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# Application of DNA-free CRISPR/Cas-mediated Genome Editing in Crops: A Review

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## ABSTRACT

Clustered regularly interspaced short palindromic repeats/CRISPR associated nuclease 9 (CRISPR-Cas9) system was instigated first into eukaryotes in the last ten years are becoming productive and worldwide application for genome modification. Genome engineering through insertion of foreign DNA insert have numerous disadvantages which could be overcome by use of DNA-free genome editing. Various ways of DNA-free genome editing mediated by CRISPR/Cas systems are CRISPR/Cas delivery as ribonucleoprotein, delivery of CRISPR/Cas as virus-like particles and agrobacterium-based delivery of CRISPR/Cas. Crop improvement through DNA-free genome editing via CRISPR/Cas have been applied in rice, wheat, maize, tomato, soybean and rare species like *Nicotiana benthamiana etc.* It is method of choice for precise genome editing without genome shuffle in an organism.

**Key words:** Cas9, CRISPR, Crop improvement, DNA-free-genome editing, Ribonucleoprotein.

DNA-free genome editing is a novel and speedy technology in biological sciences that became a method of choice as it is a means of precise genomic modification without disturbance in genome of an organism. It opened the possibility to generate genetic modified organism called as non-GMO in classical biology and biotechnology (Malzahn *et al*., 2017; Wolter and Puchta, 2017; Mao *et al.,* 2019).Conventional methods of gene editing include RNAi, zinc finger, TALENs *etc.* Breakthrough in gene editing occurs with the avent of RNA-guided endoDNAses followed by identification of Cas9 system incurred from immune system of bacterial paved novel path of targeted gene modifications. CRISPR/Cashas revolutionized the world of gene editing with surprising success in crop improvement through gene editing and alteration (Arora and Narula, 2017).

CRISPR-Cas9 can be extended as clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9. CRISPR is DNA sequences occur in the genomes of prokaryotic organism in reply of infection of phages that invades bacteria. Cas genes are essential for function of CRISPR and provide immunity in response to attack of viruses and plasmids in bacteria and archaebacteria (Barrangou and Marraffini, 2014; Sorek *et al*., 2013; Barrangou, 2013). CRISPR with Cas9 enzymes assemble as CRISPR-Cas9 system which is widely utilized to edit genome of organism (Barrangou *et al*., 2007). CRISPR is transcribed into pre-crRNA and cas genes becomes active and functional to express as cas proteins which help in processing of pre-crRNA into mature crRNA. Target nucleic acid is recognized and destroyed combinedly by crRNA and cas proteins (Koonin and Krupovic, 2014; Rath *et al*., 2015; Kumari *et al*., 2021).

CRISPR-Cas9 genome editing needs single guide RNA (sg RNA) that guide the Cas9 endonuclease to specific region of the genomic DNA, resulting in double stranded nicks in the target DNA. The CRISPR-Cas9 technique cleaves specific nucleotides *via* complementary sequence with Cas9 protein and sgRNA (Peng *et al*., 2016). Cas9 protein composed of two nucleic acid binding site like a large recognition (REC) lobe and a small nuclease (NUC) lobe that are linked by a helix bridge. REC controls Cas9 specific function and NUC integrate two nucleases, RuvC and HNH and protospacer adjacent motif (PAM). The presence of PAM flanking the target sites is required for target recognition and R-loop formation (Jinek, *et al.,* 2012; Nishimasu *et al*., 2014; Anders *et al*., 2014; Jiang and Doudna, 2017).

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Cas9 have endonuclease activity to produce double-strand breaks (DSBs) in target DNA during bacterial immune response (Mali *et al*., 2013; Bao *et al.,* 2019). DSBs can be repaired by non-homologous end joining (NHEJ) and homology directed repair (HDR) process. NHEJ uses DNA ligase IV to re-join the broken ends results in insertion or deletion mutations (indels) and can resulting in frameshift or introduction of a premature stop codon. HDR repairs the DSBs based on a homologous complementary template and results in a perfect repair. HDR is generally used for gene knock-in in plants (Schiml *et al*., 2014). A transgenic DNA can be generated by providing a donor DNA in Trans and the double strand break will be repaired by the host cell. This pathway is useful in generating loss-of-function/ knockout of the gene of interest (Costa *et al*., 2017) (Fig 1).

This technique has been applied in many species with diverse goal Gene manipulation through DNA-free CRISPR/ Cas system have been targeted in commercial crops in recent years. *viz*., *Nicotiana benthaminiana*, *Capsicum annuum*, wheat, maize, rice, potato, soybean, banana, *brassicaceae*, lettuce, tobacco *etc.* (Andersson *et al*., 2018; Murovec *et al*., 2018; Gonzalez *et al*., 2019; Hu *et al*., 2019; Park *et al*., 2019; Toda *et al*., 2019; Kim *et al*., 2020; Ma *et al*., 2020; Sant'Ana *et al*., 2020; Wu *et al*., 2020). Various approaches of Cas9/gRNA delivery have been utilized to attain editing via DNA-free system as for example, CRISPR/Cas delivering as ribonucleoprotein, virus-mediated delivery of CRISPR/Cas, *Agrobacterium tumefaciens* delivery for Cas9.

## CRISPR/Cas delivery as ribonucleoprotein

Foremost important method for DNA-free gene editing in plants are assembly of CRISPR-associated protein (Cas) ribonucleoprotein (RNP)-based genome editing. They are easy, precise and convincing technique for genome editing in crop plant which involves interaction between Cas9 and gRNA. Cas9 is expressed and purified in bacteria known as *Escherichia coli* and gRNA is generated *via* transcription or synthesized chemically. Ribonucleoprotein assembly and nanoparticles are acquired for transformation process (Park and Choe, 2019; Wang *et al*., 2019). Ribonucleoprotein and nanoparticles assembly are inserted in plant tissues *via* protoplast fusion or particle gun methods. Sometimes, PEG-mediated transfer and lipofections and electroporation are also been utilized (Liang *et al.*, 2018; Liu *et al*., 2020).

## Delivery of CRISPR/Cas as virus-like particles

Utilizing virus like particle for DNA-free genome editing by means of CRISPR/Cas system in plants has major limitations. Positive-strand RNA and DNA viruses possess limited capacity for foreign DNA insert replication renders to deletion and loss of sequence due course of replication. Furthermore, editing of small genome like CRISPR gRNA, zinc-finger nuclease, meganuclease *etc.* is easy and convenient with viral vectors. However, sequence size restrictions would pose viral infection with this large system difficult as size ofCRISPR/Cas system is more than 5.0 kb (Marton *et al*., 2010; Honig *et al*., 2015; Cody and Scholthof, 2019; Ariga *et al*., 2020; Liu and Zhang, 2020).Utilization of virus vectors for insertion of CRISPR/Cas9 in plants was applied in *Nicotiana benthamiana* and potato Virus X was employed to deliver Cas9 and gRNA to attain productive DNA-free genome edited plants. Likewise, in wheat, barley stripe mosaic virus was used to deliver guide RNA (Hu *et al*., 2019; Ariga *et al*., 2020; Ma *et al*., 2020).

## Agrobacterium based delivery of CRISPR/Cas

Agrobacterium based delivery of CRISPR/Cas cassette is method of choice of transformation in plant species.This method has been widely implicated in numerous plant species where leaf, flower, callus were used as a targeted

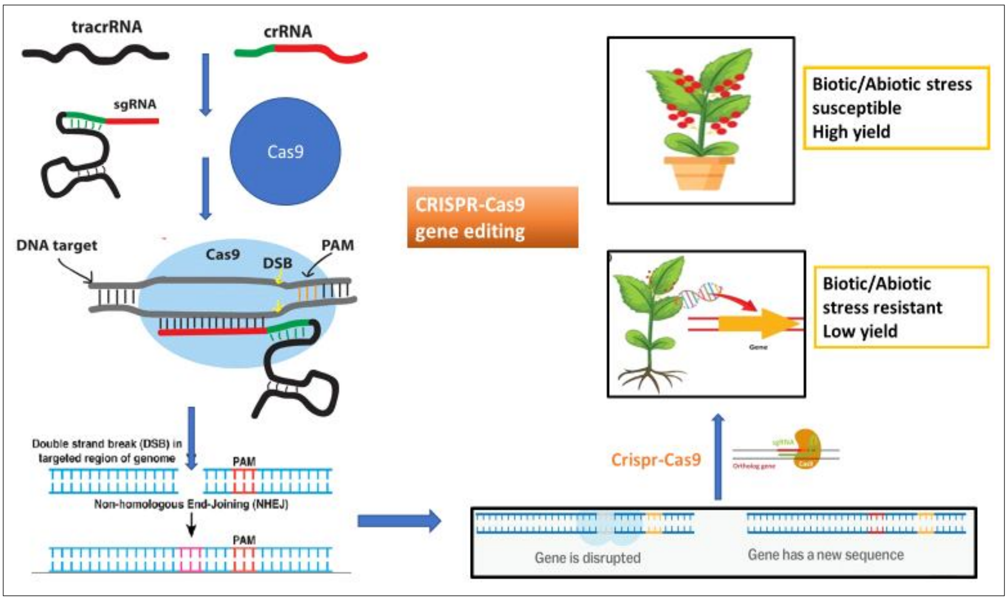


**Fig 1:** CRISPR-Cas9 knockout of target gene sequence for crop improvement.

explants usingT-DNA as integral part of Ti-plasmid consisting of Cas9 and the gRNA (Gelvin, 2003; Sandhya *et al.,* 2020). *Agrobacterium* contamination could be applied for generation of Cas9 and gRNA without antibiotics which prevents production of DNA-free plants and integration of T-DNA in genome of plant species. Transgenic-free transgenic plant in tobacco was procured by targeting phytoene desaturase (PDS) gene using this strategy (Chen *et al*., 2018; He and Zhao, 2020). This method has advantages as compared to CRISPR/Cas9 ribonucleo proteins and particle gun, though it has limitations to not applicable to all plant species. Acetolactate synthase gene was edited by cytidine editor by Agrobacterium infection in potato. Similarly, in tomato chlorsulfuron resistant plants were produced by modification in acetolactate synthase gene via point mutation through Agrobacterium mediated delivery (Danilo *et al*., 2019; Veillet *et al*., 2019).

## Crop improvement through DNA-free genome editing *via* CRISPR/Cas

### Rice

Several examples for trait improvement after utilization of CRISPR based genome editing tools are illustrated here. For example, rice genes, phytoene desaturase, betaine aldehyde dehydrogenase and mitogen activated protein kinase conferring stimuli for various abiotic stress were modified by CRISPR/Cas9 mediated genomic modification. Genes, OsDERF1, OsPMS3, OsEPSPS, OsMSH1, OsMYB5 responsible for drought tolerance were edited through inducing targeted mutation in rice. Disease susceptibility gene, OsSWEET13 has been knockout leads to bacterial blight resistance Indica rice. Annexin gene has been deactivated by CRISPR knocked out to confer cold stress in rice (Shen *et al*., 2017; Kumari and Kumawat, 2021).

Multiplexed plant genome editing and transcriptional regulation has been demonstrated in *Arabidopsis* and rice and made easier by CRISPR/Cas9.Knock-out of OsSEC3A gene increases salicylic acid content which caused resistance against blast disease in rice. Grain weight (GW) were upgraded by disruption of GW2, GW5 and GW6 genes, negative regulators of grain shape in rice is proof that grain weight is affected by grain shape. Grain size 3 (GS3) gene was knocked off in japonica varieties of rice pertains to increased grain length in T1 lines compared to wild type using CRISPR-QTL editing tool (Xu *et al*., 2016; Yuyu *et al*., 2020).

Low cesium rice plants were formulated by inactivation of the $K^+$ transporter OsHAK 1 with the CRISPR/Cas9 system and OsPRX2 for potassium deficiency tolerance. *OsRR22*, *O. sativa response regulator* 22, gene was knocked out to improve salt tolerance in rice the gene using the CRISPR/Cas9-targeted mutagenesis. SD1 and photosensitivity 5 (SE5) genes were targeted to produce semi-dwarf elite lines in rice. Genes (GS3, GW2 and GN1A) controls plant architecture, seed size, yield and erect panicle were targeted by bringing out knockouts by CRISPR/Cas9.Cooking and eating quality determines market value and consumer's preference in rice. Wx gene essential for amylose synthesis was mutated applying CRISPR/Cas9 system to produce high amylose content in rice accessions. Mutant's series with fine-tuned amylose contents was created by specific base alteration of Wx genes in rice. Targeted mutagenesis of starch branching enzyme SBEIIb leads to generation of high-amylose rice (Lacchini *et al*., 2020; Xu *et al*., 2020).

Likewise, aromatic rice was generated from unscented variety, ASD16 by targeting the OsBADH2 through CRISPR/Cas9. Knockoff of Vacuolar Iron Transporter genes, OsVIT2 to increase Fe distribution in embryo and endosperm. Sulfur metabolisms molecular switch reduces arsenic and enhance selenium in rice. Knock into the carotenoid biosynthetic pathway and integration of CrtI and PSY genes into the target spot by CRISPR/Cas9 resulted into high β carotene in rice. GABA-rich rice was created which contains seven-fold GABA by truncating the C-terminal of the OsGAD3 by means of CRISPR/Cas9 approach (Akama *et al*., 2020; Ashokkumar *et al*., 2020; Dong *et al*., 2020; Chen *et al*., 2021; Sun *et al*., 2021).

### Wheat

CRISPR/Cas9 genome editing for improvement of trait are applied in wheat *viz*., three genes knockout by CRISPR/Cas9 conferred powdery mildew resistance. Switching of the three homologs of *TaEDR1* gene leads to creation of *TaEDR1* lines in wheat having resistance to powdery mildew (Gil-Humanes *et al*., 2017; Zhang *et al*., 2017). Similarly, two genes in protoplasts were focussed to confer resistance to head blight caused by *Fusarium graminearum* (Ansari *et al*., 2020). Many genes were targeted by CRISPR/Cas9 technology for enhancing yield and protein content in wheat (Wang *et al*., 2018; Hillary and Ceasar, 2019). Knockoff of *TaGW7* gene provides grain width enhancement and weight in wheat (Wang *et al*., 2019). Likewise, gene editing using CRISPR/Cas9 has been implicated to reduce gluten content in wheat (Jouanin *et al*., 2020; Liu *et al*., 2021).

### Maize

Gene editing of PSY1 gene in maize was done through CRISPR/Cas9 resulted into mutant (psy1) with white kernels and albino seedlings. ZmTMS5, thermo-sensitive genic male sterile gene liable for male sterility in maize was selected for CRISPR/Cas9 genome editing. ARGOS, genes upgrade drought tolerance in transgenic maize because of their role in negative regulation of ethylene response and signal transduction in ethylene production pathway. Knockout of the Wx gene generated twelve elite inbred lines with waxy mutants in maize by CRISPR/Cas9 (Ansari *et al*., 2020; Gao *et al*., 2020).

### Tomato

CRISPR/Cas9 system have huge role for lengthening shelf life in tomatoes. CRISPR/Cas9 targeted mutagenesis in ALC gene was used to prolong shelf life in tomato lines. Disruption of ripening inhibitor gene, RNA recognition motif-containing gene confirms their role in fruit ripening in tomato by CRISPR gene editing. Fruit yield increases by gene knock out of flowering repressor, SP5G gene, seedless fruit by somatic mutations in the parthenocarpy related gene, SlIAA9, increased shelf life by replacement of the dominant ALC (Alcobaca) gene in tomato by CRISPR gene editing. Yellow and purple tomato was created by mutation in gene, PSY1 involves in carotene synthesis. Knockout of genes involved in carotenoid metabolic pathway leads to generation of lycopene rich tomato by CRISPR/Cas9 (Li *et al*., 2018: Vu *et al*., 2020; Wang *et al*., 2020; Chattopadhyay *et al*., 2021).

**Soybean**

CRISPR/Cas9 induced genome editing was first studied in soybean by Cai *et al*. (2015) by editing two genes (GmFEI2 and GmSHR). Bao *et al*., 2020 described construction of CRISPR/Cas9 plasmid for soybean gene editing. CRISPR/Cas9 mediated base editing tool to induce single base substitution in soybean was developed by Cai *et al*. (2020). Two genes, GmIPK1 and GmIPK2 codes for enzyme related to phytic acid biosynthesis pathway were edited by two components CRISPR/Cas9 tool (Carrijo *et al*., 2021).

**Arabidopsis**

CRISPR/Cas9 system of genome editing was firstly applied in *Arabidopsis* by Feng *et al*. (2013) where three genes, brassinosteroid insensitive1, jasmonate-zim-domain protein 1 and gibberellic acid insensitive were edited simultaneously. Similarly, Mao *et al*., (2013) utilized CRISPR/Cas9 targeted genome editing of albinism genesCHLI1 and CHLI2 with AFLP marker.CRISPR/Cas9 system was utilized to provide TuMV resistance and induced germline mutation in *Arabidopsis* (LeBlanc *et al*., 2018; Zhang *et al*., 2018).

**Potato**

Potato and their excellent nutritive value like starch, vitamin C, potassium, fibre, vitamins B, copper, tryptophan etc. help in control many of the deadly diseases. It is important crop worldwide which need recent research attention through implications of biotechnological approaches. Waxy type of potato was developed through mutation of granule-bound starch synthase gene and multi-allele mutation was also created by knocking off Acetolactate Synthase1 gene (Butler *et al*., 2016; Andersson *et al*., 2017).

**Cotton**

Targeted genome editing utilizing CRISPR/Cas9 system was first applied in cotton by Janga *et al*., (2017). Later, CRISPR induced gene truncation in two copies of Gh14-3-3d gene was used to produce *Verticillium* resistant in upland cotton germplasm (Zhang *et al*., 2018).

**Rare species**

In recent days, CRISPR/Cas9 technique has also been utilized for improvement of tree with heterozygous genome like hybrid poplar and resistant to cotton leaf curl multan virus in *Nicotiana benthamiana*, by targeting CLCuMuV genome. In, wild strawberry (*Fragaria vesca*) TAA1 genes (responsible for auxin biosynthesis and ARF8 (regulates auxin response factor 8) were edited to generate faster growth plant. These studies demonstrate the use of CRISPR/Cas9 genome editing for gene editing in wild species and creating new variants of wild species rather better in overall plant phenotype essential for the commercial cultivation (Zhou *et al*., 2018; Yin *et al*., 2019; Wang *et al*., 2020a) (Table 1).

**Table 1:** Genome editing through CRISPR/Cas9 technology in major food crops for various traits of interest.

| Crops | Target Gene (s) | Trait | References |
|---|---|---|---|
| Wheat | TaGASR7 | Grain length | Hillary and Ceasar, 2019 |
| Wheat | TaGW7 | Grain width and grain weight | Wang *et al*., 2019 |
| Wheat | Gliadin | Coeliac disease resistance in humans | Liu *et al*., 2021 |
| Rice | OsBADH2 | Aroma production | Ashokkumar *et al*., 2020 |
| Rice | CrtI and PSY | β carotene | Dong *et al*., 2020 |
| Rice | OsGAD3 | GABA content | Akama *et al*., 2020 |
| Rice | astol1 | Selenium content | Sun *et al*., 2021 |
| Maize | ZmIPK1A, ZmIPK and ZmMRP4 | Reduced phytic acid | Liang *et al*.,2014 |
| Maize | PSY1 | Seed colour | Zhu *et al*., 2016 |
| Maize | Zmzb7 | Encodes IspH protein for methyl-D-erythritol-4-phosphate (MEP) Pathway | Feng *et al*., 2016 |
| Maize | ARGOS | Drought tolerant | Adhikari and Poudel, 2020 |
| Maize | Wx | Waxy endosperm | Gao *et al*., 2020 |
| Tomato | SlAGO7, gene | Leaf traits | Brooks *et al*., 2014 |
| Tomato | SlMYB12 | Pink tomatoes | Yang *et al*., 2019b |
| Tomato | PSY1, ANT1 | Yellow and purple tomatoes | Chattopadhyay *et al*., 2021 |
| Banana | MaACO1 | Shelf life | Hu *et al*., 2020 |
| Potato | *StPPO2e* | Tuber polyphenolsoxidase | Gonzalez *et al*., 2021 |
| Cotton | GhFAD2 gene | High oleic acid | Chen *et al*., 2021 |
| *Nicotiana benthamiana* | CLCuMuV genome | Resistant to cotton leaf curl multan virus | Yin *et al*., 2019 |

## CONCLUSION

DNA-free genome editing mediated by CRISPR-Cas9 is current and rapid developing technology in plant biotechnology and biology. Various approaches rely on delivery via ribonucleoprotein or virus-like particles or Agrobacterium. Amongst all, Agrobacterium means of delivery is most viable approach for gene/genome editing.Although CRISPR-Cas9 technology has been utilized for crop plants engineering but wide implementation of this technology will require the development of protocols for plant transformation, species-specific vectors and various genomic resources in recent future.

**Conflict of interest:** None.

## REFERENCES


Adhikari, P. and Poudel, M. (2020). CRISPR-Cas9 in agriculture: Approaches, applications, future perspectives and associated challenges. Malaysian Journal of Halal Research. 3(1): 6-16.

Akama, K., Akter, N., Endo, H., Kanesaki, M., Endo, M., Toki, S. (2020). An in vivo targeted deletion of the calmodulin-binding domain from rice glutamate decarboxylase 3 (OsGAD3) increases γ-aminobutyric acid content in grains. Rice. 13(1): 20. DOI: https://doi.org/10.1186/s12284-020-00380-w.

Andersson, M., Turesson, H., Nicolia, A., Falt, A. S., Samuelsson, M. and Hofvander, P. (2017). Efficient targeted multiallelic mutagenesis intetraploid potato (*Solanum tuberosum*) by transient CRISPR-Cas9 expressionin protoplasts. Plant Cell Rep. 36: 117-128.

Anders, C., Niewoehner, O., Duerst, A, Jinek, M. (2014). Structural basis of PAM-dependent target DNA recognition by the Cas9endonuclease. Nature. 513(7519): 569-573.

Andersson, M., Turesson, H., Olsson, N. *et al.* (2018). Genome editing in potato via CRISPR-Cas9 ribonucleoprotein delivery. Physiol Plant. 164(4): 378-384.

Ansari, W.A., Chandanshive, S.U., Bhatt, V., Nadaf , B.A., Vats, S., Katara, J.L., Humira, S.H. and Deshmukh, R. (2020). Genome editing in cereals: Approaches, applications and challenges. International Journal Molecular Sciences. 21(11): 4040. doi: 10.3390/ijms21114040.

Ariga, H., Toki, S., Ishibashi, K. (2020). Potato Virus X vector mediated DNA-free genome editing in plants. Plant Cell Physiol. pcaa123 (in press). doi: 10.1093/pcp/pcaa123/5912940.

Arora, L. and Narula, A. (2017). Gene editing and crop improvement using CRISPR-Cas9 system. Front Plant Sci. 8: 1932. https://doi.org/10.3389/fpls.2017.01932.

Ashokkumar, S., Jaganathan, D., Ramanathan, V., Rahman, H., Palaniswamy, R., Kambale, R., Muthurajan, R. (2020). Creation of novel alleles of fragrance gene OsBADH2 in rice through CRISPR/Cas9 mediated gene editing. Plos One. 15(8): 1-18.

Barrangou, R. and Marraffini, L.A. (2014). CRISPR-Cas systems: Prokaryotes upgrade to adaptive immunity. Mol Cell. 54: 234-44.

Barrangou, R. (2013). CRISPR-Cas systems and RNA-guided interference. Wiley Interdisciplinary Rev: RNA. 4: 267-78.

Bao, A., Burritt, D.J., Chen, H., Zhou, X., Cao, D., Tran, L.P. (2019). The CRISPR/Cas9 system and its applications in crop genome editing. Critical Rev in Biotech. 39(3): 321-336.

Bao, A., Tran, L.S.P., Cao, D. (2020). CRISPR/Cas9-based gene editing in soybean. Methods in Molecular Biology. 2107: 349-364.

Brooks, C., Nekrasov, V., Lippman, Z.B. (2014). Eck, J.V. Efficient gene editing in tomato in the first generation using the clustered regularly interspaced short palindromic repeats/ CRISPR-associated9 system. Plant Physiology. 166(3): 1292-1297.

Butler, N.M., Baltes, N.J., Voytas, D.F. and Douches, D.S. (2016). Geminivirus-mediated genome editing in potato (*Solanum tuberosum* L.) usingsequence-specific nucleases. Front. Plant Sci. 7: 1045. https://doi.org/10.3389/fpls.2016.01045.

Cai, Y., Chen, L., Liu, X., Sun, S., Wu, C., Jiang, B., Hou, W. (2015). CRISPR/Cas9-mediated genome editing in soybean hairy roots. PLoS One. 10: e0136064.

Cai, Y., Chen, L., Zhang, Y., Yuan, S., Su, Q., Sun, S., Wu, C., Yao, W., Han, T., Hou, W. (2020). Target base editing in soybean using a modified CRISPR/Cas9 system. Plant Biotechnology Journal. 18(10): 1996-1998.

Carrijo, J., Illa-Berenguer, E., LaFayette, P., Tottes, N., Aragao, F.J.L., Parroott, W., Vianna, G.R. (2021). Two efficient CRISPR/Cas9 systems for gene editing in soybean. Transgenic Research. 30(3): 239-249.

Chattopadhyay, T., Hazra, P., Akhtar, S., Maurya, D., Mukherjee, A., Roy, S. (2021). Skin colour, carotenogenesis and chlorophyll degradation mutant alleles: Genetic orchestration behind the fruit colour variation in tomato. Plant Cell Reports. 140(5): 767-782.

Chen, Y., Fu, M., Li, H., Wang, L., Liu, R., Liu, Z., Zhang, X., Jin, S. (2021). High-oleic acid content nontransgenic allotetraploid cotton (*Gossy piunhirsutum* L.) generated by knockout of GhFAD2 gene with CRISPR/Cas9 system. Plant Biotechnology Journal. 19(3): 424-426.

Chen, L., Li, W., Katin-Grazzini, L. *et al.* (2018). A method for the production and expedient screening of CRISPR/Cas9-mediated non-transgenic mutant plants. Hortic Res. 5: 13. https://doi.org/10.1038/s41438-018-0023-4.

Cody, W.B. and Scholthof, H.B. (2019). Plant virus vectors 3.0: Transitioning into synthetic genomics. Annu Rev Phytopathol. 57: 211-230.

Costa, J.R., Bejcek, B.E., McGee, J.E., Fogel, A.I., Kyle, R., Brimacombe, M.S., Ketteler, R. (2017). Genome editing using engineered nucleases and their use in genomic screening. The Assay Guidance Manual.

Danilo, B., Perrot. L., Mara, K. *et al.* (2019). Efficient and transgene-free gene targeting using Agrobacterium-mediated delivery of the CRISPR/Cas9 system in tomato. Plant Cell Rep. 38(4): 459-462.

Dong, O.X., Yu, S., Jain, R., Zhang, N., Duong, P.Q., Butler, C., Li, Y., Lipzen, A., Martin, J.A., Barry, K.W., Schmutz, J., Tian, L., Ronald, P.C. (2020). Marker-free carotenoid enriched rice generated through targeted gene insertion using CRISPR-Cas9. Nature Communications. 11(1): 1178. doi: 10.1038/s41467-020-14981-y.

Feng, C., Yuan, J., Wang, R., Liu, Y., Birchler, J.A., Han, F. (2016). Efficienttargeted genome modification in maize using CRISPR/Cas9 system. Journal of Genetics and Genomics. 43(1): 37-43.

Feng, Z., Zhang, B., Ding, W., Liu, X., Yang, D.L., Wei, P. *et al*. (2013). Efficientgenome editing in plants using a CRISPR/Cas system. Cell Res. 23(10): 1229-1232.

Gao, H., Gadlage, M.J., Lafitte, H.R., Lenderts, B., Yang, M., Schroder, M., Farrell, J. *et al.* (2020). Superior field performance of waxy corn engineered using CRISPR-Cas9. Nature Biotechnology. 38(5): 579-581.

Gelvin, B.S. (2003). Agrobacterium-mediated plant transformation: The biology behind the "Gene-Jockeying" tool. Microbiol Mol Biol Rev. 67(1): 16-37.

Gil-Humanes, J., Wang, Y., Liang, Z., Shan, Q., Ozuna, C.V., Sánchez-León, S., Baltes, N. *et al.* (2017). High-efficiency gene targeting in hexaploid wheat using DNA replicons and CRISPR/Cas9. The Plant Journal. 89(6): 1251-1262. doi: 10.1111/tpj.13446.

Gonzalz, M.N., Massa, G.A. andersson, M. *et al*. (2019). Reduced enzymatic browning in potato tubers by specific editing of a polyphenol oxidase gene *via* ribonucleoprotein complexes delivery of the CRISPR/Cas9 system. Front Plant Sci. 10: 1649. https://doi.org/10.3389/fpls.2019.01649.

Gonzalez, M.N., Mass, G.A. andersson, M. andrea, C., Oneto, D., Turesson, H., Storani, L., Olsson, N., Falt, A.S., Hofvander, P., Feingold, S.E. (2021). Comparative potato genome editing: Agrobacterium tumefaciens-mediated transformation and protoplasts transfection delivery of CRISPR/Cas9 components directed to StPPO2 gene. Plant Cell, Tissue and Organ Culture (PCTOC). 145(2): 291-305.

He, Y. and Zhao, Y. (2020). Technological breakthroughs in generating transgene-free and genetically stable CRISPR edited plants. aBIOTECH. 1(1): 88-96.

Hillary, E.V. and Ceasar, A.S. (2019). Application of CRISPR/Cas9 genome editing system in cereal crops. The Open Biotechnology Journal. 13: 173-179 doi: 10.2174/1874070701913010173.

Honig, A., Marton, I., Rosenthal, M. *et al.* (2015). Transient expression of virally delivered meganuclease in planta generates inherited genomic deletions. Mol Plant. 8(8): 1292-1294.

Hu, J., Li, S., Li, Z. *et al.* (2019). A barley stripe mosaic virus based guide RNA delivery system for targeted mutagenesis in wheat and maize. Mol Plant Pathol. 20(10): 1463-1474.

Hu, C., Sheng, O., Deng, G., He, W., Dong, T., Yang, Q., Dou, T., Li, C., Gao, H., Liu, S., Yi, G., Bi, F. (2020). CRISPR/Cas9-mediated genome editing of MaACO1 (aminocyclopropane-1-carboxylate oxidase1) promotes the shelf life of banana fruit. Plant Biotechnology Journal. 19(4): 654-656.

Janga, M.R., Campbell, L.M. and Rathore, K.S. (2017). CRISPR/Cas9-mediatedtargeted mutagenesis in upland cotton (*Gossypium hirsutum* L.). Plant Mol. Biol. 94: 349-360.

Jiang, F. and Doudna, J.A. (2017). CRISPR-Cas9 Structures and Mechanisms. Annu Rev Biophysic. 46: 505-529.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M.H. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science. 337: 816-821.

Jouanin, A., Schaart, J.G., Boyd, L.A., Cockram, J, Leigh, F.J., Bates, R., Wallington, E.J., Visser, R.G.F and Smulders, M.J. (2019). Outlook for coeliac disease patients: towards bread wheat with hypoimmunogenic gluten by gene editing of α and γ-gliadin gene families. BMC Plant Biology. 19(1): 1-16, doi: 10.1186/s12870-019-1889-5.

Kim, H., Choi, J., Won, K.H.A. (2020). Stable DNA-free screening system for CRISPR/RNPs-mediated gene editing in hot and sweet cultivars of *Capsicum annuum*. BMC Plant Biol. 20(1): 449. DOI: https://doi.org/10.1186/s12870-020-02665-0.

Koonin, E.V. and Krupovic, M. (2014). Evolution of adaptive immunity from transposable elements combined with innate immune systems. Nat Rev Genet. 16(3): 184-192.

Kumari, V., Kumawat, P., Yeri, S., Rajput, S.S. (2021). CRISPR-Cas9: A genome editing tool in crop plants: A review. Agricultural Reviews. Doi: 10.18805/ag.

Kumari, V. and Kumawat, P. (2021). Domestication to *De novo* domestication: Step towards gene editing revolution. Agricultural Mechanization in Asia, Africa and Latin America. 52(3): 1-26.

Lacchini, E., Kiegle, E., Castellani, M., Adam, H., Jouannic, S., Gregis, V. and Kater, M.M. (2020). CRISPR-mediated accelerated domestication of African rice landraces. PLoS One. 15(3): 1-12.

LeBlanc, C., Zhang, F., Mendez, J., Lozano, Y., Chatpar, K., Irish, V.F. and Jacob, Y. (2018). Increased efficiency of targeted mutagenesis by CRISPR/Cas9 in plants usingheat stress. The Plant Journal. 93(2): 377-386.

Li, X., Wang, Y., Chen, S., Tian, H., Fu, D., Zhu, B., Luo, Y., Zhu, H. (2018). Lycopene is enriched in tomato fruit by CRISPR/Cas9-mediated multiplex genome editing. Frontiers in Plant Science. 9: 559. https://doi.org/10.3389/fpls.2018.00559.

Liang, Z., Zhang, K., Chen, K., Gao, C. (2014). Targeted mutagenesis in *Zea mays* using TALENs and the CRISPR/Cas system. Journal of Genetics and Genomics. 41(2): 63-68.

Liang, Z., Chen, K., Zhang, Y. *et al*. (2018). Genome editing of bread wheat using biolistic delivery of CRISPR/Cas9 *in vitro* transcripts or ribonucleoproteins. Nat Protoc. 13(3): 413-430.

Liu, W., Rudis, M.R., Cheplick, M.H. *et al*. (2020). Lipofection-mediated genome editing using DNA-free delivery of the Cas9/gRNA ribonucleoprotein into plant cells. Plant Cell Rep. 39(2): 245-257.

Liu, Q., Yang, F., Zhang, J., Liu, H., Rahman, S., Islam, S., Ma, W. and She. M. (2021). Application of CRISPR/Cas9 in crop quality improvement. International Journal Molecular Sciences. 22: 4206. doi.org/10.3390/ijms22084206.

Liu, H. and Zhang, B. (2020). Virus-based CRISPR/Cas9 genome editing in plants. Trends Genet. 36(11): 810-813.

Liu, Q., Yang, F., Zhang, J., Liu, H., Rahman, S., Islam, S., Ma, W., She, M. (2021). Application of CRISPR/Cas9 in crop quality improvement. International Journal Molecular Sciences. 22: 4206. doi: 10.3390/ijms22084206.

Ma, X., Zhang, X., Liu, H. *et al*. (2020). Highly efficient DNA-free plant genome editing using virally delivered CRISPRCas9. Nat Plants. 6(7): 773-779.

Malzahn, A., Lowder, L., Qi, Y. (2017). Plant genome editing with TALEN and CRISPR. Cell Biosci. 7, 21.

Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E., Norville, J.E., Church, G.M. (2013). RNA-guided human genome engineering via Cas9. Science. 339(6121): 823-826.

Mao, Y., Botella, J.R., Liu, Y. *et al.* (2019). Gene editing in plants: Progress and challenges. National Science Review. 6(3): 421-437.

Mao, Y., Zhang, H., Xu, N., Zhang, B., Gou, F. and Zhu, J.K. (2013). Application ofthe CRISPR-Cas system for efficient genome engineering in plants. Molecular Plant. 6(6): 2008-2011.

Marton, I., Zuker, A., Shklarman, E. *et al*. (2010). Nontransgenic genome modification in plant cells. Plant Physiol. 154(3): 1079-1087.

Murovec, J., Gucek, K., Bohanec, B. *et al.* (2018). DNA-free genome editing of *Brassica oleracea* and B. rapa protoplasts using CRISPR-Cas9 ribonucleoprotein complexes. Front Plant Sci. 9, 1594. https://doi.org/10.3389/fpls.2018.01594.

Nishimasu, H., Ran, F.A., Hsu, P.D., Konermann, S., Shehata, S.I., Dohmae, N., Ishitani, R., Zhang, F., Nureki, O. (2014). Crystal structure of Cas9 in complex with guide RNA and target DNA. Cell. 156(5): 935-949.

Park, J., Choi, S., Park, S. *et al.* (2019). DNA-free genome editing via ribonucleoprotein (RNP) delivery of CRISPR/Cas in lettuce. Methods Mol Biol. 1917: 337-354.

Park, J. and Choe, S. (2019). DNA-free genome editing with preassembled CRISPR/Cas9 ribonucleoproteins in plants. Transgenic Res. 28: 61-64.

Peng, R., Lin, G., Jinming, L. (2016). Potential pitfalls of CRISPR/Cas9-mediated genome editing. The FEBS Journal. 283: 1218-1231.

Rath, D., Amlinger, L., Rath, A., Lundgren, M. (2015). The CRISPR-Cas immune system: Biology, mechanisms and applications. Biochimie. 117: 119-128.

Sant'Ana, R.R.A., Caprestano, C.A., Nodari, R.O. *et al*. (2020). PEG-delivered CRISPR-Cas9 ribonucleoproteins system for gene-editing screening of maize protoplasts. Genes (Basel). 11(9): 1029. https://doi.org/10.3390/genes11091029.

Sandhya, D., Jogam, P., Allini, V.R. *et al.* (2020). The present and potential future methods for delivering CRISPR/Cas9 components in plants. J. Genet Eng Biotechnol. 18(1): 25. doi: 10.1186/s43141-020-00036-8.

Schiml, S., Fauser, F., Puchta, H. (2014). The CRISPR/Cas9 system can be used as nuclease for in planta gene targeting and as paired nickases for directed mutagenesis in Arabidopsis resulting in heritable progeny. The Plant Journal. 80(6): 1139-1150.

Shen, C., Que, Z., Xia, Y., Tang, N., Li, D., He, R., Cao, M. (2017). Knock out of the annexin gene OsAnn3 via CRISPR/Cas9-mediated genome editing decreased cold tolerance in rice. Journal of Plant Biology. 60: 539-547.

Sorek, R., Lawrence, C.M., Wiedenheft, B. (2013). CRISPR-mediated adaptive immune systems in bacteria and archaea. Annu Rev Biochem. 82: 237-66.

Sun, S.K., Xu, X.J., Tang, Z., Tang, Z., Huang, X.Y., Wirtz, M., Hell, R., Zhao, F.J. (2021). A molecular switch in sulfur metabolism to reduce arsenic and enrich selenium in rice grain. Nature Communications. 12(1): 1392. DOI: 10.1038/s41467-021-21282-5.

Toda, E., Koiso, N., Takebayashi, A. *et al.* (2019). An efficient DNA-and selectable-marker-free genome-editing system using zygotes in rice. Nat Plants. 5(4): 363-368.

Veillet, F., Perrot, L., Chauvin, L. *et al*. (2019). Transgene-free genome editing in tomato and potato plants using Agrobacterium-mediated delivery of a CRISPR/Cas9 cytidine base editor. IJMS. 20(2): 402. https://doi.org/10.3390/ijms20020402.

Vu, T.V., Sivankalyani, V., Kim, E.J., Doan, D.T.H., Tran, M.T., Kim, J., Sung, Y.W., Park, M., Kang, Y.J., Kim, J.Y. (2020). Highly efficient homology-directed repair using CRISPR/Cpf1-geminiviral replicon in tomato. Plant Biotechnology Journal. 18(10): 2133-2143.

Wang, P., Zhao, F.J., Kopittke, P.M. (2019). Engineering crops without genome integration using nanotechnology. Trends Plant Sci. 24(7): 574-577.

Wang, R., Lammers, M., Tikunov, Y., Bovy, A.G., Angenent, G.C., de Maagd, R.A. (2020). The rin, nor and Cnr spontaneous mutations inhibit tomato fruit ripening in additive and epistatic manners. Plant Science. 294: 110436.

Wang, J., Wu, H., Chen, Y., Yin, T. (2020a). Efficient CRISPR/Cas9- mediated gene editing in an interspecific hybrid poplar with highly heterozygous genome. Frontiers in Plant Sciences. 11, 996. https://doi.org/10.3389/fpls.2020.00996.

Wang, W., Pan, Q., He, F., Akhunova, A., Chao, S., Trick, H. and Akhunov, E. (2018). Tran generational CRISPR-Cas9 activity facilitates multiplex gene editing in allopolyploid wheat. The CRISPR Journal. 1(1): 65-74. doi: 10.1089/crispr.2017.0010.

Wang, W., Pan, Q., Tian, B., He, F., Chen, Y., Bai, G., Akhunova, A., Trick, H.N. and Akhunov, E. (2019). Gene editing of the wheat homologs of TONNEAU1-recruiting motif encoding gene affects grain shape and weight in wheat. The Plant Journal. 100(2): 251-264. doi: 10.1111/tpj.14440.

Wolter, F. and Puchta, H. (2017). Knocking out consumer concerns and regulator's rules: Efficient use of CRISPR/Cas ribonucleoprotein complexes for genome editing in cereals. Genome Biol. 18(1): 43. DOI: 10.1186/s13059-017-1179-1.

Wu, S., Zhu, H., Liu, J. *et al.* (2020). Establishment of a PEGmediated protoplast transformation system based on DNA and CRISPR/Cas9 ribonucleoprotein complexes for banana. BMC Plant Biol. 20(1): 425. DOI: https://doi.org/10.1186/s12870-020-02609-8.

Xu, R., Yang, Y., Qin, R., Li, H., Qiu, C., Li, L., Wei, P., Yang, J. (2016). Rapid improvement of grain weight via highly efficient CRISPR/Cas9-mediated multiplex genome editing in rice. Journal of Genetics and Genomics. 43(8): 529-532.

Xu, Y., Lin, Q., Li, X., Wang, F., Chen, Z., Wang, J., Li, W., Fan, F., Tao, Y., Jiang, Y., Wei, X., Zhng, R., Zhu, Q.H., Bu, Q., Yang, J., Gao, C. (2020). Fine-tuning the amylose content of rice by precise base editing of the Wx gene. Plant Biotechnology Journal. 19(1): 11-13.

Yang, T., Deng, L., Zhao, W., Zhang, R., Jiang, H., Ye, Z., Li, C.B.; Li, C. (2019b). Rapid breeding of pink-fruited tomato hybrids using the CRISPR/Cas9 system. Journal of Genetics and Genomics. 46(10): 505-508.

Yin, K., Han, T., Xie, K., Zhao, J.;, Song, J., Liu, Y. (2019). Engineer complete resistance to Cotton Leaf Curl Multan virus by the CRISPR/Cas9 system in Nicotiana benthamiana Phytopathology Research. 1(1): 1-9.

Yuyu, C., Aike, Z., Pao, X., Xiaoxia, W., Yongrun, C., Beifang, W., Yue, Z., Liaqat, S., Shihua, C., Liyong, C., Yingxin, Z. (2020). Effects of GS3 and GL3.1 for grain size editing by CRISPR/Cas9 in rice. Rice Science. 27(5): 405-413.

Zhang, T., Zheng, Q., Yi, X., An, H., Zhao, Y., Ma, S. and Zhou, G. (2018). Establishing RNA virus resistance in plants by harnessing CRISPR immune system. Plant Biotechnology Journal. 16(8): 1415-1423.

Zhang, Y., Bai, Y., Wu, G., Zou, S., Chen, Y. and Gao, C., Tang, D. (2017). Simultaneous modification of three homoeologs of TaEDR1 by genome editing enhances powdery mildew resistance in wheat. The Plant Journal. 91(4): 714-724. doi: 10.1111/tpj.13599.

Zhou, J., Wang, G., Liu, Z. (2018). Efficient genome editing of wild strawberry genes, vector development and validation. Plant Biotechnology Journal. 16(11): 1868-1877.

Zhu, J., Song, N., Sun, S., Yang, W., Zhao, H., Song, W., Lai J. (2016). Efficiency and inheritance of targeted mutagenesis in maize using CRISPR-Cas9. Journal of Genetics and Genomics. 43(1): 25-36.

Zhang, Z., Ge, X., Luo, X., Wang, P., Fan, Q., Hu, G. *et al*. (2018). Simultaneous editing of two copies of Gh14-3-3d confers enhanced transgene-clean plantdefense against *Verticillium dahliae* in allotetraploid upland cotton. Front. Plant Sci. 842. doi: 10.3389/fpls.2018.00842.