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CRISPR: Next-generation tool used in plant biotechnology

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Abstract
Genomic editing is the modification of the genomic DNA of an organism at a specific place with the aim of inserting, deleting or replacing one or more nucleotides resulting in the activation or inactivation of a gene and the acquisition of new characteristics. The system derived from prokaryotic cells, CRISPR (clustered regularly interspaced short palindromic repeat) - CAS (CRISPR-associated nuclease) has the ability to revolutionize the way genetic modifications are made. The genetic modification technique CRISPR is extremely promising and versatile for ensuring a productive and sustainable agriculture to feed the rapidly growing population. Through this tool, more and more plants have been subjected to gene editing, this technique is gaining momentum in the field of plant biotechnology. Even though the CRISPR method was developed later than other gene editing methods such as Zinc Finger Nucleases (ZFNs) and Transcription activator-like effector nucleases (TALENs), CRISPR has become a more popular method, being increasingly used and more successful than other gene editing methods due to its precision. Due to the applicability of the CRISPR-CAS system, being able to sequence the genome of living cells in various species, the ease of application of these methods as well as the economic efficiency, the CRISPR-CAS system has changed the way genome editing is done, having an influence in different fields research as well as in the field of medicine. In recent years (over a decade), with the development of the CRISPR genetic editing system, different types of nucleases associated with this system, such as Cas9, Cas12 and Cas13, have been discovered, each being used in different scenarios. The main objective of this review is to provide an overview of the CRISPR system, how it works and its application in plant biotechnologies.

Keywords: CRISPR, Biotechnology, Plants, Genetic engineering

Introduction

A variety of products offered by plants, such as food, medicinal products and other products, have a very important role in human life and beyond. Plant traits can be improved through genetic modification as well as through plant breeding [26]. Through the classic method of plant modification, a gene is randomly integrated into the plant's genome, the results having low predictability and unexpected variations. Gene editing technology can modify the genome very precisely, producing deletions, insertions or replacements of DNA sequences [14]. Genetic engineering of plants can be done using different methods, such as Zinc Finger Nucleases (ZFNs) being an artificial nuclease. Transcription activator-like effector nucleases (TALENs) is a more flexible engineered nuclease, and CRISPR (Clustered Regularly Interspaced Short Palindromic Repeat) is a technology with a simpler and more flexible nuclease to engineer [29,26].

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeat) is a vast class of short repeat sequences which are found in prokaryotes, including bacteria and archaea. These sequences are complementary with foreign DNA such as virus DNA. The bacteria infected with viruses produce this type of DNA that binds to the virus DNA, the Cas (Crispr-associated nucleases) enzyme cleaves the invaded

DNA into pieces. The CRISPR array is included in both chromosomal and plasmid DNA [25]. The CRISPR/Cas system is a defensive mechanism of prokaryotes against viruses. Even if the CRISPR/Cas system is an acquired immune system of prokaryotes, it can also be used as a tool in the genetic editing of different organisms [32].

In the year 1987, a team of researchers observed a repetitive segment of a neighbouring bacterial gene. Many researchers thought that these types of segments were junk [19]. CRISPR was first time detected in 1993 in archaea, especially in *Haloferax mediterranei* [15]. In 2005 three groups reported that the segments matched the sequences of phages or plasmids which indicates the possible role that CRISPR has in immunity [19].

CRISPR/Cas system mechanisms

The CRISPR-Cas system has 3 stages that has to follow to function. The first stage is adaptation, in this stage, new spacers are inserted from exogenous nucleic acid in the CRISPR locus. The second stage is expression, this stage is focused on expressing the cas genes and biogenesis of CRISPR RNA (crRNA), in which the CRISPR array is processed into small interference crRNA. The third and final stage is targeting, In this stage, the complex crRNA and Cas nucleases recognise the target nucleic acid and cleave at a specific homologous sequence [25,6].

The stages that the CRISPR-Cas system follows are represented in Figure 1. Which was inspired by 2 models from 2 different papers: Rath D, Amlinger L, Rath A, Lundgren M., (2015) The CRISPR-Cas immune system: Biology, mechanisms and applications; and Barrangou R, Marraffini LA. (2014), CRISPR-cas systems: Prokaryotes upgrade to adaptive immunity.

Classification of the CRISPR system

The CRISPR/Cas family is distinguished into 2 classes (class 1 and 2) , 6 types and over 30 subtypes, this classification is based on several criteria [32,13].

The main difference between Class 1 and Class 2 is that in Class 1, the functional complex is composed of multiple Cas proteins that bind with crRNA and cleave the target DNA sequences together, while Class 2 uses a single protein to function [32,13].

CRISPR class 1

The Crispr class 1 is classified into 3 types: type I, type III and type IV [32]. The effect complexes have an elaborated architecture composed of multiple Cas proteins [21].

Type I is divided into 7 subtypes (I-A, I-B, I-C, I-D, I-E, I-F, and I-U). The Cas 3 gene (or its Cas3 variants) is encoded into all type I loci which encodes single-stranded DNA (ssDNA) [21].

The type III system contains a cas10 gene signature, which encodes a multi-domain protein containing a variant of RNA recognition motif that is homologous to the nucleic acid polymerases and cyclases being the largest subunit in the effector complexes [21].

The Type IV CRISPR system lacks the adaptation encoding genes (Cas1, Cas 2 and Cas 4) and is divided in IV-A, IV-B and IV-C. All subtypes have coding genes for Csf2 (Cas 7), Csf3 (Cas 5) and Csf1 to form the complex [33].

CRISPR class 2

The Crispr class 2 system is divided into 3 types: type II, type V and type VI. This class requires only one large protein to function as the complex composed of the multiple proteins in class 1 [32].

Type II system is the most different type from type I and III, having the least number of genes [21]. This type uses the protein Cas9 which is composed of two subunits responsible for cutting the target DNA, the NHN nuclease is inserted in the RuvC-like domain sequence [22]. The CRISPR type II loci also contain genes for the proteins Cas1 and Cas2. Type II system is classified into 3 subtypes (subtype II-A, II-B and II-C) [24].

Type V system uses the protein Cas12a, the CRISPR array is composed of Cas12a, Cas4, Cas1, Cas2. The protein Cas12a is similar in shape and size to Cas9. Cas12a contains a RuvC-like nuclease domain similar to the Cas9 domain. However, Cas12a does not have an HNH nuclease domain which is present in Cas9 proteins [28, 21].

Type VI complex contains Cas13 with its derivates (Cas13a, Cas13B, Cas13c and Cas13d). the difference between Cas13 and other CRISPR-cas systems is that Cas13 targets single-stranded RNA [14]. All effectors from this group have the HEPN domain as the recognizable feature, being unrelated to type II and type V effectors [22].

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The CRISPR system is divided in two classes, each class having three types. Class 1 contains type I, type III, and Type IV, and class 2 contains type II, type V, and type VI. Each type uses one or more Cas (CRISPR associates proteins) units. This classification is represented in Figure 2, which is inspired by the model from the research paper Gostimskaya I., CRISPR–Cas9: A History of Its Discovery and Ethical Considerations of Its Use in Genome Editing.

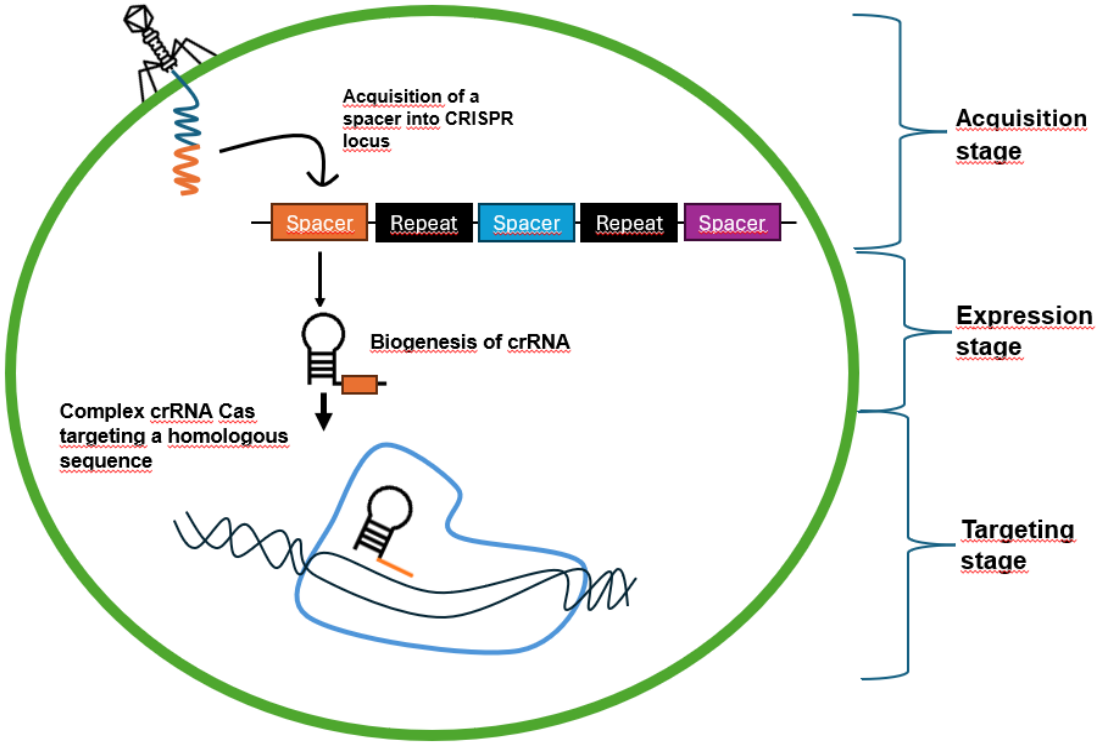

Figure 1. The 3 stages that the CRISPR system follows

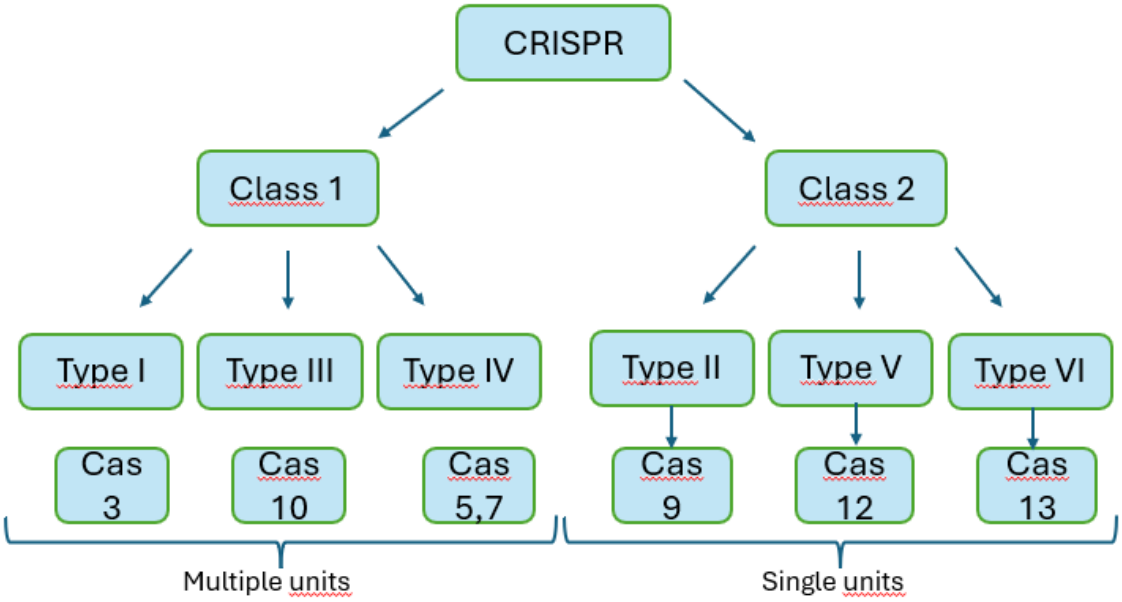

Figure 2. Classification of CRISPR system

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CRISPR application in plants

The CRISPR/Cas9 system, since it was recognized as a cutting-edge tool for genome editing, has been successfully applied in many plant species. This includes model plants such as *Arabidopsis thaliana, Nicotiana benthamiana, and Nicotiana tabacum* as well as in crops such as rice, soybean, maize, tobacco, potato, wheat, sorghum, tomato, poplar, apple, grapes, banana, etc; medicinal plants such as *Salvia. miltiorrhiza, Dendrobium officinale, Cannabis sativa* and ornamental plants namely *Petunia, Chrysanthemum and Phalaenopsis orchid*, etc [8, 14, 29, 7].

CRISPR/Cas9 application on crops

CRISPR/Cas9 is a powerful tool to improve crops by being simple, efficient, and highly specific. The biotic and abiotic stresses are greatly affecting the crops, making it more difficult for the plants to develop. The CRISPR/Cas9 system can improve these crops to better tolerate different types of stresses for the benefit of humans.

Plant susceptibility (S) and resistance (R) genes must be identified and functionally annotated in order to use genome editing to improve disease resistance. Due to their dual functions in plant physiology and pathogen susceptibility, many S genes are often crucial for the host's survival. While deactivating these genes frequently results in resistance to disease, it also has pleiotropic effects such as reduced plant development, abnormal phenotypes, and heightened vulnerability to abiotic stresses and other pathogenic agents [10].

Improvement in crop quality

Physical characteristics namely colour, texture, size and fragrance, are key factors in crop quality. Also, the internal quality is influenced by the content of nutrients (proteins, lipids, etc.) and by the bioactive substances (flavonoids, carotenoids, etc.) [18].

Tomatoes have different shapes and sizes, these traits can be altered with CRISPR/Cas9 technology by modifying the expression of OVATE, CLV, fas and lc, and ENO genes. OVATE and SUN genes are involved in controlling the symmetry of the fruits [20]. In Asia, the consumption of pink-coloured tomatoes is higher compared to that of red tomatoes, while other parts of the globe prefer the red tomatoes. The pink fruit has the decisive y (yellow) locus in chromosome 1 compared to the red-coloured tomato fruit which have the dominant Y allele [5]. Knocking out SlMYB12 results in the production of pink-coloured tomato fruits. Different coloured tomatoes can be produced namely yellow, pink and purple by targeting phytoene synthase 1(PSY1) MYB transcription factor 12 (MYB12), and Anthocianin 2 (ANT2) and Anthocianin 1 (ANT1) [20, 30].

Improvement of Crop Abiotic Stress Tolerance

Most of the plants have to support the harsh environmental conditions, suffering from different stresses. Abiotic stress refers to the stress produced by non-living factors such as extreme temperature (heat and cold), drought, oxidative stress, salinity, heavy metals, environmental pollution (soil pollution, water pollution, air pollution). These factors have a negative impact on plant growth, development, yield and the quality of crops. This type of stress caused to plants can lead to a 50% crop yield reduction [32, 8, 18, 9, 17].

Global warming is causing more drought stress on crops with a major loss of productivity. The stress caused by drought is causing yield losses between 30% and 90%, the effect is different for each species [12, 17]. CRISPR/Cas9 has been used in rice to modify OsERA1 which showed a big improvement in drought stress tolerance. In wheat, the genes TaDREB2 and TaERF3 edited with CRISPR showed an improvement in drought resistance. The genes SRL1 AND SRL2 edited with CRISPR/Cas9 in rice achieved a phenotype with curled leaves and drought tolerance. Using CRISPR/Cas9 to edit GID1, tomato plants with a high leaf water content in a drought environment were created, resulting in plants with increased drought resistance [17].

Zeng et. al. research showed that by editing 3 genes, OsPIN5b, GS3 and OsMYB30, simultaneously and individually in wild rice, obtaining several mutants, all mutants showed a high yield and with better low-temperature tolerance [31].

A research study demonstrated that the salinity stress is connected with oxidative stress. They were able to edit the gene OsbHLH024 with CRISPR, creating a mutant rice phenotype with a salinity tolerance by negatively regulating the function of Na^+ and K^+ transporter genes. The new phenotype ion

homeostasis of Na^+ and K^+ resulted in a higher tolerance and antioxidants control ROS by suppressing the high accumulation of H_2O_2 and MDA [2].

Improvement of Crop Biotic Stress Tolerance

Biotic stress factors such as viruses, bacteria, fungus, weeds and insects cause more than 42% losses of the global crop yield (15% attributable to insects, 13% to weeds, and 13% to other pathogens) [4].

Fungal pathogens cause Powdery mildew which is a common disease in many species. Mildew resistance Locus (mlo) genes are responsible to the response of the plant to this disease. Tomatoes have 16 mlo genes (SlMlo1 to SlMlo16) SlMlo1 has the highest contribution to Powdery Mildew susceptibility. Tomato plants modified with CRISPR where the gene SlMlo1 is knocked out are fully resistant to Powdery Mildew disease [32].

CRISPR/Cas9 can be used to improve bacterial resistance in plants. In rice plants, targeting the gene OsSWEET13 results in phenotypes with mutations (knock-out), the plant has resistance to bacterial blight [17]. Other studies showed that by using CRISPR to target DIPM-1, DIPM-2 and DIPM-4 to induce mutations is increasing the resistance to fire blight disease in apples [1].

Improvements in medicinal plants

Medicinal plants differentiate themselves from the other types of plants because of their chemical content, having different types of bioactive compounds and secondary metabolites. Until 2020 more than 30 medicinal plants have been edited with CRISPR/Cas9 system. The use of this system in medicinal plants is still limited [3].

Salvia miltiorrhiza is used in cardiovascular diseases, the roots consist of various bioactive compounds, the majority are grouped in hydrophilic and lipophilic compounds. A group of scientists in 2017 used CRISPR to edit *Salvia miltiorrhiza,* they knocked out the gene SmCPS1, this gene is involved in the biosynthesis of tanshinone. By knocking out this gene and analysing the mutants show that SmCPS1 can change the synthesis of secondary metabolites [14,3].

Dendrobium officinale has multiple pharmaceutical effects including cardioprotective, anti-tumour, gastrointestinal protective, antidiabetic, immunomodulatory, anti-ageing, and anti-osteoporosis [11]. In 2021, CRISPR/Cas9 was used to remove large genomic fragments of *Dendrobium officinale* terminal flower 1 (DOTFL1). The low expression of DOTFL1 makes plants bloom faster, formation of pseudobulbs and differentiation of inflorescence meristems [14].

Improvements in ornamental plants

The colour of the flower in ornamental plants is among the most important characteristics and is primarily produced by flavonoids, carotenoids, anthocyanins and betalains. In *Ipomea nil* Watanabe et al. used CRISPR system to edit the Carotenoid Cleavage Dioxygenase (CCD) gene which resulted in mutants with pale yellow petals [27, 16].

In the petunia cultivar "Mirage Rose," the ethylene biosynthesis enzyme coding gene 1 aminocyclopropane-1-carboxylate oxidase1 (PhACO1) was edited through CRISPR/Cas9. The transgenic petunias' flowers showed delayed senescence that was associated with decreased ethylene production [23].

Conclusions

The CRISPR/Cas system is a powerful tool in genome editing with precise gene editing through knock-out or knock-in of a gene, can induce insertions, deletions in a cell genome, replacement of a DNA sequence, gene regulations, point mutations and activation of gene expression. All the benefits that CRISPR genome editing has can be applied in the field of plant biotechnology by improving all types of plants, from crops (genetically modifying a species of plants to have a bigger production or to be resistant to stress), medicinal plants (modifying the synthesis of secondary metabolites), to ornamental plants (modifying the colour of the petals).

CRISPR (clustered regularly interspaced short palindromic repeat) is a relatively new method used in genome editing with a rapid spreading throughout the scientific community because of its simplicity and its versatility. It is recommended to study more this method to better understand the safety and effectiveness of this method and the impact that it can have on human life in different domains (food, medicine, pharmaceutical, etc).

References

[1] Ahmad S, Wei X, Sheng Z, Hu P, Tang S. (2018), CRISPR/Cas9 for development of disease resistance in plants: Recent progress, limitations and future prospects. Vol. 19, Briefings in Functional Genomics. Oxford University Press. p. 26–39.
[2] Alam MS, Kong J, Tao R, Ahmed T, Alamin M, Alotaibi SS, et al. (2022), CRISPR/Cas9 Mediated Knockout of the OsbHLH024 Transcription Factor Improves Salt Stress Resistance in Rice (Oryza sativa L.). Plants. 1;11(9).
[3] Alok A, Jain P, Kumar J, Yajnik K, Bhalothia P. (2020), Genome engineering in medicinally important plants using CRISPR/Cas9 tool. In: Genome Engineering via CRISPR-Cas9 System. Elsevier; p. 155–61.
[4] Balconi C, Stevanato P, Motto M, Biancardi E. (2012), Breeding for biotic stress resistance/tolerance in plants. In: Crop Production for Agricultural Improvement. Springer Netherlands; p. 57–114.
[5] Ballester AR, Molthoff J, de Vos R, Hekkert B te L, Orzaez D, Fernández-Moreno JP, et al. (2010), Biochemical and molecular analysis of pink tomatoes: Deregulated expression of the gene encoding transcription factor SLMYB12 leads to pink tomato fruit color. Plant Physiol. 152(1):71–84.
[6] Barrangou R, Marraffini LA. (2014), CRISPR-cas systems: Prokaryotes upgrade to adaptive immunity. Vol. 54, Molecular Cell. Cell Press; p. 234–44.
[7] Belhaj K, Chaparro-Garcia A, Kamoun S, Patron NJ, Nekrasov V. (2015), Editing plant genomes with CRISPR/Cas9. Vol. 32, Current Opinion in Biotechnology. Elsevier Ltd; p. 76–84.
[8] Bhat MA, Bhat MA, Kumar V, Wani IA, Bashir H, Shah AA, et al. (2020), The era of editing plant genomes using CRISPR/Cas: A critical appraisal. J Biotechnol. 20:324:34–60.
[9] Biswas D, Saha SC, Dey A. (2021), CRISPR-Cas genome-editing tool in plant abiotic stress-tolerance. Plant Gene. 1;26.
[10] Cardi T, Murovec J, Bakhsh A, Boniecka J, Bruegmann T, Bull SE, Eeckhaut T, Fladung M, Galovic V, Linkiewicz A, Lukan T, Mafra I, Michalski K, Kavas M, Nicolia A et al. (2023), CRISPR/Cas-mediated plant genome editing: outstanding challenges a decade after implementation. Vol. 28, Trends in Plant Science. Elsevier Ltd; p. 1144–65.
[11] Chen W, Lu J, Zhang J, Wu J, Yu L, Qin L, et al. (2021), Traditional Uses, Phytochemistry, Pharmacology, and Quality Control of *Dendrobium officinale* Kimura et. Migo. Vol. 12, Frontiers in Pharmacology. Frontiers Media S.A.
[12] Dietz KJ, Zörb C, Geilfus CM. (2021), Drought and crop yield. Vol. 23, Plant Biology. John Wiley and Sons Inc; p. 881–93.
[13] Gostimskaya I. (2022), CRISPR–Cas9: A History of Its Discovery and Ethical Considerations of Its Use in Genome Editing. Vol. 87, Biochemistry (Moscow). p. 777–88.
[14] Guo M, Chen H, Dong S, Zhang Z, Luo H., (2022), CRISPR-Cas gene editing technology and its application prospect in medicinal plants. Vol. 17, Chinese Medicine (United Kingdom). BioMed Central Ltd;
[15] Ishino Y, Krupovic M, Forterre P., (2018), History of CRISPR-Cas from encounter with a mysterious repeated sequence to genome editing technology. J Bacteriol. ;200(7).
[16] Khan Z, Razzaq A, Sattar T, Ahmed A, Habibullah Khan S, Zubair Ghouri M., (2021), Understanding floral biology for CRISPR-based modification of color and fragrance in horticultural plants. F1000Res. 11:854.
[17] Li Y, Wu X, Zhang Y, Zhang Q., (2022), CRISPR/Cas genome editing improves abiotic and biotic stress tolerance of crops. Vol. 4, Frontiers in Genome Editing. Frontiers Media S.A.
[18] Liu H, Chen W, Li Y, Sun L, Chai Y, Chen H, et al., (2022), CRISPR/Cas9 Technology and Its Utility for Crop Improvement. Vol. 23, International Journal of Molecular Sciences. MDPI.
[19] Liu L, Fan XD., (2014), CRISPR-Cas system: A powerful tool for genome engineering. Vol. 85, Plant Molecular Biology. Kluwer Academic Publishers; p. 209–18.
[20] Liu Q, Yang F, Zhang J, Liu H, Rahman S, Islam S, et al., (2021), Application of crispr/cas9 in crop quality improvement. Vol. 22, International Journal of Molecular Sciences. MDPI.
[21] Makarova KS, Wolf YI, Alkhnbashi OS, Costa F, Shah SA, Saunders SJ, et al. (2015), An updated evolutionary classification of CRISPR-Cas systems. Nat Rev Microbiol.;13(11):722–36.

[22] Makarova KS, Wolf YI, Iranzo J, Shmakov SA, Alkhnbashi OS, Brouns SJJ, et al. (2020), Evolutionary classification of CRISPR–Cas systems: a burst of class 2 and derived variants. Vol. 18, Nature Reviews Microbiology. Nature Research; p. 67–83.
[23] Mekapogu M, Song HY, Lim SH, Jung JA., (2023), Genetic Engineering and Genome Editing Advances to Enhance Floral Attributes in Ornamental Plants: An Update. Vol. 12, Plants. Multidisciplinary Digital Publishing Institute (MDPI);
[24] Nidhi S, Anand U, Oleksak P, Tripathi P, Lal JA, Thomas G, et al., (2021), Novel crispr–cas systems: An updated review of the current achievements, applications, and future research perspectives. Vol. 22, International Journal of Molecular Sciences. MDPI AG.
[25] Rath D, Amlinger L, Rath A, Lundgren M., (2015) The CRISPR-Cas immune system: Biology, mechanisms and applications. Vol. 117, Biochimie. Elsevier B.V.; p. 119–28.
[26] Sandhya D, Jogam P, Allini VR, Abbagani S, Alok A., (2020), The present and potential future methods for delivering CRISPR/Cas9 components in plants. Vol. 18, Journal of Genetic Engineering and Biotechnology. Springer;
[27] Sirohi U, Kumar M, Sharma VR, Teotia S, Singh D, Chaudhary V, et al., (2022), CRISPR/Cas9 System: A Potential Tool for Genetic Improvement in Floricultural Crops. Vol. 64, Molecular Biotechnology. Springer; p. 1303–18.
[28] Tang Y, Fu Y., (2018), Class 2 CRISPR/Cas: An expanding biotechnology toolbox for and beyond genome editing 06 Biological Sciences 0604 Genetics. Vol. 8, Cell and Bioscience. BioMed Central Ltd.
[29] Wada N, Ueta R, Osakabe Y, Osakabe K., (2020), Precision genome editing in plants: State-of-the-art in CRISPR/Cas9-based genome engineering. Vol. 20, BMC Plant Biology. BioMed Central Ltd.; 2020.
[30] Wang T, Zhang H, Zhu H., (2019), CRISPR technology is revolutionizing the improvement of tomato and other fruit crops. Vol. 6, Horticulture Research. Nature Publishing Group.
[31] Zeng Y, Wen J, Zhao W, Wang Q, Huang W. (2020), Rational Improvement of Rice Yield and Cold Tolerance by Editing the Three Genes OsPIN5b, GS3, and OsMYB30 with the CRISPR–Cas9 System. Front Plant Sci.
[32] Zhang D, Zhang Z, Unver T, Zhang B., (2021), CRISPR/Cas: A powerful tool for gene function study and crop improvement. Vol. 29, Journal of Advanced Research. Elsevier B.V.; p. 207–21.
[33] Zhou Y, Bravo JPK, Taylor HN, Steens JA, Jackson RN, Staals RHJ, et al. (2021), Structure of a type IV CRISPR-Cas ribonucleoprotein complex. iScience.24(3).