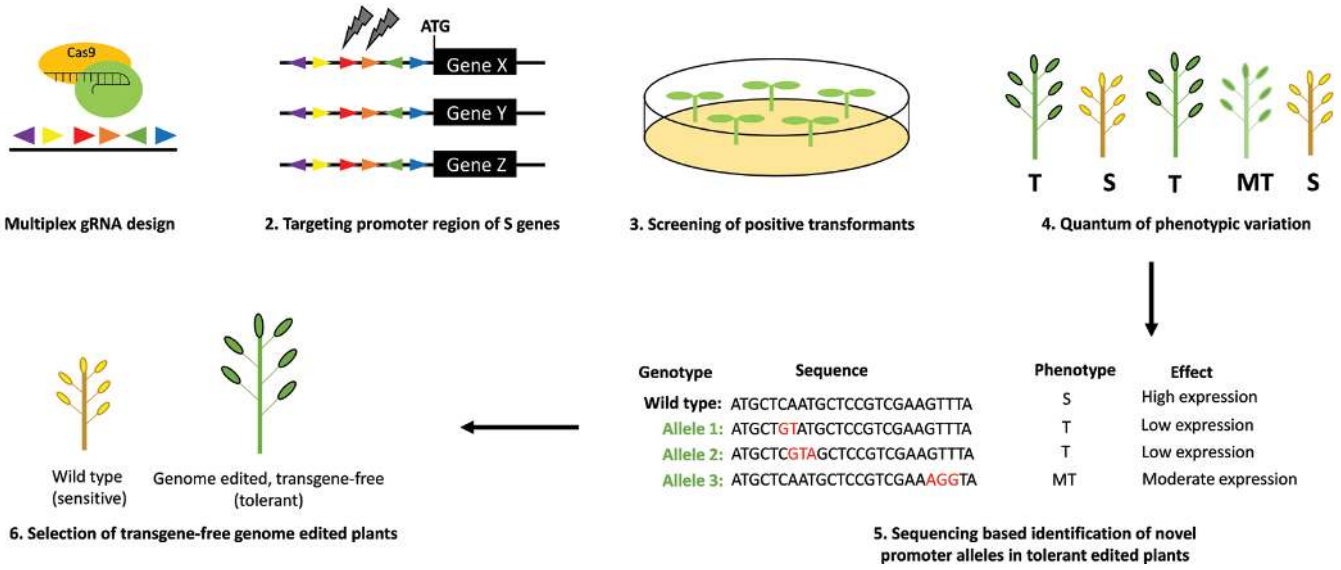

Fig. 1. CRISPR-mediated genome-wide targeting of promoters recreated novel alleles. A pool of short guide RNAs (sgRNAs) is designed to target promoter region of multiple *S* genes. This pool of sgRNAs, along with the CRISPR/Cas9 machinery, is delivered into the target cells. The edited cells are then screened on antibiotic selection medium, and seeds are harvested at maturity. Transgenic plants are evaluated for the target phenotype in the field or the greenhouse, and tolerant plants are sequenced to identify novel allelic variations. Novel alleles or quantitative trait loci causing stress tolerance are identified, and transgene-free genome-edited stress-tolerant plants are selected in segregating generations. T, tolerant; S, sensitive; MT, moderately tolerant.

(Bortesi and Fischer, 2015). Therefore, it has the potential to replace standard GM-based overexpression and gene silencing methods. Because of the precise modification or regulation of a gene of interest, genome editing tools have added benefit over conventional genetic engineering methods where integration of the transgene is random (in the case of overexpression) which can repress or activate other genes, and unwanted silencing of other genes can occur by siRNA-mediated RNAi. In CRISPR, appropriate designing of gRNA can overcome this limitation of off-targeting. Though there are successful examples of CRISPRa/i applications in animal cells (Gilbert *et al.*, 2013; Zheng *et al.*, 2018; La Russa and Qi, 2015), examples from plant systems are are relatively less (Piatek et al., 2015). In plant species, CRISPR/Cas9-mediated genome editing has been successfully applied in diverse crops, including wheat, rice, maize, and cotton. However, reports regarding targeting of abiotic stress tolerance genes are scanty. This is primarily because of the fact that such studies so far have focused on biotic stresses such as diseases and insect pests. Recently, CRISPR/Cas9-mediated genome editing for heat tolerance has been achieved by targeting an *S* gene, *SlAGAMOUS-LIKE 6* (*SlAGL6*) in tomato, which improved fruit setting under heat stress (Klap *et al.*, 2017). In addition, targeting multiple genes in a single organism using CRISPR/Cas9 has also been introduced and successfully optimized for different crops such as rice (Miao *et al.*, 2013), wheat (Wang *et al.*, 2018), maize (Char *et al.*, 2017), and cotton (Gao *et al.*, 2017). Thus, CRISPR/Cas9 holds great potential to develop crops tolerant to multiple stresses by targeting several *S* genes simultaneously in an elite high-yielding but sensitive cultivar (Fig. 2). Also, *T* genes, as previously discussed, can also be overexpressed using CRISPR-mediated gene activation (CRISPRa). Recently, the introduction of a CRISPR/Cas base-editing system further extended the applications of CRISPR/Cas9 to genome-wide screening for targeted trait improvement (Rodríguez-Leal *et al.*, 2017; Mahas and Mahfouz, 2018). Precise base changes at some of important sites in a gene can result in 'loss' or 'gain of function' mutants. It is believed that this technique may substitute traditional plant breeding approaches, which mostly rely on finding plant populations with sufficient genetic variation to introduce desirable traits into elite crop cultivars. CRISPR/Cas9 base editing could generate novel allelic variants in a population, and thus the novel alleles corresponding to a particular desirable phenotype can then be identified by sequencing of the gRNA (Eid *et al.*, 2018). Thus, CRISPR/Cas9 base editing has remarkable potential for the programs targeting the creation of novel traits for speedy crop improvement.

Transgene-free genome-edited tolerant cultivars

Despite the technological advancements being reported in CRISPR/Cas9 system for crop improvement, not much progress has been made towards the application of these tools for improvement of abiotic stress tolerance in crops. This is primarily because the response of plants to these abiotic stresses is highly complex, with hundreds or thousands of genes being up- or down-regulated under stress conditions (Kumari et al., 2009; Blum, 2011; Joshi et al., 2016). Nevertheless, drought tolerance in maize has been recently achieved and tested at field level by precise gene editing of AGROS8 using CRISPR/Cas9 (Shi *et al.*, 2017). It was highly encouraging for the molecular breeders working towards abiotic stress tolerance; however, being transgenic in nature, it still faced several concerns related to its societal and public acceptance. Thus, avoidance of transgene integration and off-target mutations are the most important challenges for the utilization of this robust tool. In the last quarter of 2015, DNA-free genome editing

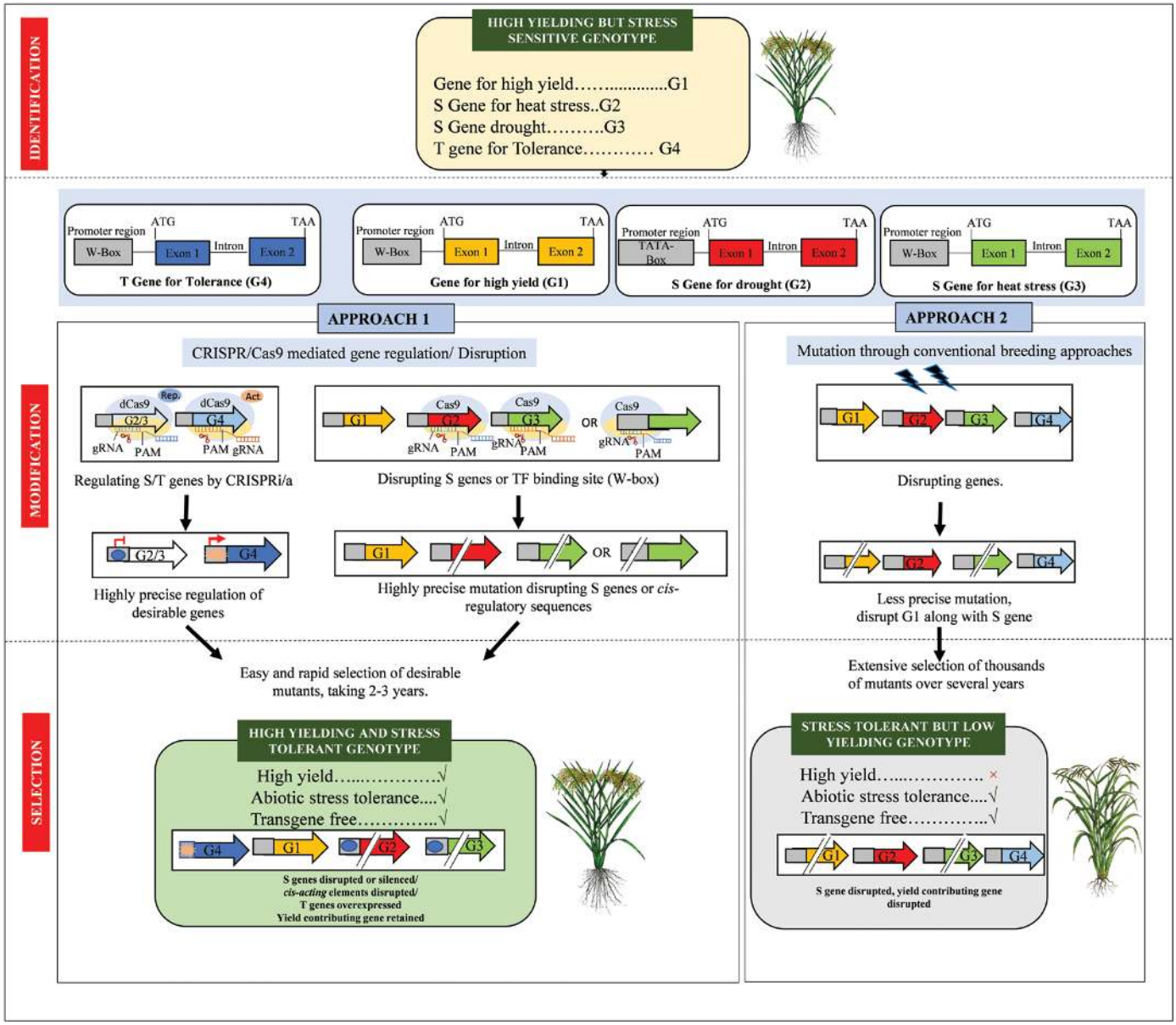

Fig. 2. Rapid and transgene-free development of abiotic stress-tolerant crops using the multiplex CRISPR/Cas9 technique in comparison with conventional mutation breeding techniques. Conventional mutation breeding techniques (e.g. ethyl methanesulfonate, X-rays, and γ-rays) could be used to disrupt the *S* genes with a lower efficacy. Moreover, there are chances of other essential genes (yield-contributing gene here) becoming disrupted. In contrast, CRISPR/Cas9 could be used to precisely disrupt several sensitivity genes (*S* genes) and *cis*-elements (W-box here) using highly specific guide RNAs (gRNAs). The gRNA binds just before the protospacer adjacent motif (PAM) sequence on these genes and introduces double-stranded breaks which are later repaired by a non-homologous end joining (NHEJ) mechanism. This NHEJ mechanism imparts mutations (insertion or deletion) while repairing, thus causing a shift in the ORF, leading to the disruption of *S* genes. Alternatively, these genes can preferably be silenced by the CRISPR interference (CRISPRi) technique where catalytically inactive Cas9 (dCas9) can be fused with a repressor to bind to the target gene. Similarly, genes conferring tolerance (the *T* genes) can be overexpressed using the CRISPR activation (CRISPRa) technique. Because of the precise targeting by CRISPR techniques, desirable combinations (gene disruption or regulation) of genes can be obtained in a much shorter time as compared with conventional breeding techniques. In addition, by using these methods, crop varieties can be developed which are transgene free and may be exempt from GMO regulations.

with pre-assembled CRISPR/Cas9 ribonucleoproteins (RNPs) was introduced in plants, including Arabidopsis, rice, tobacco, and lettuce (Woo *et al.*, 2015). Although it was a breakthrough study, the plants produced were regenerated from protoplasts, which is still a daunting task for many crop species. Soon after, CRISPR/Cas9 RNP-based transgene-free genome editing was demonstrated in maize (Svitashev *et al.*, 2016) and wheat (Liang *et al.*, 2017) by bombarding immature embryos. Later on, a more efficient, callus-based and transgene-free genome editing protocol with transient expression of CRISPR/Cas9 DNA or RNA (called TECCDNA or TECCRNA, respectively) was successfully introduced in wheat (Zhang *et al.*, 2016), and this may be applied to other crops for which the regeneration from callus has been optimized. The frequency of genome-edited mutant plants was found to be higher in the TECCDNA-based method than in TECCRNA. However, due to possible integration of

transgenes in theTECCDNA method, it was recommended to use TECCDNA for functional characterization and optimization of the genome editing protocol for callus as regeneration material and the TECCRNA method for development of commercial varieties (Zhang *et al.*, 2016). Since, in CRISPR/Cas9 RNP-mediated as well as TECCRNA-based genome editing, the crops developed are completely transgene free, they could probably be exempted from GMO regulations. Undoubtedly these technological advancements have opened up new avenues for the speedy improvement of important and complex traits such as drought, salinity, and heat tolerance in crop plants which is the need of the hour, particularly in the era of changing climate and declining arable lands and resources.

Limitations and proposed solutions

The *S* and *T* genes could be beneficially exploited to achieve abiotic stress tolerance; however, disrupting the *S* genes may sometimes have some fitness cost that may be due to disturbed biological processes and metabolic pathways. It is possible that the effects may not be drastic for the plants, but could affect the desirable phenotype. A possible solution to overcome this challenge could be the intermediate expression of such *S* genes rather than their complete knockout. This can be achieved by targeting their promoter region or potential *cis*-regulatory sequence, as shown recently for the *SWEET14* gene to induce rice resistance to bacterial leaf blight (Blanvillain-Baufumé *et al.*, 2017). Recently, a CRISPR/Cas9-based targeting of *cis*-regulatory sequences in the promoter of several genes was shown to create a novel phenotypic variation for some quantitative traits in tomato (Rodríguez-Leal *et al.*, 2017). This approach could also be applied in the context of targeting promoters of *S* genes to engineer abiotic stress-tolerant crops with a minimum compromise on their fitness levels.

Although several *cis*-regulatory sequences could be targeted to achieve abiotic stress tolerance, some of these sequences also serve as a potential binding site for TFs which positively regulate abiotic stress tolerance and other metabolic pathways. For example, GmWRKY13 causes sensitivity to salt stress by binding to the W-box (TTGAC), and targeting this *cis*-element enhances salt tolerance in soybean (Zhou *et al.*, 2008). However, some other TFs such as GmWRKY21 and GmWRKY54 also bind to this sequence and positively regulate stress tolerance. Thus, editing such *cis*-regulatory sequences would undoubtedly lead to salt tolerance in plants, but it may also make them sensitive to other stresses. Therefore, the utilization of such *cis*-regulatory sequences would be tricky and may need some additional genetic modifications in the organism. One possible solution to this issue could be the overexpression of some alternative/additional genes to compensate for the effect of such mutated *cis*-regulatory sequences.

Despite numerous encouraging reports about the successful application of CRISPR/Cas9 for crop improvement, a major bottleneck with its full application is the inefficient regeneration ability, via tissue culture, of some crop species. Another limitation in this context is the use of traditional tissue culture protocols, which are not only labor intensive but also cause random somatic mutations that reduce the gain efficiency of CRISPR. Possible solutions may include the use of regeneration boosters such as exploiting the gene *ARR10*, which enhances the regeneration ability in tissue culture by optimizing the cytokinin concentration (Hill and Schaller, 2013; Hill *et al.*, 2013). For this purpose, novel methods for gene delivery need to be developed for recalcitrant species such as the direct delivery of edited pollen, which can bypass the route of tissue culture (Gao, 2018).

Conclusion and prospects

CRISPR/Cas has been substantially adopted in plant developmental biology to characterize genes and to underpin the molecular mechanisms of various traits. However, attention is now shifting towards its application in agriculture. In this regard, we have discussed the potential use of the CRISPR/Cas9 technique for the development of abiotic stress-tolerant crops via targeting the key sensitivity (*S* genes and *cis*-regulatory sequences) and tolerance (*T* genes) players. As a general approach, *T* genes are deployed to achieve abiotic stress tolerance in plants; however, the expression of *S* genes sometimes interferes with the biological function of these *T* genes. Therefore, silencing *S* genes to disturb their function may help plants to adjust their physiological and biochemical pathways for abiotic stress tolerance. Although there are numerous reports of success in achieving biotic stress resistance in plants, the same is not true for abiotic stresses. A representative list of *S* genes is presented in Table 1. However, for a comprehensive account of these genes, a review by Nguyen *et al.* (2018) is recommended, which shows those genes that could potentially be targeted via the transgene-free CRISPR/Cas9 approach for the development of non-transgenic cultivars tolerant to abiotic stresses such as drought, salinity, cold, and heavy metals. Using multiplex genome editing, crops could be developed with improved tolerance to multiple abiotic stresses with a single transformation event. In addition to the *S* genes, several *cis*-regulatory sequences have also been identified, which negatively regulate abiotic stress tolerance (Table 2). These *cis*-regulatory sequences are highly conserved in their nature and function in the regulation of gene expression by interaction with specific TFs. Thus, editing these *cis*-regulatory sequences may also serve as a potential approach for improving tolerance towards abiotic stress. At the time of its discovery, base editing via CRISPR/Cas9 was limited to the conversion of cytosine to thymine, which has been a major bottleneck for the extensive application of this technology in crop improvement. Nevertheless, recent advancements in nucleotide base editing has overcome this barrier by using an adenine base editor which is based on a tRNA adenosine deaminase fused to the nickase CRISPR/Cas9, enabling A–T to G–C conversion at higher frequencies (Li *et al.*, 2018). This could further expand the applications of CRISPR/Cas9 for the development of novel quantitative traits with a gain-of-function mutation via single nucleotide replacements. In addition to its numerous advantages over other techniques, certain limitations restrict its adaptability in genome editing. One of the major limitations is the off-target mutations caused by Cas9 in transgenic plants. Nevertheless, this could now be overcome to a certain extent via a stress-inducible CRISPR/Cas9 technique which can significantly reduce the rate of off-target mutations even down to the negligible level, as has been shown successfully in rice (Nandy *et al.*, 2019). Thus,

stress-inducible CRISPR/Cas9 will be a promising tool for efficient and precise genome editing in plants for numerous traits, including abiotic stresses.

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