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CRISPR: The New frontier of Genome Engineering

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Introduction

In the current scenario, the most critical challenge faced by the human race is to provide food security for a growing population. By 2050, the human population will reach 10 billion and to feed the world, global food production needs to increase by 60–100% (FAOSTAT, 2016). Besides the growing population rate, extreme weather, reduced agricultural land availability, increasing biotic and abiotic stresses are significant constraints for farming and food production. Development of technologies that can contribute to crop improvement can increase production to some extent. Genetic manipulation techniques using physical, chemical and biological (T-DNA insertion/transposons) mutagenesis have contributed majorly in studying the role of genes and identifying the biological mechanisms for the improvement of crop species in the past few decades (Ma et al., 2016). For the past three decades, transgenic techniques have been used to understand basic plant biology and also used for crop improvement. However, the integration of transgenes into the host genome is non-specific, sometimes unstable and is a matter of public concern when it comes to edible crop species (Stephens and Barakate, 2017). In the last decade, the use of genome editing technologies with site-specific nucleases (SSNs) has successfully demonstrated precise gene editing in both animal and plant systems. These SSNs create double-stranded breaks (DSB) in the target DNA. The DSBs are repaired through non-homologous end joining (NHEJ) or homology-directed recombination (HDR) pathways resulting in insertion/deletion (INDELS) and substitution mutations in the target region(s), respectively (Jinek et al., 2012). In contrast to the transgenic approach, which leads to random insertions and very often random phenotypes, genome editing methods produce defined mutants, thus becoming a potent tool in functional genomics and crop breeding. Genome edited crops have an additional advantage over

transgenic plants since they 'carry' their edited DNA for the desired trait (Malzahn et al., 2017). Such improved crops can be used in breeding programs and the resulting varieties can be used directly with lesser acceptability/consumption issues and relatively lesser regulatory procedures compared to conventional genetically modified (GM) crops (Waltz, 2018).

Engineered nucleases contain a non-specific nuclease domain fused with a sequence-specific DNA binding domain. Such fused nucleases can precisely cleave the targeted gene and the breaks can be repaired through NHEJ or HDR and hence the term 'genome editing' (Gaj et al., 2013).

Clustered Regularly Interspaced Palindromic Repeats (CRISPR /Cas9)

The discovery of CRISPR/Cas9 gene editing system has revolutionized research in animal and plant biology with its utility in genome editing being first demonstrated in 2012 in mammalian cells (Jinek et al., 2012). Unlike ZFNs and TALENs, CRISPR genome editing is more straightforward and involves designing a guide RNA (gRNA) of about 20 nucleotides complementary to the DNA stretch within the target gene. The acronym CRISPR, (first coined in 2002; Jansen et al., 2002) refers to tandem repeats flanked by non-repetitive DNA stretches that were first observed in the downstream of Escherichia coli iap genes. In 2005, these non-repetitive sequences were found to be homologous with foreign DNA sequences derived from plasmids and phages. Subsequently, the mechanism of homology-dependent cleavage was explored for genome editing and the technology of CRISPR/Cas9 cleavage 'arrived' as a promising genome editing tool.

The CRISPR cleavage methodology requires:

(i) a short synthetic gRNA sequence of 20 nucleotides that bind to the target DNA and (ii) Cas9 nuclease enzyme that cleaves 3–4 bases after the protospacer adjacent motif (PAM; generally, 50 NGG; Jinek et al., 2012). The Cas9 nuclease is composed of two domains, (a) RuvC-like domains and (b) a HNH domain, with each domain cutting one DNA strand. Following the development of the CRISPR cleavage methodology, it has been widely applied in plant and animal genome editing. Between 2010 and 2018, nearly 5000 articles have been published detailing the use of CRISPR1. Implementing a CRISPR project involves simple steps viz. (i) identifying the PAM sequence in the target gene, (ii) synthesizing a single gRNA (sgRNA), (iii) cloning the sgRNA into a suitable binary vector, (iv) introduction into host species/cell lines transformation followed by (v) screening and (vi) validation of edited lines (Figure 1).

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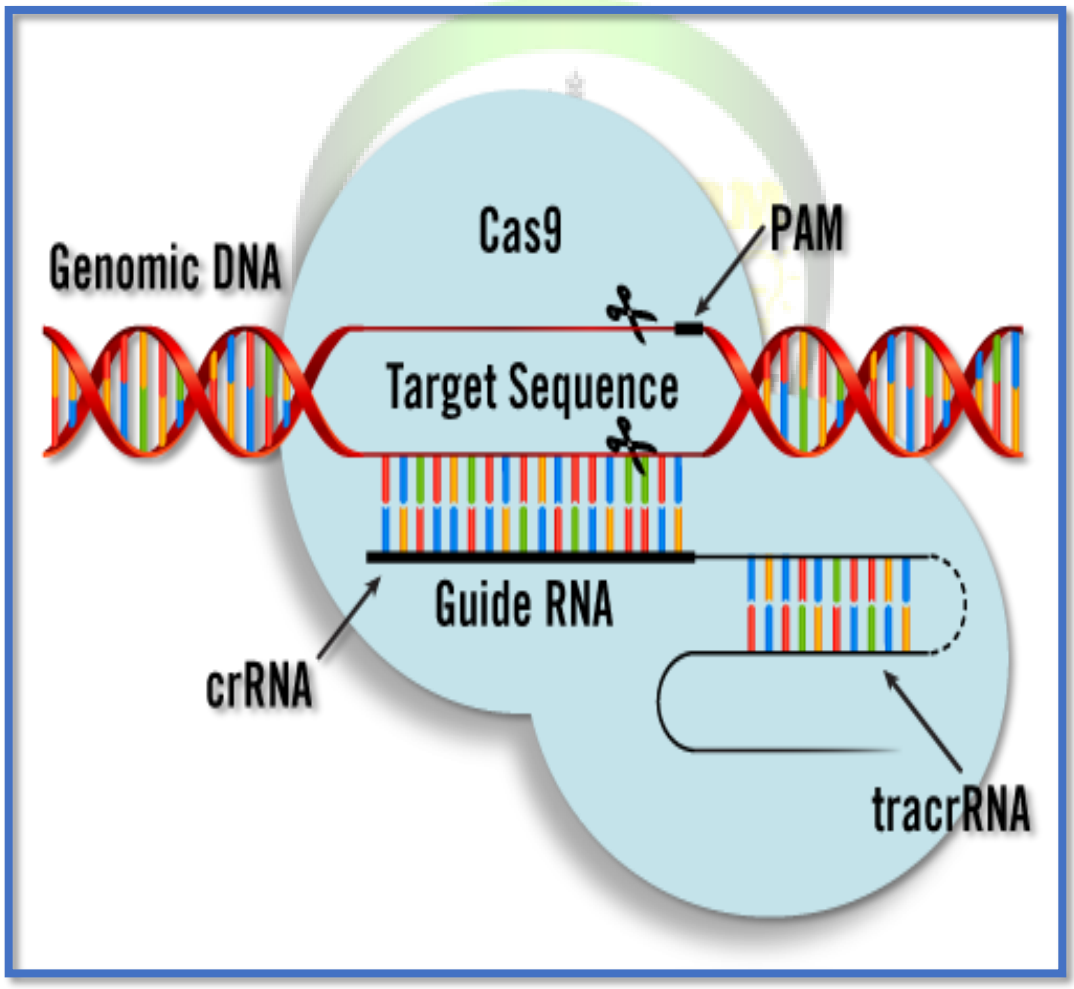
Components of CRISPR

- ✓ **crRNA-** Contains the guide RNA that locates the correct section of host DNA along with a region that binds to tracrRNA (generally in a hairpin loop form).
- ✓ **tracrRNA-** Binds to crRNA and forms an active complex.
- ✓ **sgRNA-** Single guide RNAs are a combined RNA consisting of a tracrRNA and at least one crRNA.
- ✓ **Cas9-** Protein whose active form is able to modify DNA. Many variants exist with differing functions (i.e., single strand nicking, double strand break, DNA binding) due to Cas9's DNA site recognition function.
- ✓ **Protospacer adjacent motif (PAM)**



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CRISPR-Cas Defense Mechanism

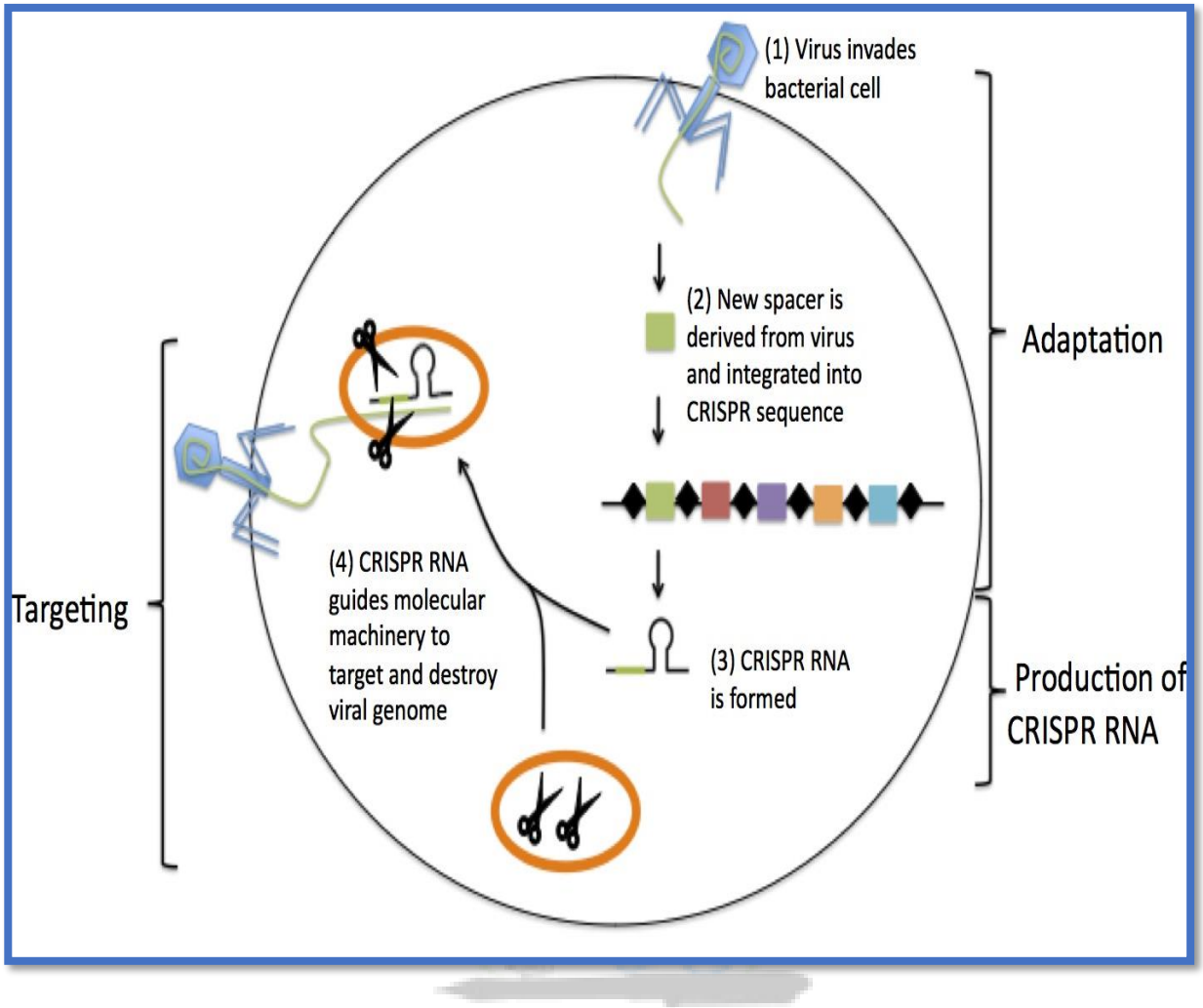

Figure 1: Three stages of CRISPR-Cas mediated defense process.

The CRISPR-Cas mediated defense process can be divided into three stages:

First stage- Adaptation, leads to insertion of new spacers in the CRISPR locus.

Second stage- Expression, the system gets ready for action by expressing the Cas genes and transcribing the CRISPR into a long precursor CRISPR RNA (pre-crRNA). The pre-crRNA is subsequently processed into mature crRNA by Cas proteins and accessory factors.

Third stage- Interference, target nucleic acid is recognized and destroyed by the combined action of crRNA and Cas proteins complex.

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crRNA generation and target interference in type I, II and III CRISPR/Cas systems

(a)Transcription of the CRISPR array into a pre-crRNA (b) Processing of the pre- crRNA into mature short crRNAs. In type I, RNA cleavage is performed by Cas6-homologues, which bind the repeat stem-loop and stay associated for Cascade formation. In type II, tracrRNA is required for binding and processing of the pre-crRNA by Cas9 and RNaseIII. In type III, Cas6 binds to non-structured repeats and processes the pre-crRNA into crRNA and

then dissociates; (c) Target binding and cleavage. Type I Cascade binds the DNA target before recruiting Cas3 for degradation. In type II, Cas9 stays associated with the tracrRNA:crRNA complex after processing and subsequently binds and cleaves target DNA. The type III-B CMR-complex binds spacer sequence and targets RNA. It is hypothesized that a type III-A Csm complex forms and this system targets DNA.

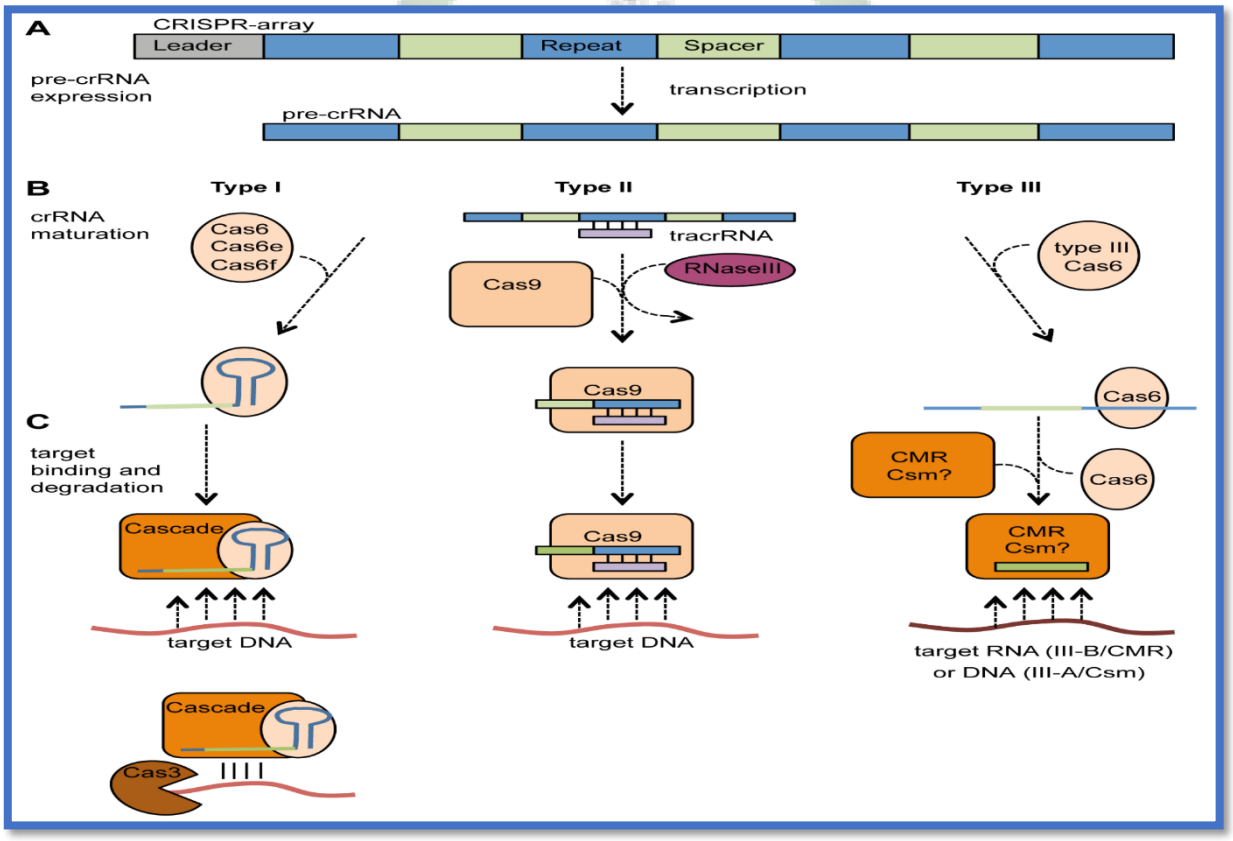

Figure 2: Type I, II and III CRISPR/Cas systems

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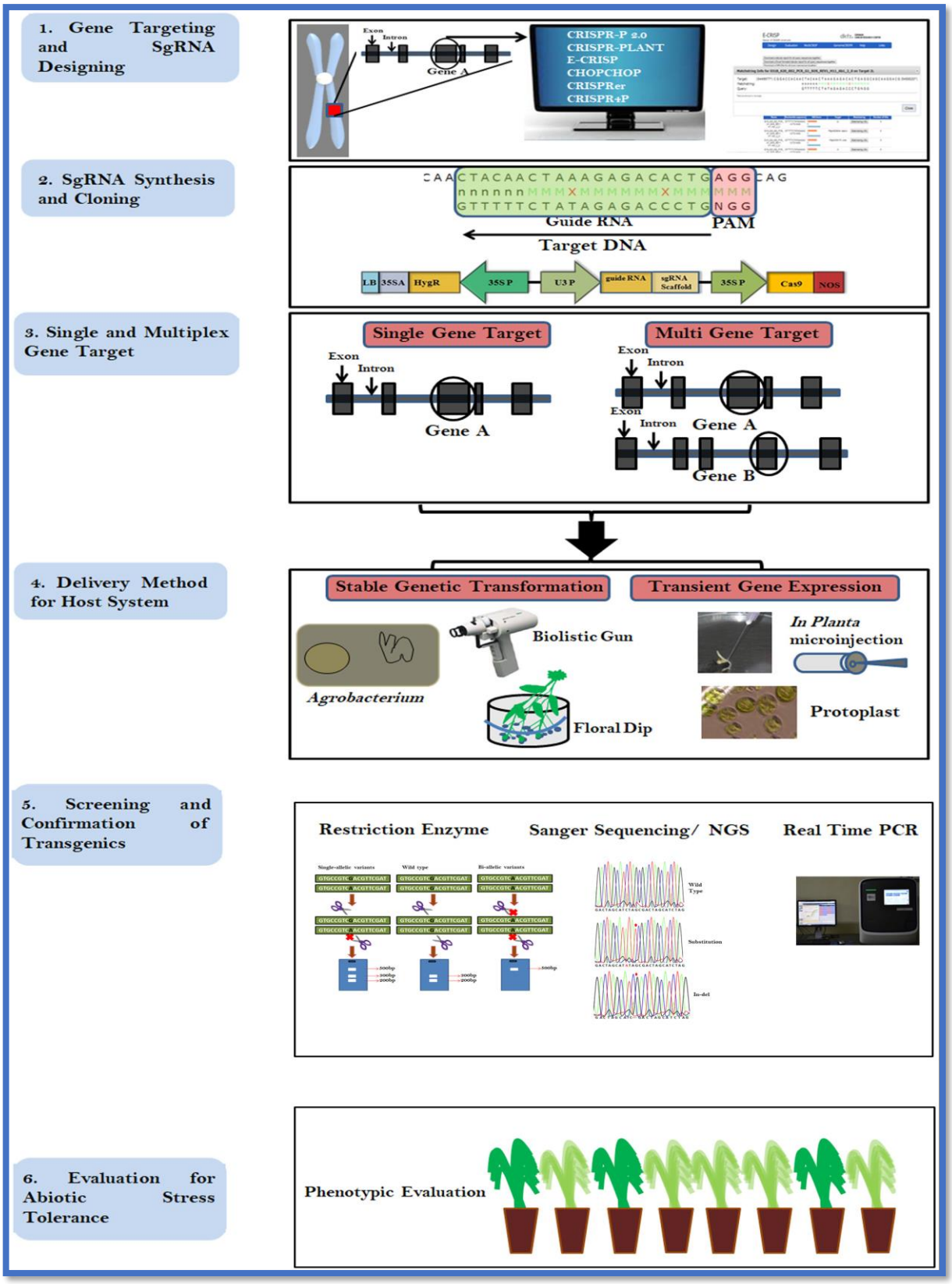

Figure 3: Flow chart describing the steps involved in CRISPR/Cas9 based genome editing.

Step 1 describes the selection of gene and designing of gRNA, Step 2 describes the cloning of the gRNA in a suitable binary vector. Step 3 Shows the availability single and multiplex editing. Step 4 describes methods of transformation; Step 5 explains screening methods of edited crops and Step 6 demonstrates the evaluation and selection of the desirable transgene-free plant for the target trait.

CRISPR/Cas-based applications for plant breeding

The improvement mainly focuses on yield, quality, and biotic and abiotic resistance.

- ✓ CRISPR/Cas-mediated mutation can achieve indels, gene deletions, and multiplex gene knockout.
- ✓ Gene insertion and replacement mediated by either homology-directed repair or nonhomologous end joining can achieve gene stacking for multiple traits, gene correction for gain-of-function, and gene insertion or replacement to produce new traits in breeding.
- ✓ Applications of base editing for crop trait improvement, such as precise amino acid substitution, gene disruption by introducing a stop codon, gene regulation, and whole-gene screening.
- ✓ CRISPR/Cas system-based gene regulation by editing the regulatory site in the untranslated region, promoter, or enhancer region.
- ✓ CRISPR/Cas-based antiviral breeding strategies. The CRISPR system with a guide RNA targeting DNA or RNA viruses is integrated into the plant genome, conferring resistance to invading viruses.
- ✓ CRISPR/Cas-based genome-wide screening, a valuable technique for functional genomics and genetic improvement.

Regulatory concerns for the crops developed using genome editing tools

New breeding technologies like ZFNs, TALENs, and CRISPR does not fall under the definition of a GMO under regulatory regimes in many countries. The United States Department of Agriculture (USDA) has stated that CRISPR/Cas9 edited crops can be cultivated and sold free from regulatory monitoring (Waltz, 2018). This can save several million dollars on getting regulations of GMO crops for the field test and data collections.

In addition, it also reduces time as it usually takes numerous years to release a GMO crop. It also will remove the doubt of consuming GMO crops among the community.

Table 1: Application of CRISPR based genome editing approach in plants for biotic, abiotic, and nutritional traits.

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Crop	Method	Target gene	Stress/trait	Reference
Biotic Stress				
Rice	NHEJ	OsERF922	Blast Resistance	Wang F. et al., 2016
	NHEJ	OsSWEET13	Bacterial blight resistance	Zhou et al., 2015
Bread Wheat	NHEJ	TaMLO-A1, TaMLO-B1 & TaMLOD1	Powdery mildew resistance	Wang et al., 2014
Abiotic stress				
Maize	HDR	ARGOS8	Increased grain yield under drought stress	Shi et al., 2017
Tomato	NHEJ	SlMAPK3	Drought tolerance	Wang et al., 2017
A. thaliana	NHEJ	UGT79B2, UGT79B3	Susceptibility to cold, salt, and drought stresses	Zhao et al., 2016
	HDR	MIR169a	Drought tolerance	Zhao et al., 2016
	NHEJ	OST2 (OPEN STOMATA 2) (AHA1)	Increased stomatal closure in response to abscisic acid (ABA)	Osakabe et al., 2016
Rice	HDR, NHEJ	OsPDS, OsMPK2, OsBADH2	Involved in various abiotic stress tolerance	Shan et al., 2013
	NHEJ	OsMPK5	Various abiotic stress tolerance & disease resistance	Xie and Yang, 2013
Rice	NHEJ, HDR	OsMPK2, OsDEP1	Yield under stress	Shan et al., 2014
	NHEJ	OsDERF1, OsPMS3, OsEPSPS, OsMSH1, OsMYB5	Drought tolerance	Zhang et al., 2014
Rice	NHEJ	OsAOX1a, OsAOX1b, OsAOX1c, OsBEL	Various abiotic stress tolerance	Xu et al., 2015

	NHEJ	OsHAK-1	Low cesium accumulation	
	NHEJ	OsPRX2	Potassium deficiency tolerance	Mao et al., 2018
Nutritional and other traits				
Maize	NHEJ	ZmIPK1A ZmIPK & ZmMRP4	Phytic acid synthesis	Liang et al., 2014
Wheat	HDR	TaVIT2	Fe content	Connorton et al., 2017
Soybean	NHEJ	GmPDS11 and GmPDS18	Carotenoid biosynthesis	Du et al., 2016
Tomato	NHEJ	Rin	Fruit ripening	Ito et al., 2015
Potato	HDR	ALS1	Herbicide resistance	Butler et al., 2016

Conclusion

New breeding techniques provide scientists the ability to precisely and quickly insert the desired traits than conventional breeding. CRISPR/Cas9 based genome editing is a fundamental breakthrough technique. Application of genome editing tools in crop improvement to enhance yield, nutritional value, disease resistance and other traits will be a prominent area of work in the future. In the last 5 years, it is being applied vigorously in many

plant systems for functional studies and combating biotic and abiotic stresses as well as to improve other important agronomic traits. Though several modifications to this technology have to lead to increasing on-target efficiency, most work carried is preliminary and needs further improvement. Nevertheless, CRISPR/Cas9 based genome editing will gain popularity and be an essential technique to obtain 'suitably edited' plants that will help achieve the zero-hunger goal and maintain feed the growing human population.

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