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Applications of CRISPR-Cas mediated genetic engineering in crop breeding

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Abstract: The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) is the most widely used approach nowadays in major plant breeding applications mainly against DNA and RNA viral diseases. The CRISPR-Cas is actually an adaptive immune response protecting the cells from viral infections in prokaryotes. CRISPR technology and its variants are being used in improving many of the traits in plants to enhance yield, quality, and nutritional value, to introduce or enhance tolerance to biotic and abiotic stresses and majorly resistance against viral infections. The widespread dissemination and use of CRISPR-Cas technology to deal with diverse agricultural problems could be a key indicator of its future potential. In this review the status and future prospects of CRISPR-Cas applications, and current advances in crop breeding improvement have been focused.

Keywords: CRISPR-Cas; Crops; Genome editing; Trait improvement

1. Introduction

Plant viruses infect many economically important crops including cucumber, wheat, cotton, maize, cassava and other vegetables. This results in a major decrease in crop yields worldwide as they're obligate parasites and rely on the host cell to finish their life cycle. In keeping with reports, it's estimated that 15 to 17% reduction in global crop yields every year occur thanks to plant diseases, and 47% of this loss is caused by plant viruses only (1,2,3). Plant viruses possess serious threat to world agriculture and also the food security of the rapidly increasing world population causing decrease in cropland area per capita resulting in shortage. Major vectors of viruses infecting plants are insects (whiteflies, hoppers, thrips, beetles, etc.), mites, nematodes, and plasmodiophorids (4). Virus-infected plants exhibits a range of symptoms betting on the pathogen; these symptoms often include leaf yellowing, leaf distortion, leaf curling, and other growth distortions like stunting of the full plant, abnormalities in flower or fruit formation etc (5).

Viruses affecting plants are classified into six major groups supported their genome material: double-stranded DNA (dsDNA) viruses, single stranded DNA (ssDNA) viruses, reverse-transcribing viruses, double-stranded RNA (dsRNA) viruses, negative sense single stranded RNA (ssRNA-) viruses, and positive sense single stranded RNA (ssRNA+) viruses (6,7).

Genome-engineering strategies have recently emerged as a crucial tool in introducing desirable traits in many eukaryotic species, including plants. Among these genome engineering technologies, the CRISPR (clustered regularly interspaced palindromic repeats)/CRISPR-associated (CRISPR-Cas) (mainly CRISPR-Cas 9 and its variant is receiving interest globally due of its high simplicity, user friendliness, high efficiency and precision further as reproducibility. in step with recent studies CRISPR- Cas9 will be accustomed to engineer virus resistance in plants, either by directly targeting and cleaving the viral

genome, or by modifying the host plant genome to introduce viral immunity. Recent advancement and development of the CRISPR-Cas technology made it the most promising and very versatile tool for crop improvement for providing sustainable productive agriculture for better feeding of rapidly growing population amidst of constantly changing climatic condition (8,9,10).

This phenomenal use of CRISPR-Cas9 technology for editing of genome for combating plant virus infection in order to destruct the invading viral DNA for virus resistance development in plants is said to be firstly reported in 2015 by three independent communications; Two in Nature Plants by Ji et al. (11,12) & Baltes et al. (12) followed by one in Genome Biology by Ali et al. (13) against geminiviruses. In recent, many research groups also experimented on this and have reported the feasibility of viable virus resistance development in numerous plants using CRISPR-Cas9 technology. In plants, major application of CRISPR technology is to improve certain genetic traits which includes nutrition enhancement, resistance against citrus canker, rice blast, powdery mildew as well as against DNA viruses and RNA viruses.

2. Overview of CRISPR-Cas technology

The CRISPR-Cas systems in prokaryotes incorporate short, foreign transcripts from invading nucleic acids into a CRISPR array within the host genome to supply immunity (14,15).There are three separate CRISPR-Cas systems I, II and III, which use separate molecular mechanisms for recognizing and initiating cleavage of target nucleic acids (16,17).The type II system isolated from Streptococcus pyogenes and widely remarked as CRISPR-Cas9 is that the most typically used CRISPR-Cas system in genome editing today because of the benefit of modifying the targeted site by changing a brief sequence of roughly 20 nucleotides (nt) (18).

A short sequence motif adjacent to the targeted sequence called protospacer adjacent motif (PAM) is critical for target recognition and cleavage in type I and II systems (19). The transcripts of invading nucleic acids from the CRISPR array later transcribed as CRISPR RNA (crRNA) are post-transcriptionally processed into a posh with trans-activating RNA (tracRNA) by the widely conserved endogenous RNase III in type II systems (20).

The development of an artificial “linker loop” or scaffold that fuses the crRNA and tracRNA into one small guide RNA (sgRNA) has greatly facilitated design and simple construction of multiple sgRNA iterations (21). CRISPR-Cas systems exploit widely conserved double strand breaks (DSB) repair pathways to induce desirable changes at specific chosen loci. CRISPR-Cas systems could also be engineered to require advantage of the non-homologous end-joining (NHEJ) pathway to affect the error-prone repair of DSB often leading to frameshift mutations. Additionally, CRISPR-Cas systems could also be engineered to attain the homology-directed repair (HDR) of a DNA template on an exogenous donor vector effectively allowing the insertion of foreign genes or DNA sequences (22). Since CRISPR-Cas9 are sequence specific, its advantageous to use over RNA interference as off target effects of RNAi can be prevented and viral resistance durability of amiRNA- mediated silencing is avoided (23,24).

The general procedure of genome editing using CRISPR-Cas is basically divided into four major steps. In the first step a gene specific sgRNA is designed and constructed. These days many online tools have been developed for designing of sgRNAs, but it has not been widely adapted for plants as large-scale data collection and systematic study of sgRNA efficiencies in plant cells are needed to increase its accuracy. Step 1 takes 1-3 weeks. The second step transformation of protoplast using CRISPR is done and selection of active CRISPR is done using PCR/RE. This step takes two weeks. In the third step callus is transformed stably with active CRISPR using Agrobacterium-mediated transformation or particle bombardment. The time duration of this step varies according to different plants. In the fourth and final step, using PCR/RE the transformed or regenerated plants with desired modifications are identified and confirmed via sequencing (25).

3. General application of CRISPR-Cas in major agricultural plant breeding

3.1 Use of CRISPR-Cas9 in wheat plant

In wheat TaEDR1 gene which has role in negatively regulating the defense response against powdery mildew is targeted using CRISPR-Cas9 to knock down all the three homologs of TaEDR1 resulting in powdry mildew resistance (26). The TaGW2-A1, -B1 and –D1 genes of wheat which are responsible for controlling weight and protein contents of grain are targeted by CRISPR-Cas9 by gene knockout leading to increase in grain weight and protein content (27). The Ms1 gene responsible for male fertility in wheat is also targeted by CRISPR-Cas for gene knockout (28).

3.2 Use of CRISPR-Cas9 in rice

The OsRR22 gene, which is a transcription factor, is inactivated by mutations using CRISPR-Cas for enhanced salinity tolerance (29). The CAO1 and LAZY1 genes responsible for Synthesis of Chl b from Chl a and regulating shoot gravitropism, respectively were disrupted using CRISPR-Cas to target the defective synthesis of Chlorophyll b and tiller-spreading phenotypes (30). The gene disruption was done using CRISPR-Cas9 in SBEI and SBEIIb genes responsible for determining the amylose content, fine structure of amylopectin, and physiochemical properties of starch, resulting in higher proportion of long chains in amylopectin (31).

The Gn1a, DEP1, GS3 and IPA1 genes in rice which are regulators of grain number, panicle architecture, grain size and plant architecture, respectively were targeted using CRISPR-Cas9 for genes disruption resulting in enhanced grain number, dense erect panicles, and larger grain size, respectively (32). OsERF922 gene which are the negative regulator of Rice blast resistance were targeted using CRISPR-Cas9 for Gene disruption which led to enhanced rice blast resistance (33). The gene OsMATL which encodes a pollen-specific phospholipase were knocked out using CRISPR-Cas leading to Haploid seed formation by Yao et al. in 2018 (34).

Endo et al. in 2016 showed herbicide resistance can be instilled in rice plant by the Acetolactate synthase (ALS) encoding gene disruption using CRISPR-Cas9, while Sun et al. in 2016 used CRISPR-Cas for gene replacement of the same gene for herbicide resistance in the rice plant (35,36). The CRISPR-Cas was used for Thermo-sensitive genic male sterility Gene knockout to target Thermo-sensitive genic male sterility (37).

3.3 Use of CRISPR-Cas9 in tomato plant breeding

In Tomato plant the SlJAZ2 gene which is Important repressor in jasmonate signaling pathway and key regulator of stomatal aperture during biotic stresses was targeted using CRISPR-Cas for Gene knock in, for Bacterial speck resistance in tomato plants (38).

The SlMlo1 gene of tomato which Confers susceptibility to fungi, causing the powdery mildew disease is targeted using CRISPR-Cas9 for gene disruption which gives resistance to Powdery mildew disease (39). In Tomato the SlAGL6, a transcription factor, playing essentials roles, especially in flower meristem and floral organ development was targeted by CRISPR-Cas9 for gene knockout resulting in parthenocarpic phenotype (40). The SP5G, Florigen paralog and flowering repressor in tomato plant is targeted using CRISPR-Cas9 for Gene knockout which gives Rapid flowering and Early yield (41). SlIAA9, the key gene controlling parthenocarpy is targeted using CRISPR-Cas for gene knockout which gives parthenocarpy phenotype in the tomato plant. (42)

The gene SlMAPK3, a Mitogen-activated protein kinases 3 encoding gene, which responds to drought stress was knocked out using CRISPR-Cas9 for Drought tolerance in the tomato plant (43). The genes CrtR-b2 and Psy1, which are the key genes of carotenoid biosynthesis, CRISPR-Cas was used for Genes knockout results in changes on carotenoids profile (44). In Stress-tolerant wild-tomato the SP, SP5G, SlCLV3, SlWUS and SlGGP1 genes having role as Flowering repressors, small-peptide-encoding gene, homeobox-encoding gene and vitamin C–biosynthetic enzyme encoding gene were targeted using CRISPR-Cas for genes disruption, Insertions, deletions and inversion. This gives domesticated phenotypes yet retained parental disease resistance and salt tolerance (45).

4. Applications of CRISPR-cas9 in other major crops

In Maize ZmTMS5 gene was targeted by using CRISPR-Cas9 for gene knockout to target thermosensitive genic male sterility (46). In sorghum plant, a monocot the k1C gene was targeted using CRISPR for genes disruption in N-terminal ER signal peptide region which results into high Lysine content and increased protein digestibility (47). In the Monocot plant Cavendish banana (*Musa acuminata*), The PDS, Phytoene desaturase encoding gene was targeted using CRISPR-Cas by Naim et al. for gene knockout to target Albinism phenotype (48). In Banana (Musa spp.), the Integrated endogenous banana streak virus (eBSV) in the B genome of plantain activates into infectious viral particles under stress. CRISPR-Cas was used to knockout the integrated dsDNA of BSV from the banana genome which results into Asymptomatic plants to banana streak virus (49).

In dicot plants *Camelina sativa* the FAD2 gene responsible for Fatty acids biosynthesis was targeted using CRISPR-Cas9 for Genes knockout to Improve seed Oleic acid content (50). In Dicot *Arabidopsis thaliana*, the FWA and the SUPERMAN promoters, which are the Flowering time gene and a transcriptional regulator of floral homeotic genes, Genes knock in was done with the help of CRISPR-

Table 1: Targeted genes using CRISPR-cas9 in another plants

Plants	Target Genes	Target Traits	References
Glycine Max (Soybean)	Gmpds11 And Gmpds18	Carotenoid Biosynthesis	(65)
Solanum lycopersicum (Tomato)	Rin (Ripening Inhibitor)	Fruit Ripening	(66)
Manihot esculenta (Cassava)	Phytoene desaturase (MePDS) gene	Carotenoid Biosynthesis	(67)
Zea mays (Maize)	ARGOS8	Increased Grain Yield Under Drought Stress	(68)
Zea mays (Maize)	Zmipk1a Zmipk Andzmmrp4	Phytic Acid Synthesis	(69)
Arabidopsis thaliana	Dsdna Of Virus (A7, B7, And C3 Regions)	Beet Severe Curly Top Virus Resistance	(11)
Nicotiana benthamiana	Beydv	Bean Yellow Dwarf Virus (Beydv) Resistance	(12)
Nicotiana Benthamiana	Orfs And The IR Sequence Sdna Of Virus	Tomato Yellow Leaf Curl Virus (TYLCV) And Merremia Mosaic Virus (Memv)	(13)
Linum usitatissimum (Flax Plant)	5'ENOLPYRUVYLSHIKIMATE-3-PHOSPHATE SYNTHASE (EPSPS) gene	Herbicide Glycophospate Tolerance	(70)
Malus domestica (Apple)	Dspe-Interacting Proteins of Malus 1, 2, 4	Resistance Against Fire Blight (Erwinia Amylovora), host Susceptibility Factor For fire Blight Disease Induced	(71)
Hordeum vulgare (Barley)	*MP/CP*, *Rep/Repa* Genes And LIR	Against *Wheat Draft Virus*	(72)
Manihot Esculenta (Cassava)	*AC2* And *AC3* Genes Encoding Viral Proteins Trap and Ren, Respectively	Against *African Cassava Mosaic Virus*	(73)
Musa acuminata (Banana)	Phytoene desaturase	For decreasing chlorophyll and carotenoid contents	(74,75)
Gossypium hirsutum (cotton)	CLCuD IR and Rep regions	Resistance to Cotton leaf curl disease	(76)
Marchantia polymorpha (common liverwort)	ARFI (Auxin response factor 1)	For gene disruption by targeted mutagenesis in the liverwort *Marchantia polymorpha* L., as a model species for studying land plant evolution.	(77)
Citrus sinensis (Sweet Orange)	CsPDS gene	Cas9-mediated, sequence-specific genetic modification for CRISPR trail	(78)

Cas9 resulting in Targeted gene activation and DNA methylation in Arabidopsis (51). In the Potato plant, the GBSS gene which is Granule-bound starch synthase encoding gene, is responsible for amylose synthesis was targeted using CRISPR-Cas for Gene knockouts which resulted into product with Increased amylopectin content (52).

In Grape plants the gene VvWRKY52, a Transcription factor gene playing important roles in plant defense regulatory networks. Gene knockout was done using CRISPR-Cas9 to obtain *Botrytis cinerea* resistance (53).

The CsLOB1 gene in oranges plays a critical role in promoting pathogen growth and erumpent pustule formation. The CRISPR-Cas9 is used in Disruption of CsLOB1 promoter Deletions, insertions and substitutions leading to resistance against Citrus canker disease (54). The CsLOB1 gene (Critical citrus disease susceptibility gene) for citrus canker in the Grapefruit plant is targeted for disruption of the coding regions of both alleles of CsLOB1, this gives Citrus canker resistance in the plant (55). In grapefruits The CsLOB1 gene Plant-specific transcriptional factor in the lateral organ boundaries (LOB) domain family, the Disruption of the PthA4 effector binding elements in the Type I CsLOB1 Promoter is done using CRISPR-Cas9 which leads to the Citrus canker alleviated (56)

5. Conclusion

Even though this technique has great application against many plant breeding purposes and is already being used in several crop species but still factors like low stability, high costs and high levels of technical requirements, are the major drawbacks in its applicability and works needs to be done for its improvement (57,58,59).

Using CRISPR-Cas technology to confer resistance to both DNA and RNA viruses is now available for transgenic and non-transgenic plants. Site-specific insertion of transgenic DNA through CRISPR-Cas systems engineered to use the HDR pathway offers advantages over the random insertion methods commonly employed in plants (Agrobacterium-mediated transformation and microprojectile bombardment).

The CRISPR-Cas system can be engineered to insert transgenes at chosen loci, avoid unwanted positional effects and forestall the disruption of native gene function. The widespread dissemination and use of CRISPR-Cas technology to deal with diverse agricultural problems could be a key indicator of its future potential. The extent of the potential of CRISPR technology in applied plant research and crop breeding benefit for the food security of the world will depend upon the performance and public perception of the genetically modified crop varieties (60,61,62). This technique is liable to become more and more precise and efficient day by day, as the future opportunities like inducible Cas9 expression and direct delivery of Cas9 protein, are being researched upon in various organisms and cell types among the biologists all over the world (63,64).

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