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ROLE OF CRISPR-CAS9 AND AMIRNA IN CONFERRING ABIOTIC AND BIOTIC STRESS TOLERANCE TO PLANTS

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Keywords: CRISPR-Cas9, Abiotic and Biotic Stress, amiRNA, Zinc Finger, Transcription activator-like effector nucleases (TALEN), cis-sequences, NHEJ

Introduction. Global warming leads to the occurrence of a number of abiotic and biotic stresses which affect the agricultural productivity. Presence of abiotic stresses lead to changes in plant pest interactions by strengthening the host plant sensitivity to pathogenic organisms and insects that lead to a decline in the competitive ability with weeds. On the contrary, some pests can change the plant response to abiotic stress factors. Hence, various systematic studies are essential to understand the effect of concurrent abiotic and biotic stress conditions on crop productivity.

Abiotic stresses such as drought, salinity, temperature, and heavy metals, imperil a major challenge for crop production leading to substantial yield reduction worldwide. Thus, breeding tolerant cultivars against these abiotic and biotic stresses is the most credible and eco-friendly approach to deal with this challenge. Improvement in genome editing techniques, gives new prospects in crop improvement by using precision genome engineering for targeted crop traits.

CRISPR-Cas9 a new era in the genome engineering. CRISPR (clustered regularly-interspaced short palindromic repeats) technologies provide an effective and straight forward means to perform targeted genetic manipulations in plant and animal cells. At present, the ever expanding CRISPR toolbox allows genomic knock-ins/-outs , gene silencing and activation epigenetic reprogramming , as well as single-base editing .

The CRISPR-Cas9 technology originates from type II CRISPR-Cas systems, which provide bacteria with adaptive immunity to viruses and plasmids. The CRISPR-associated protein Cas9 is an endonuclease that uses a guide sequence within an RNA duplex, tracrRNA:crRNA, to form base pairs with DNA target sequences, enabling Cas9 that introduces a site-specific double-strand break in the DNA. The dual tracrRNA: crRNA was engineered as a single guide RNA (sgRNA) that contains two important features:

- A sequence at the 5′ side that determines the DNA target site by Watson-Crick base-pairing.
- A duplex RNA structure at the 3′ side that binds to Cas9.

This lead to creation of a simple two-component system in which changes in the guide sequence of the sgRNA program Cas9 to target any DNA sequence of interest.

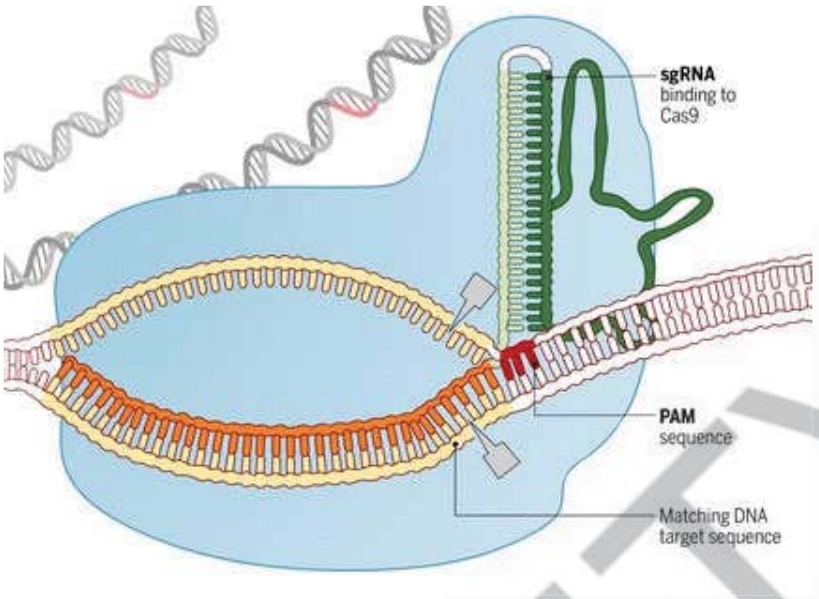

Fig. 1: The Cas9 enzyme (blue) causes breaks in double-stranded DNA by utilising its two catalytic centers (blades) to cleave each strand of a DNA target site (gold) next to a PAM sequence (red) and matching the 20-nucleotide sequence (orange) of the single guide RNA (sgRNA). The sgRNA includes a dual-RNA sequence derived from CRISPR RNA (light green) and a separate transcript (tracrRNA, dark green) that will bind and stabilize the Cas9 protein. Cas9-sgRNA–mediated DNA cleavage produces a blunt double-stranded break that triggers repair enzymes to disrupt or replace DNA sequences at or near the cleavage site. Catalytically inactive forms of Cas9 can also be used as well programmable regulation of transcription and visualization of genomic loci. DOI: 10.1126/science.1258096

CRISPR/Cas9 genome editing involves easy and straightforward designing and cloning methods, with the same Cas9 being potentially available for use with different guide RNAs targeting multiple sites in the genome. Various modified Cas9 cassettes have been used in crop plants for enhancing target specificity and reducing off-target cleavage (e.g., Nmcas9, Sacas9, and Stcas9).

CRISPR/Cas 9 can be used to induce either (CRISPR activation or CRISPRa) or repress (CRISPR interference or CRISPRi) gene expression by merging the catalytically inactive Cas9 (dCas9) with a transcriptional activator or repressor ***(Bortesi and Fischer, 2015).*** interference. In CRISPR,correct designing of gRNA can help in overcoming the limitation of off-targeting, hence is advantageous over the conventional gene editing methods. As opposed to ZFNs and TALENs, which use protein motifs for target identification, CRISPR-Cas9 depends on RNA-DNA recognition to create the double-strand break.

Advantages of CRISPR-Cas9 over ZFNs and TALENs are

(i) Ease in target design,

(ii) Efficacy of introduction of mutations by directly injecting the RNAs encoding Cas9 protein and guide RNA,

(iii) The ease of multiplexing, causing targeted mutations in multiple genes in a single event ***(Ma et al., 2015; Malzahn et al., 2017)*** ,

(iv) Designing of CRISPR-Cas9 vector is relatively less tricky compared to

designing ZFNs or TALENS due to the availability and easy access to the enhanced bioinformatics tools which are used to distinguish the most appropriate sequences to design the guide RNAs with no further need of screening libraries to find out the most efficient target.

Methods of Action

Selection of the Structural Genes to achieve Abiotic Stress Tolerance. The target genes help in altering plant immune response, oxidative stress response, hormone signaling pathway, plant development, and expression of transport proteins.

Reactive oxygen species (ROS) play a pivotal role 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 plant and soil rhizobia ***(Sinharoy et al., 2016)***.

Although over-production of ROS which is a usual response of plants towards abiotic and oxidative stresses can impart different types of growth abnormalities like the reduction in photosynthesis rate, enhanced cell death, and even male sterility, leading to a reduction in crop yield ***(Hu et al., 2011; Zafar et al., 2019).*** Therefore, keeping a check on ROS production and its scavenging is vital to maintain redox balance in cells ***(Mittler, 2017).***

Various genes encoding antioxidant enzymes such as catalases (CAT), superoxide dismutase (SOD), glutathione reductases (GR), glutathione-S-transferases (GST), and many peroxidases (POD) participate in the scavenging of ROS molecules. These genes can be called as the 'Tolerance genes' (T genes) which contribute to abiotic stress tolerance ***(Hu et al., 2011; Mittler, 2017)***.

For instance, Papain like Cysteine proteases (PLCPs) are found to be improved under abiotic stress conditions in various plant species like Sweet potato (*SPCP2*), 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).***

Melatonin biosynthetic genes: Melatonin is an antioxidative molecule which helps plants in scavenging ROS and reactive nitrogen species (RNS) .Plants which overexpress melatonin biosynthesis genes were found to be tolerant to different abiotic stresses ***(Zou et al., 2014; Byeon and Back, 2016; Antoniou et al., 2017).***

Nonetheless, various Sensitivity genes (*S* genes) genes cause increased production of ROS, (which is called as oxidative stress), reduced antioxidant activity, enhanced programmed cell death (PCD) and the genes that cause derangement in the hormonal homeostasis makes plants sensitive to abiotic stresses have been reported ***(Fang et al., 2015; Liu et al., 2016; Zhao et al., 2017).***

Knocking out the *S* genes contributes towards stress tolerance by disrupting the involved pathways. For instance, *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 H2O2 (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 the H2O2 biosynthesis and positively regulating the antioxidant activities ***(Fang et al., 2015).***

Various sensitivity genes such as *OsDIS1* (*O. sativa* drought-induced SINA protein 1) and *DST* (drought and salt tolerance) have been tried for such functional transformation via RNAi-mediated gene silencing ***(Huang et al., 2009; Ning et al., 2011).***

Selection of Regulatory genes to enhance Abiotic Stress tolerance. Regulatory genes like transcription factors, phosphatases, kinases are yet another important class of targets for modulating the level of expression of several downstream genes and activating many stress signals.

For instance, In *Arabidopsis*, a NAM-ATAF1/2 and CUC2 (NAC) transcription factor, *ANAC069*, functions as negative regulator of abiotic stresses (S gene). *ANAC069* regulate the expression of multiple stress responsive genes by binding specifically to the core motif sequence of C[A/G]CG[T/G] in their promoter region which leads to a decline in ROS scavenging capability and high 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).***

Likewise, overexpression of T genes like *AtMYB44* confers drought and salt tolerance via enhancing ABA-induced stomatal closure ***(Jung et al., 2008).***

Similarly, overexpression of ZmWRKY106 enhances drought and heat tolerance in transgenic plants by monitoring the expression of stress-related genes, reducing the ROS content and increased activities of antioxidant enzymes ***(Wang et al., 2018).***

Importance of *cis*-regulatory sequences in addressing Abiotic stress tolerance in plants. *Cis*-regulatory sequences are of immense importance in regulation of the expression of genes as these sequences assist in the recruitment of the specific transcription factors (TFs). These sequences are usually 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 where it can lead to the induction or reduction or even no expression of the gene. Several *cis*-sequences such as W-box (TTGACC) and GCC box (AGCCGCC) function as a negative regulator of abiotic stress response/tolerance by providing binding sites for particular transcription factors such as GhWRKY17 and OsERF922, respectively. Thus, these *cis*-sequences could serve as a proper target for creating nucleotide level mutations using recent genome editing tools that may improve tolerance to abiotic stress tolerance in crops.

For instance, *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*-element and binds specifically to DNA sequence C[A/G]CG[T/G] ***(He et al., 2017).***

The mutation in this main sequence causes the failure of gene regulation by ANAC069 leading to stress tolerance. CRISPR-Cas9 drove 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 QTL and improved tomato size and yield ***(Rodríguez-Leal et al., 2017).***

Role of miRNA and amiRNA. MicroRNAs (miRNAs), a class of endogenous small noncoding RNAs with the size of 21–24 nucleotides that can mediate post-transcriptional and translational gene regulation. miRNAs play an important role in diverse aspects of plant development and plant responses to biotic and abiotic stresses.

The biogenesis of miRNA is a multi-tier process that begins with the transcription of a miRNA gene into a primary transcript (pri-miRNA) .

Pri-miRNA is sequentially processed into a stem-loop structured precursor (pre-miRNA) by DICER-LIKE1 (DCL1), and pre-miRNA is then processed into miRNA/miRNA* duplex and stabilized by methyltransferase HUA ENHANCER1 (HEN1).

The methylated miRNA duplex is eventually loaded into the ARGONAUTE (AGO) protein to form the so-called RNA-induced silencing complexes (RISCs), followed by the release and degradation of miRNA.

By targeting complementary sequences, RISCs negatively regulate gene expression through mRNA degradation and/or translation inhibition.

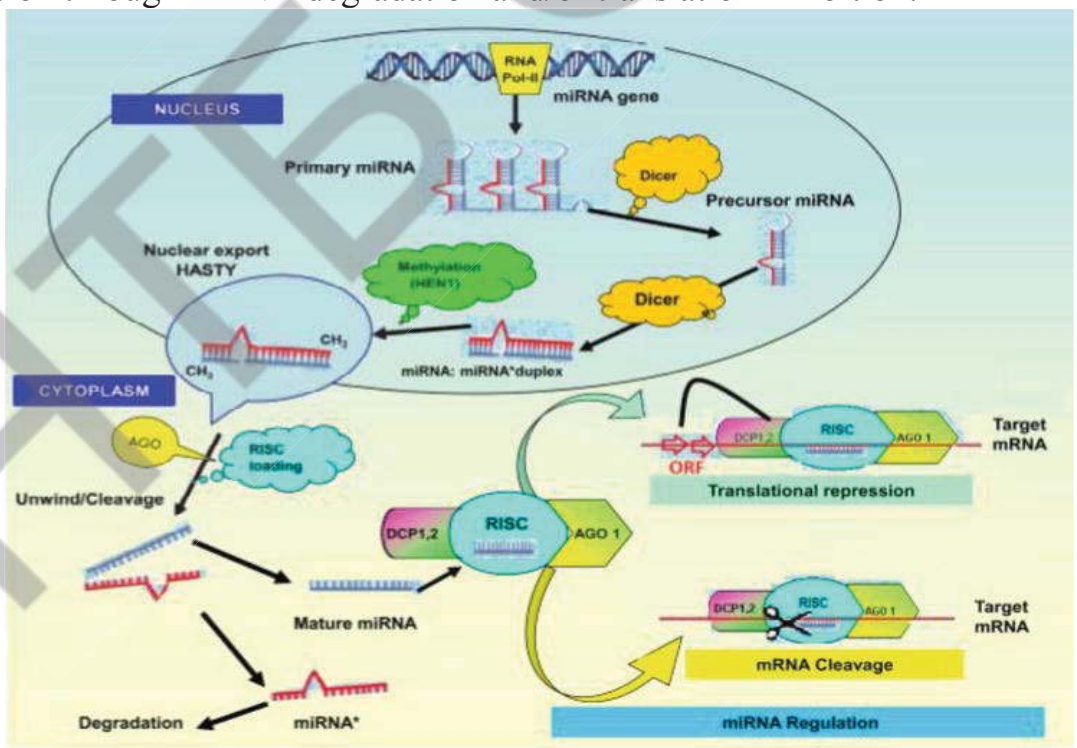

Fig. 2: Biosynthesis and mode of action of miRNA. Source: Djami-Tchatchou et al.

Artificial microRNA (amiRNA) technology has already been triumphantly made to silence target gene expression by producing artificially designed miRNAs using the naturally existing miRNA precursor as a backbone . When comapared to various genome editing tools and techniques , the amiRNA technology is more flexibile and reversible in generating loss-of-function mutants without altering DNA sequences. As the expression of amiRNAs can be regulated by chemical-inducible or cell/tissue-specific promoters , amiRNAs are extensively utilized for examining the gene functions associated with mutant lethality. amiRNA has a high silencing specificity and only recognizes target sequences with less than 5 mismatches , making it an ideal tool to silence individual AS isoforms or multiple genes sharing short conserved sequences.

CRISPR/Cas9 nonhomologous end joining (NHEJ; Figure 3a) can be accomplished by the presentation of indels at pre-miRNA arrangements or the miRNA handling locales of MIR qualities, which hinders or retards miRNA biogenesis ***(Chang et al., 2016; Zhou et al., 2017).***

Similarly, indel addition in target qualities can meddle with miRNA-target mRNA blending and lead to the resulting disappointment of mRNA cleavage into RISC.

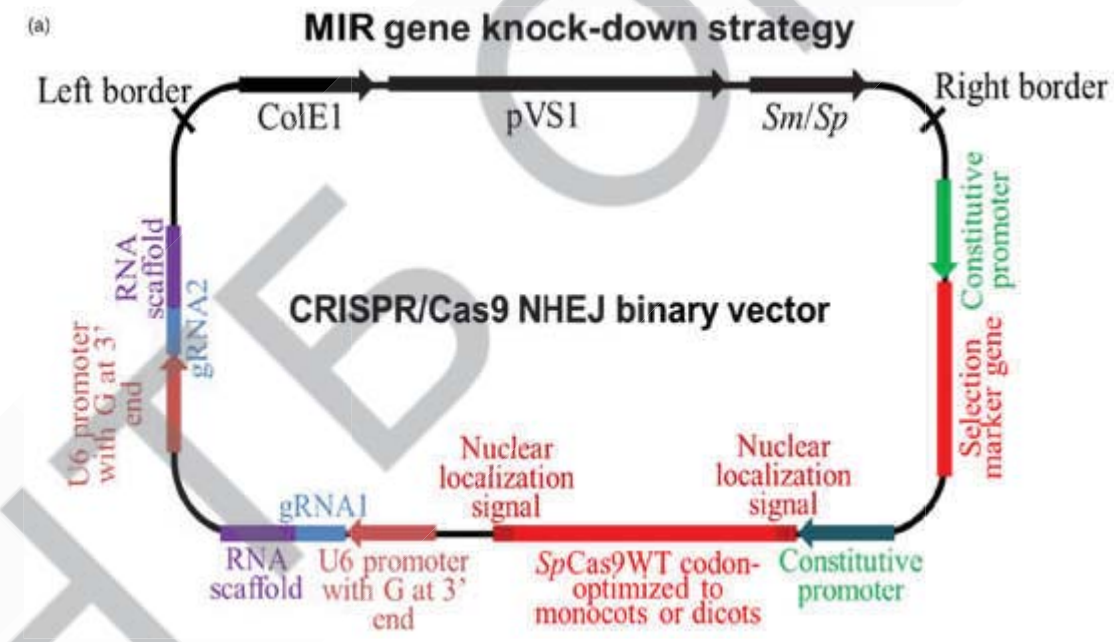

Fig. 3a

The CRISPR/Cas9 system to target promoter sequences or generate MIR gene knock-down using nonhomologous end joining (NHEJ) strategy. Above, a typical CRISPR/Cas9 NHEJ binary vector carrying a T-DNA that contains a selection marker gene under control of a constitutive promoter, *Streptococcus pyogenes* Cas9 wild-type gene (*Sp*Cas9WT) codon-optimized to monocots or dicots and containing one or two nuclear localization signals under control of a constitutive promoter, one

or two RNA guide RNAs (gRNA1 or gRNA2) and an RNA scaffold *in tandem* under control of the U6 RNA polymerase III promoter containing a guanine (G) extra at the 3′ end.

Below, an overview of the NHEJ strategy in plants containing T-DNA from the CRISPR/Cas9 binary vector integrated into the genome (constitutive expression) or by transient expression (e.g. in protoplasts) via biolistic approach or the type III secretion system of *Agrobacterium tumefaciens* ***(Chang et al., 2016; Zhou et al., 2017)***.

Below, the complex gRNA:RNA scaffold is transcribed, associated with Cas9 nuclease in the nucleus and directed to the target sequence in genomic DNA. The gRNAs match the target sequence (promoter sequence or MIR gene) and mediate its cleavage by Cas9 nuclease next to the protospacer adjacent motif (PAM), generating a double-strand DNA break (DSB). Post DNA cleavage, the damage is rectified by the DNA repair mechanism of the plant cell, but errors (insertion or deletions of any nucleotides, named *indels*) can be inserted in the repaired DSB sequence, resulting in *indels* within the transcription start site, cis-regulatory elements or other binding sites of trans-acting factors, leading to the up- or down-regulation of MIR gene expression. In addition, *indels* in miRNA processing sites prevent the biogenesis of these molecules.

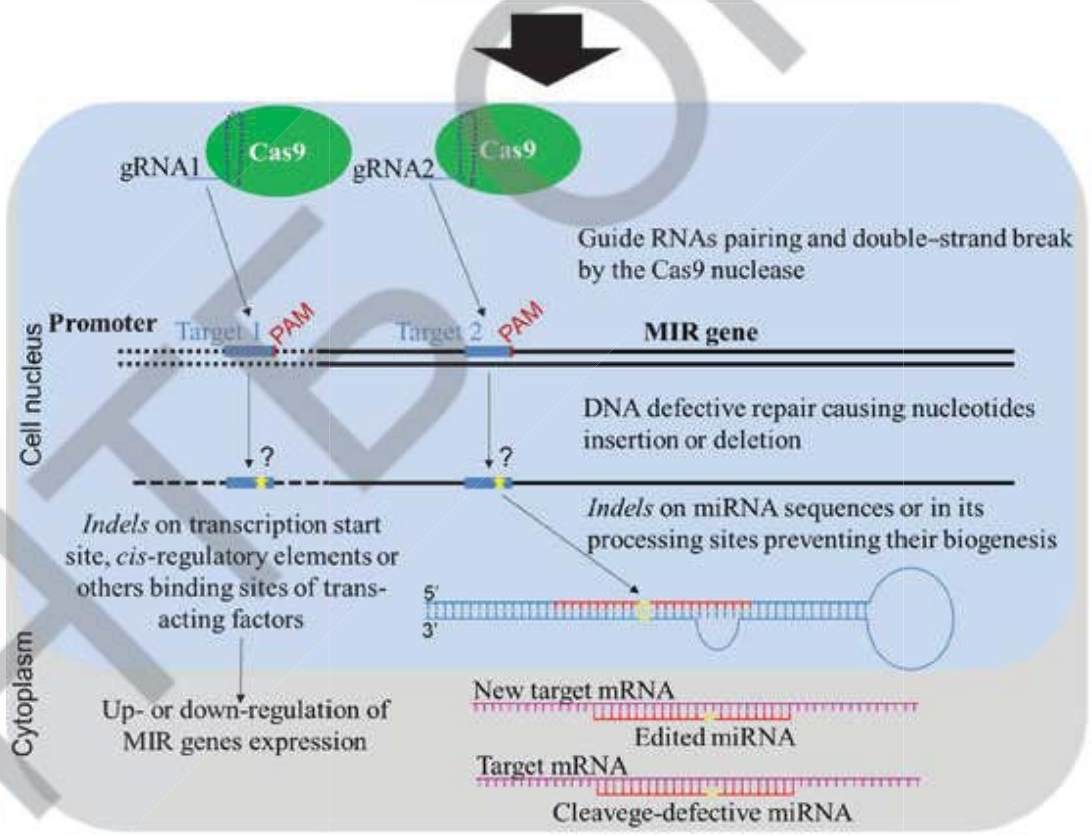

Fig. 3b

Biotic Stress Tolerance. The recognition of biotic stress-associated miRNA loci involves

(1) The selection of putative biotic stress-associated miRNA target transcripts, (2) The mapping of miRNA-directed cleavage sites

(3) A bioinformatic analysis to retrieve potential stress-associated miRNA-precursors and miRNA mature sequences

For instance, Arabidopsis miR393 is a stress-responsive miRNA that contributes to resistance against virulent Pseudomonas syringae pv. tomato strain DC3000 (Pto DC3000), presumably by repressing auxin-signaling

In various plants it was studied that the overexpression of miR393 effectively blocks the microbial growth and provides a disease resistant tool.

Some studies have shown that the miR160a overexpression positively regulate callose deposition induced by MAMP, while miR773 and miR398b negatively control MAMP-induced callose deposition and provide specific protection to bacterial infection.

Conclusion and Future Prospects. In plants the conventional genetic tools such as T-DNA insertion and ion/chemical-induced mutagenesis, which usually cause random gene inactivation are now being replaced by the CRISPR-Cas technology that confers a sequence-specific gene knockout as proposed by ***Zhang et al., 2016a***. Here the potential use of CRISPR-Cas9 technique for the development of abiotic stress-tolerant crops *via* targeting the key sensitivity (*S* genes and *cis*-sequences) and tolerance players (T genes) has been discussed. From a general perspective, *T* genes are deployed to achieve abiotic stress tolerance in plants. Eventhough the expression of *S* genes sometimes hiders with the biological function of these *T* genes. Hence, silencing *S* genes to disrupt their function might help plants to adjust their physiological and biochemical pathways for abiotic stress tolerance. These *cis*-sequences are highly conserved in their nature and function for the regulation of gene expression by interaction with specific transcription factors. Hence, editing these *cis*-sequences serves as a potential approach for improving tolerance towards abiotic stress. Likewise the amiRNA technology is not only a strong genetic tool for production of loss-of-function mutants in plant research, and enhanced disease resistance against various abiotic and biotic stresses such as pathogens or pests.

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