A Seminar Paper
On

CRISPR/Cas9 GENOME EDITING for STRESS TOLERANCE in PLANTS

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CRISPR/Cas9 Genome Editing for Stress Tolerance in Plants [1]

By

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ABSTRACT

Progression in genome editing technology has changed the functional genomics and crop improvement fields dramatically. CRISPR/Cas9 (clustered regularly interspaced short palindromic repeat) is a genome editing technology depends on Cas9 endonuclease and complementarity of gRNA to a specific sequence. It has widened the research area in agriculture by improving crop plants with the deletion of harmful characters and addition of beneficial traits. This genome editing technology has become ground breaking innovation in various sectors of plant biology. CRISPR technology makes genetic manipulation by activation and repression of target genes, making precise modification, multiplex genome engineering and generating knockouts. CRISPR/Cas9 is new and constantly advancing technology. Apart from plant nutritional improvement, it has contribution in biotic and abiotic stress tolerance in plants. The review highlights the contribution of CRISPR/Cas9 technology in biotic and abiotic stress tolerance in plants.

Key Words: Genome Editing, CRISPR/Cas9, Cas9endonuclease

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Chapter 1

Introduction

1.1. Rationale and Background

Genome editing refers to one type of genetic engineering in which DNA is inserted, deleted, modified or replaced in the genome of a living organism. In agricultural crops, processes for precise genome editing are of great importance to functional characterization of plant genes and genetic improvement. Due to low frequency of homologous recombination, successful gene targeting in plants is very inefficient and difficult from microbial system (Hanin and Paszkowski, 2003).

To increase the efficiency of gene targeting in plants and animals, recently sequence specific nucleases have been developed. Among this most commonly used methods are ZFNs (zinc finger nucleases) and TALENs. These are sequence specific chimeric protein (Pabo et al., 2001; Wood et al., 2001).When ZFN and TALEN are introduced and expressed in cell, their DNA binding domain binds to corresponding sequence and guide chimeric nucleases (e.g. Fok I nucleases) to cleave DNA strand specifically. Normally, single zinc finger motif recognizes specifically 3 bp and engineered ZFN having tandem repeats can recognize 9-36bp. But it is time consuming and tedious to screen and identify a desirable ZFN (Pabo et al., 2001).

Plant pathogenic bacteria *Xanthomonas* from which TALENs are derived and contain 34 amino acid and tandem repeats in which at 12 and 13 positions, DNA binding Specificity are determined by RVD(repeat variable diresidues)(Boch et al., 2009).During double strand breaks TALEN activates error-prone-DNA repairing system(Gorbunova et al., 1999).

A new gene targeting tool, most recently has been developed in mammalian and systems using CRISPR associated nuclease system. It stands for Clustered Regularly Interspaced Short Palindromic Repeat. CRISPR-Cas9 is a popular technology that is used to edit parts of the genome by removing, adding or altering sections of the DNA sequence. It is an adaptive immunity in bacteria and archaea (Deveau et al., 2010). Cas9 endonuclease is a part of the

Streptococcus pyogenes CRISPR Cas type II system. Two short RNA molecule called CRISPR RNA (CrRNA) and trans-activating cr RNA, with whom it form complex which guide the nucleases to cut non self DNA at specific site on both strands (Gasiunas et al., 2012).The gRNA (guide RNA) can replace crRNA and transcrRNA heteroduplex and it can be programmed to target specific sites (Jinek et al., 2014).The genome editing using CRISPR/Cas9 is more precise, predictable, rapid and efficient.

The CRISPR/Cas system has been used for human (Cho et al., 2013; Cong et al., 2013; Mali et al., 2013), mice (Shen et al., 2013), zebra fish (Chang et al., 2013), yeast (Dicarlo et al., 2013) and bacteria (Jiang et al., 2013).

'Stress' in plants can be defined as any exterior factor that negatively affects plant growth, reproduction, productivity or survival. Plant stress can be divided into two primary categories. Abiotic stress is a physical (e.g., light, temperature, salinity, flooding, ion toxicity, drought) or chemical's harmful effect that the environment may impose on a plant. Biotic stress is a biological negative effect, (e.g., insects, pathogen) to which a plant may be exposed during its lifetime. Some plants may be injured by a stress, which means that they exhibit one or more metabolic dysfunctions. If the stress is medium and short term, the injury may be not lengthy and the plant may recover when the stress is removed. If the stress is severe enough, it may prevent flowering, seed formation, and induce senescence that leads to plant death. Such plants are considered to be susceptible. Success in generating stress tolerant plant has resulted in enhancement in crop yield (Mickelbart et al., 2015). To overcome the limitation of the classical method, such as time consuming and lack of precision, novel strategies are used to increase crop production in the dynamic climatic situation and increasing population scenario. This genome editing tools provide huge opportunity for modification in the genome effectively to produce stress tolerant crop plants.

1.2. Objectives of the study

- To review about the contribution of CRISPR/Cas9 genome editing for biotic stress tolerance in plants
- To review about the contribution of CRISPR/Cas9 technique for abiotic stress tolerance in plants.

Chapter 2

METHOD AND MATERIALS

This seminar paper is exclusively a review paper, so all the information has been collected from secondary sources. During the preparation of the review paper, I collected various information from various relevant books, journals, proceedings, reports and internet. Apart from those, my major professor as well as course instructors also provided much valuable suggestion for the preparation of this seminar paper. After collecting all the available information, I myself compiled the collected information and finally prepared this seminar paper.

CHAPTER 3

REVIEWS OF FINDINGS

The details on the assigned topic so far extracted and reviewed are discussed below under different sub headings.

3.1 CRISPR/Cas9 technology in case of biotic stress tolerance

3.1.1CRISPR/Cas9 in rice blast resistance

Rice (*Oryza sativa* L.) is one of the most important food crops in the world, feeding nearly 50% of the world's population. Rice blast, caused by the *Magnaporthe oryzae*, is one of the most destructive diseases in rice and often causes serious damage to global rice production. The most economical and effective approach for controlling rice blast is enhancing the resistance of rice to M. oryzae. Wang F. et al., (2016) set an experiment where knockdown of expression of the rice ERF (ethylene responsive factors) gene OsERF922 by RNA interference (RNAi) enhanced rice resistance to M. oryzae, where, for blast resistance in rice OsERF922 acts as a negative regulator. Using electroporation, the Cas9/sgRNA-expressing binary vectors (pC-ERF922S1S2, pC-ERF922 and pC-ERF922S1S2S3) were transformed into an *Agrobacterium tumefaciens* strain EHA105. Transformation of the embryogenic calli derived from the japonica rice variety Kuiku131 was performed through Agrobacterium-mediated Transformation. After 2–3 months of cultivation, transgenic seedlings were transferred to a field during the rice growing season. To characterize the blast resistance phenotype of the rice mutants, 6 homozygous mutant T2 lines (Fig.1) with different types of allelic mutations were inoculated with the fungal pathogen *M. oryzae* isolate 47-6 06-at the seedling stage. The leaves of wild-type plants nearly died due to pathogen infection, likely because the pathogenicity of isolate 06-47-6 was very strong, and the wild-type variety was highly susceptible (Fig.2).Also, the lesion areas were significantly reduced in all mutant rice lines compared with wild-type plants.

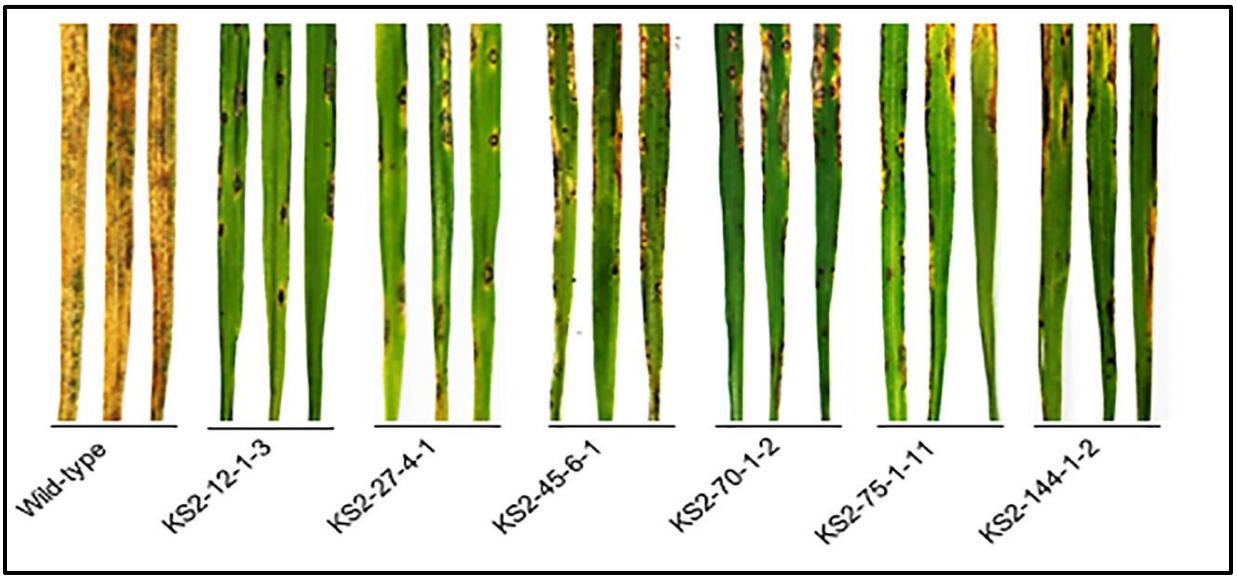

Figure1: The blast resistance phenotypes of the mutant rice lines and wild-type plants at the seedling stage. (Leaves were detached from the inoculated plants at 7 dpi for photography) (Source: Wang F. *et al.*, 2016)

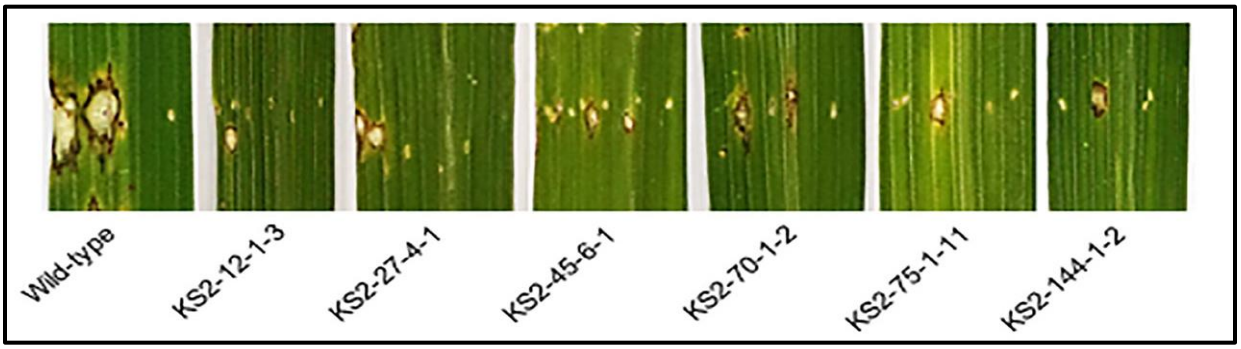

Figure2: Blast resistance phenotypes of the mutant rice lines and the wild-type plants at the tillering stage (Source: Wang F. *et al*., 2016)

In this experiment, it was found that, the lesion length of the mutant types was much lower than wild type (Fig.3) and the lesion area of the wild type also higher than the mutants (Tab.1).

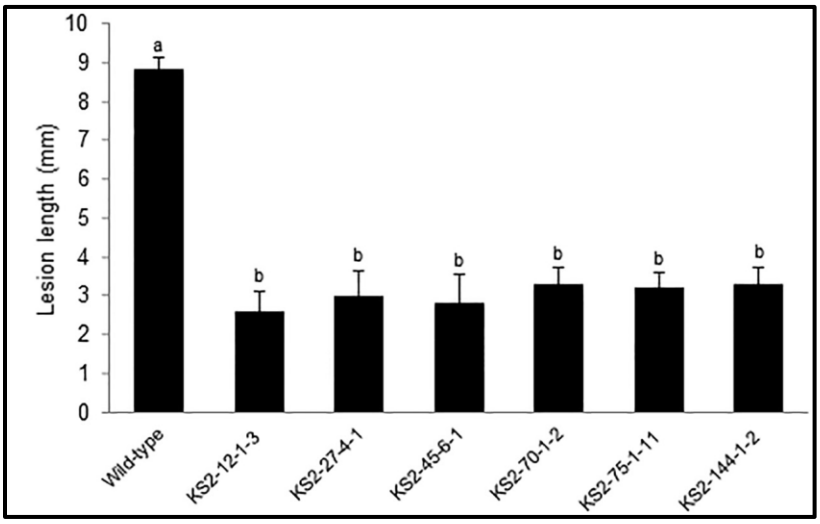

Figure3. Histograms showing the average length of lesions formed on the inoculated leaves tillering stage for each line. The values marked with different letters are significantly different ($P < 0.01$, Student's t-test) (Source: Wang F. *et al*., 2016)

Table1. Lesion area (%) on the third leaf of wild type and mutants

Variant	Lesion area (%)
Wild type	96
KS2-12-1-3	46
KS2-27-4-1	51
KS2-45-6-1	60
KS2-70-1-2	57
KS2-75-1-11	52
KS2-144-1-2	62

(Source: Wang F. *et al*., 2016)

3.1.2CRISPR/Cas9 mediated resistance to Citrus canker

Citrus canker, caused by *Xanthomonas citri subsp. citri (Xcc),* is severely damaging to the global citrus industry. Targeted editing of host disease-susceptibility genes represents an interesting and potentially durable alternative in plant breeding for resistance. Peng A. et al. (2017) used five sgRNA constructs were designed to modify the EBEPthA4 in Wanjincheng orange. Sixteen lines that harboured EBEPthA4 modifications were identified from 38 mutant plants. Four mutation lines (S2-5, S2-6, S2-12 and S5-13), showed enhanced resistance (Fig4) to citrus canker compared with the wild type. No canker symptoms were observed in the S2-6 and S5-13 lines. Promoter editing of CsLOB1G alone was sufficient to enhance citrus canker resistance in Wanjincheng orange and it contains the effector binding element (EBEPthA4).The transformation method was plasmid transmitted.

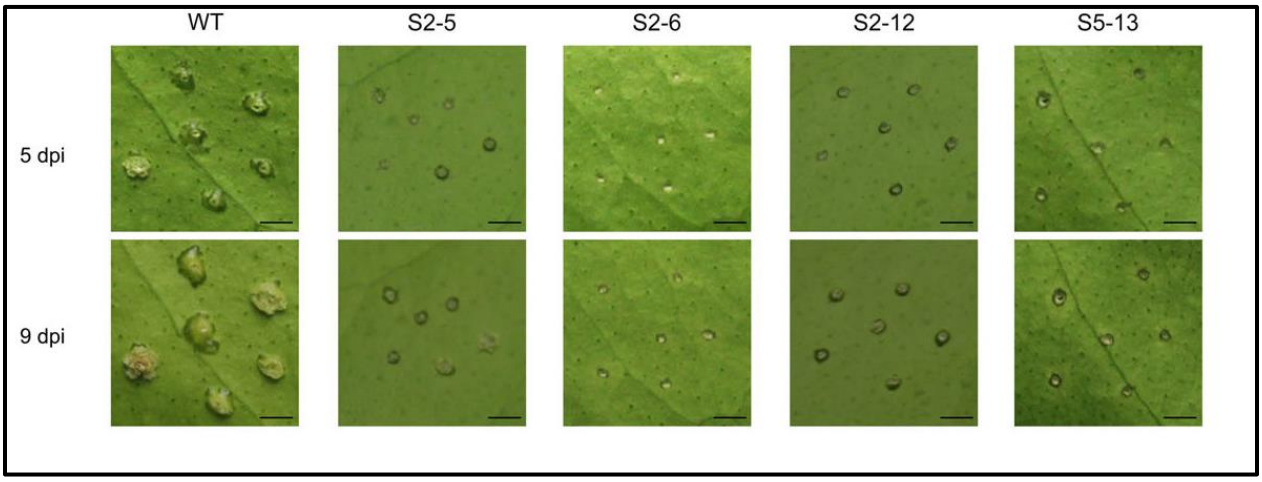

Figure4. Identification of citrus canker resistance in Wanjincheng orange (*Citrus sinensis*) mutants. Citrus canker symptoms were recorded by photographing 5 and 9 days post inoculation (dpi) (Source: Peng A. et al., 2017)

In this experiment disease area of leaf (Fig.5) and disease index (Tab.2), in both case mutant shows positive result.

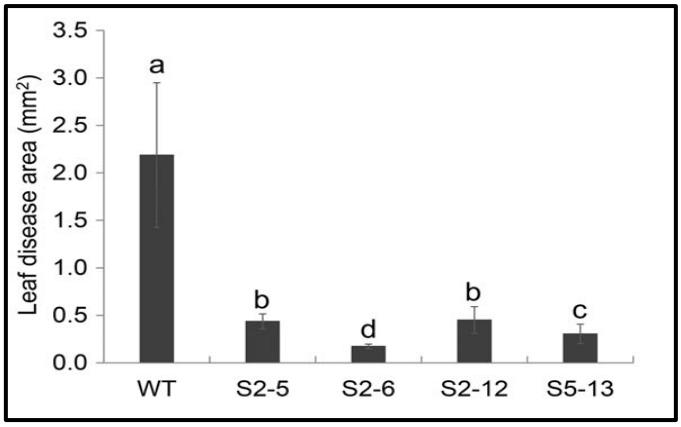

Figure5. Disease lesion area of leaves of each mutation line of citrus canker resistance in Wanjincheng orange (*Citrus sinensis*) mutants investigated at 9 dpi. Different letters above bars represent significant differences from the wild type based on Duncan's multiple range test ($P < 0.05$) WT, wild type. (Source: Peng A. *et al*., 2017)

Table2. Disease index of leaves of each mutation line of citrus canker resistance in Wanjincheng orange (*Citrus sinensis*) mutants investigated at 9 dpi

Variants	Disease index (%)
WT	84
S2-5	13
S2-6	2
S2-12	16
S5-13	8

(Source: Peng A. et al., 2017)

3.1.3CRISPR/cas9 for *potyvirus* resistance in Arabidopsis

Plant viruses are ubiquitous in natural environments and can severely limit plant growth and fertility. Globally, viruses are a significant economic burden to both well-developed and under-developed agriculture because of absolute yield losses in the field and decreased marketability of harvested crops. The Potyvirus genus contains a greater number of virus species than any other plant virus genus, and certain species within this genus [notably its type member, *Potato virus Y*

(PVY)] are particularly damaging to economically important crops. In this study, CRISPR/Cas9 technology was utilized to successfully engineer complete resistance to Turnip Mosaic Virus (TuMV) in arabidopsis thaliana by introducing sequence-specific deleterious point mutations at the eIF(iso)4E locus through agrobacterium mediated transformation, This Turnip Mosaic Virus (TuMV) is a major pathogen in field grown vegetable crops. Different genotypes (#44, #65, #68, #98, #105(WT) were rub inoculated with a green fluorescent protein (GFP)-expressing TuMV clone (hereafter TuMV-GFP). At 7(Fig.6) and 14(Tab.3) days post-inoculation (dpi), TuMV infection was assessed by monitoring the expression of GFP in inoculated and systemic leaves. GFP expression (indicative of TuMV-GFP infection) was clearly visible at 14 dpi and expression was higher in wild-type plants (#105) and in mutants no expression (Tab.3). TuMV-GFP titre (pg) in inoculated (left) and systemic (right) leaves was calculated at 7dpi (Fig.7), where viral titre was higher in wild type. When dry weight of this mutant and wild type plants was calculated, it was higher in mutant (Tab.4).

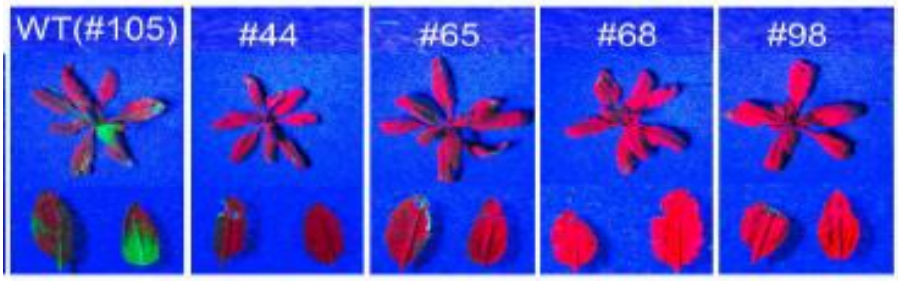

Figure6. A Representative photos of TuMV-GFP infected plants, imaged under UV light, 7 days post infection (An enlarged image of inoculated (left) and systemic (right) leaves are shown below each rosette) (Source: Pyott E. et al., 2016)

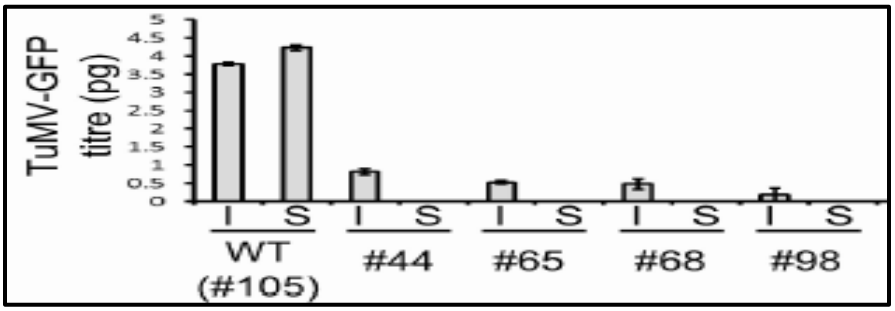

Figure7.TuMV-GFP titre (pg) in inoculated (left) and systemic (right) leaves at 7dpi (Source: Pyott E. et al., 2016)

Table3. GFP expression (%) of variants at 14 dpi

Variant	GFP-expression (%)
WT	92.5
#44	0
#65	0
#68	0
#98	0

(Source: Pyott E. et al., 2016)

Table4. Dry weight (mg) of mutants and wild type

Genotype	Dry weight(mg)
WT	450
#44	620
#65	580
#68	700
#98	600

(Source: Pyott E. et al., 2016)

3.1.4 CRISPR/Cas9 mediated resistance to powdery mildew in wheat

Bread wheat (*Triticum aestivum* L., 2n = 42, AABBDD) is a major staple crop worldwide. It provides approximately 20% of all calories consumed by humans. In wheat, powdery mildew is caused by *Blumeria graminis* f. sp. *tritici* (Bgt), which is one of the most harmful plant pathogens worldwide. Wang Y. *et al*., (2014) chose to target three MLO loci by CRISPR/Cas9 through plasmid transfer transformation method, which encode proteins that were shown to repress defenses against powdery mildew diseases in other plants. In this experiment the among mutants and wild type plant after powdery mildew inoculation, no microcolony forms in mlo-aabbdd mutant (Fig.8a &8b, Tab.5)

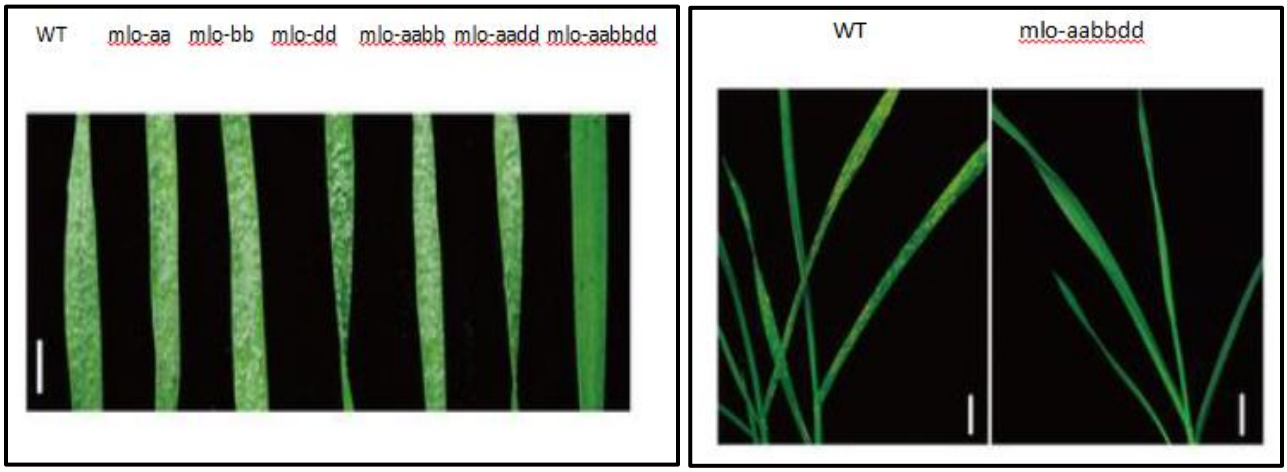

Figure (8a &8b):a. Disease symptoms of wild-type (WT) and mutant plants, b. Disease symptoms of wild-type (WT) and mlo-aabbdd mutant plants (The photograph was taken 7 d after inoculation in plant Scale bars, 2 cm) (Source: Wang Y. *et al*., 2014)

Table5. Microcolony formation of Bgt on the surfaces of leaves of the indicated genotypes 3 d post inoculation

Variants	Microcolonies /total no. germinated spore
WT	20
mlo-aa	18
mlo-bb	19
mlo-dd	16
mlo-aabb	15
mlo-aadd	17
mlo-aabbdd	0

(Source: Wang Y. *et al*., 2014)

3.2 CRISPR/Cas9 technology in case of abiotic stress tolerant

3.2.1 Increasing Maize Yield under Drought Stress

Developing more drought-tolerant crops in a sustainable manner is one means to meet the demand of an increasing human population that will require more food, feed and fuel. Improvement in drought tolerance of crops is ultimately measured by an increase in grain yield under water-limiting conditions. The physiological processes and metabolic networks underlying drought tolerance are complicated and often difficult to delineate. Nevertheless, the phyto-hormone ethylene is known to play an important role in regulating plant response to abiotic stress, including water deficits and high temperature (Hays et al., 2007). Field studies have shown that reducing ethylene biosynthesis by silencing 1-aminocyclopropane-1-carboxylic acid synthase6 in transgenic maize plants improves grain yield under drought stress conditions (Habben et al., 2014). A higher yield also can be achieved by decreasing the sensitivity of maize to ethylene (Shi et al., 2016). ARGOS genes are negative regulators of the ethylene response and modulate ethylene signal transduction, enhancing drought tolerance when overexpressed in transgenic maize plants (Guo et al., 2014). Constitutively overexpressed ARGOS likely counteracts the effect of water deficiency by promoting cell expansion and/or division, mitigating the yield loss by enhancing plant growth under drought stress.

ARGOS8 gene of maize is a negative regulator of ethylene responses. A previous study has shown that under drought stress conditions, transgenic plants which constitutively overexpressing ARGOS8 have reduced ethylene sensitivity and improved grain yield. Then a CRISPR-Cas-enabled advanced technology was employed to generate novel variants of ARGOS8. In the 5′-untranslated region of the native ARGOS8 gene, the native maize GOS2 promoter was inserted or was used to replace the native promoter of ARGOS8, from relatively low mRNA expression levels to significantly increased ARGOS8 expression levels. Precise genomic DNA modification at the ARGOS8 locus was verified by PCR and sequencing. A field study showed that compared to the WT, the ARGOS8 variants shows over expression (Fig.9). In this study two plants for WT controls and six individual plants were analyzed for the genome-edited variants. DAP, days after pollination (Shi et al., 2016).

Relative expression

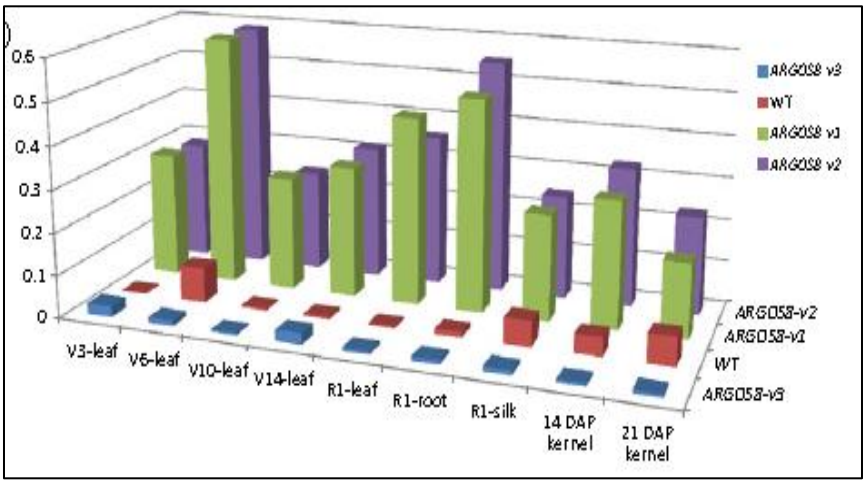

Figure9. Relative expression of ARGOS8 variants compared to WT (Wild type) (Source: Shi et al., 2016)

In another experiment, where two ARGOS8 variant were used against wild type in two condition, those are flowering stress, and optimal (well watered) conditions (Tab.6)

Table6. Grain yield of ARGOS8 genome-edited variants and wild type under different condition (source: Shi et al., 2016)

.

Variant	flowering stress (bushel/ acre)	optimal (well watered) (bushel/ acre)
ARGOS8-v1	138.0	209.0
ARGOS8-v2	138.0	210.0
WT	132.8	207.1

(Source: Shi et al., 2016)

In this case also ARGOS8 variant showed positive result compared to wild type. ARGOS8 gene generated by altering its regulatory elements can deliver a significant increase in grain yield under a flowering stress condition, with no yield loss under an optimal condition (Shi et al., 2016).

3.2.2 Herbicide stress tolerance in Maize

Sulfonylurea herbicides prevent branched amino acid biosynthesis in plants due to the inhibition of the enzyme acetolactate synthase (ALS). Resistance to one of these herbicides, chlorsulfuron, has been described as a result of single amino acid changes in the ALS protein at position 197 in Arabidopsis (Pro to Ser) and tobacco (Pro to Gln or Ala) and at a corresponding location, position 178, in soybean (Proline to Ser).

ALS was chosen for gene editing to demonstrate that RNA-guided Cas9 can facilitate specific DNA sequence changes in a native maize gene. There are two ALS genes in maize, ALS1 and ALS2, located on chromosomes 4 and 5, An ALS2 specific ALS-CR4 gRNA was designed based on the polymorphisms between ALS1 and ALS2 nucleotide sequences, and tested. ALS2 was highly preferred over ALS1 when ALS-CR4 gRNA was used. Acetolactate synthase (ALS) is the first common enzyme in the biosynthetic pathways to valine, isoleucine, and leucine.

To test whether the edited ALS plants offers herbicide resistance, Four different concentrations of chlorsulfuron (50, 100 (1x), 200, and 400 mg/liter) were sprayed on selected four-week old segregating plants with edited and wild-type ALS2 alleles. Three weeks after treatment, plants with an edited allele showed normal phenotype, while plants with only wild-type alleles demonstrated strong signs of senescence (Fig.10).

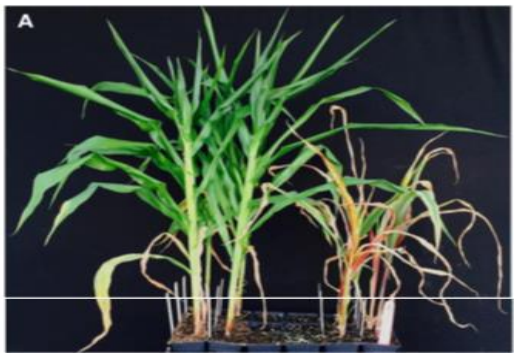

Figure10.Plant with edited ALS and without edited ALS (Source: svitashev et al., 2015)

In addition, embryos isolated from seed of mutant plant and wild-type, were germinated on media with 100 ppm of chlorsulfuron. Fourteen days after germination, the condition of ALS edited plants was positive (Tab.7, Fig.11).

Table7. Condition of wild type and (ALS edited) plants after 14 d of germination grown in chlorsulfuron (100ppm)

Plant part	Plants (ALS edited)	Wild type
Shoot	Normal height	short
Root	Well developed	Not developed

(Source: svitashev et al., 2015)

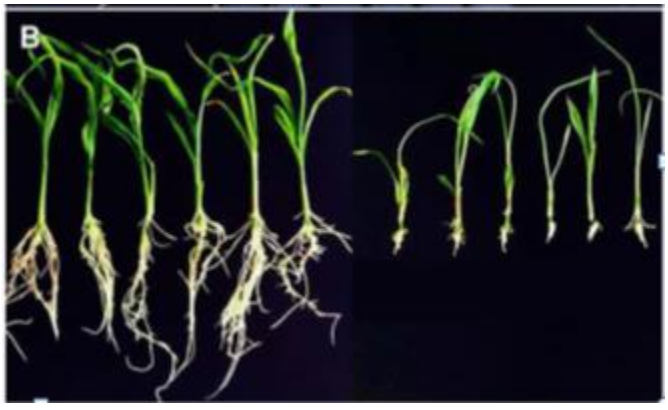

Figure11. T1 generation embryos germinated on media with chlorosulfuron; 14 days after germination (source: svitashev et al., 2015)

3.2.3 Herbicide resistance in rice

A P178S mutation in the Acetolactate synthase 1(ALS1), a key enzyme for the biosynthesis of branched chain amino acids and a major target for important herbicides including chlorosulfuron and bispyribac sodium (BS) (Mazur et. al., 1987). Svitashev S.et. al., (2016) introduced ALS1 gene in rice using CRISPER/Cas9-mediated homologous recombination with two gRNA. The CRISPER/Cas9 construct then transferred to the plasmid, and later the plasmids are introduced in rice (Japonica cv. Nipponbare) calli through particle bombardment. 116 independent lines survived and among this, from which 52 were selected randomly for further analysis.

In this experiment, rice plant with ALS edited and wild type without ALS were taken. They are being sprayed with 100 mM bispyribac sodium at the five-leaf stage. The condition of ALS edited plants was positive (Tab.8, Fig.12). (Sun Y. et al., 2016)

Table8.The Condition of the plants are given in the table at 10 and 36 days after being sprayed with 100 mM bispyribac sodium at the five-leaf stage

Days	Plants (ALS edited)	Wild type
After 10	Normal leaf	Withered leaf
After 36	Survived	Died

(Source: Sun Y. et al., 2016)

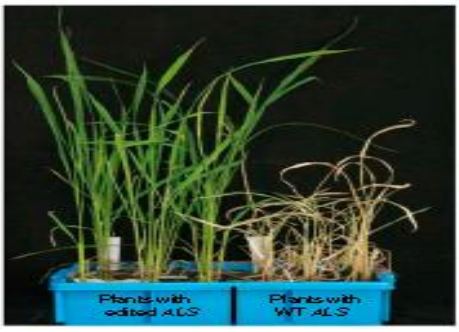

Figure12. Herbicide-Resistant Rice Plants (ALS edited) through CRISPR/Cas9 technique with wild type plant (Source: Sun Y. et al., 2016)

CHAPTER 4

CONCLUSIONS

- Biotic stresses occur due to the damage done to plants by other living organisms such as fungi, bacteria, virus, nematodes, harmful insects etc. The most prominent biotic stress that the plants experience is due to disease outbreak. Various diseases of important plants can be controlled precisely through CRISPR/Cas9 technology.

- Plant faces various abiotic stress such as drought, flooding, salinity, heat, ion toxicity, chemical effect etc. which causes alteration in membrane stability, respiration rate, phyto-hormone secretion, ion instability, oxidative burst, inhibition of photosynthesis, growth reduction and improper development ultimately yield reduction occur. Through CRISPR/Cas9 technology, stress of drought, chemical effects can be solved efficiently and hopefully others stresses will be solved in future.

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