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Applications of CRISPR/Cas9-based genome editing in the plant biology

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Abstract: Clustered regularly interspaced short palindromic repeats/CRISPR-associated proteins (CRISPR/Cas) is an acquired immune system found in bacteria and archaea that can specifically silence or degrade a foreign single or double strand nucleic acid to protect it from infection. In recent years, the CRISPR/Cas9 system has rapidly been evolved into a genome editing technology, in which the Cas9 endonuclease can be targeted to specific DNA sequences by guide RNAs (gRNAs) that are easily programmable. Due to simplicity, specificity and high efficiency, CRISPR/Cas9 is gradually replacing other gene editing technologies and has been implemented in basic and applied plant sciences to boost yield, regulate metabolic process, and increase stress resistance in different varieties. In current review, we introduced its application scope in scientific research and practical application. We summarized the procedure of target plant generation by CRISPR/Cas9 method. We mainly reviewed the applications of CRISPR/Cas9 and its recent advances in model plants and other crop plants, attempting to provide a related general information to researchers. Further, we also included the inadequacies and concerns of CRISPR/Cas9 that have emerged so far.

Key words: CRISPR/Cas9, applications, model plants, plant biology

1. Introduction

Over the past years, engineered nucleases, including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats/CRISPR-associated proteins (CRISPR/Cas) have provided a much simpler and more economical method for gene-targeted modification in both animals and plants. However, due to ZFNs and TALENs having their own limitations, for example requirement of many protein engineering steps, their application in gene editing in either animals or plants is limited. In comparison, the simplicity, specificity, and high efficiency of the CRISPR/Cas system have made it an increasingly popular genetic engineering tool to modify target genes for biological applications.

As a part of the adaptive immune system, the CRISPR/Cas system was first observed in bacteria in 1987 (Ishino et al., 1987); however, as a gene editing tool, the CRISPR/Cas system was independently reported in 2013 (Cong et al., 2013; Hwang et al., 2013; Jiang et al., 2013a; Jinek et al., 2013; Mali et al., 2013). In 2020, the Nobel Prize in Chemistry was awarded to Dr. Emmanuelle Charpentier and Dr. Jennifer Doudna for their pioneering work in accurate genome editing using CRISPR method. According to the Cas genes and the nature of the interference complex, CRISPR/Cas system has been divided into two classes (class 1 and class 2), which have been further subdivided into six types (types I to VI) based on their signature Cas genes (Koonin et al., 2017). At present, the type II CRISPR/Cas9, an RNA guided endonuclease protein encoded by *Streptococcus pyogenes*, has been rapidly developed in animal cells and plant cells (Figure 1), and has been widely used for biological science, medical science, and agricultural science (Figure 2) due to its high efficiency and precision. In general, gene modification by CRISPR/Cas9 is achieved by cutting the double strand DNA at target sites first and then repairing the double-stranded break (DSB) through nonhomologous end-joining (NHEJ) or homology-directed repair (HDR) mechanisms (Shalem et al., 2015).

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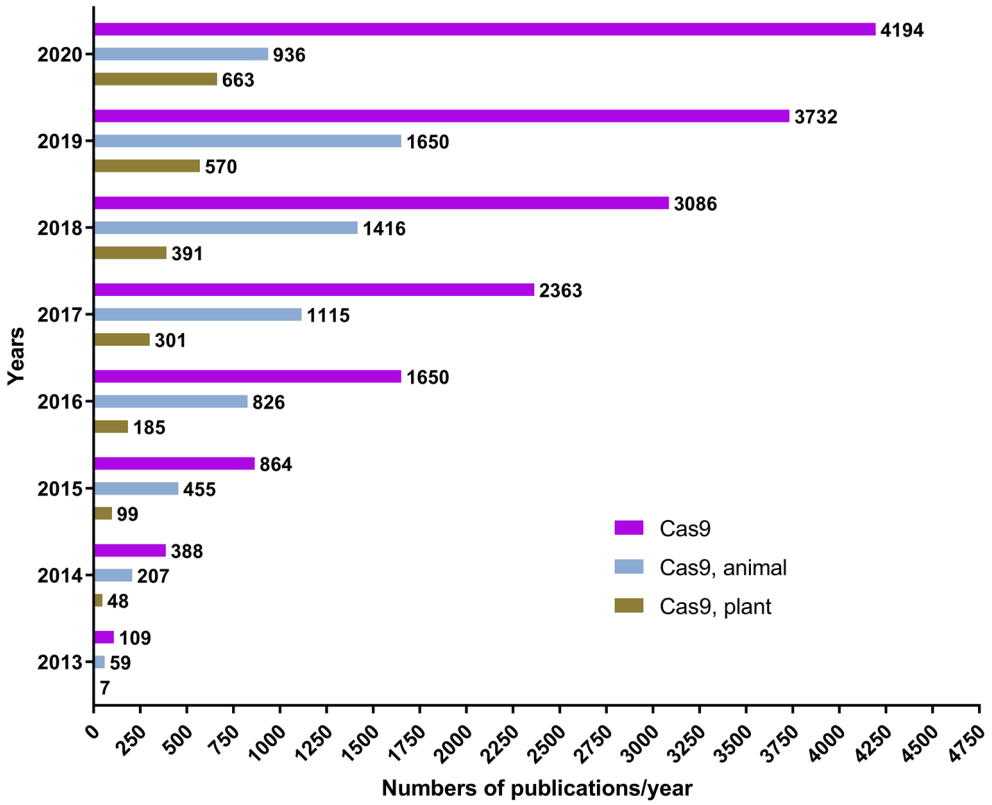

Figure 1. Number of published articles searched by key words "Cas9", "Cas9, animal" or "Cas9, plant" on PubMed.

In animal systems, ranging from cells (in vitro) to animals (in vivo), the CRISPR/Cas9 is successfully applied for gene editing, transcriptional regulation, epigenetic regulation, large-scale genetic screens, generation of animal models, genomic imaging, and lineage tracing (Wang and Qi, 2016). In the medical science field, CRISPR/Cas9 mediated gene editing has been increasingly used to study and even cure human diseases. For example, CRISPR/Cas9, in combination with induced pluripotent stem (iPS) cells, plays a key role in gene modification for creating disease models, producing biologic medical materials or the preparation of donor-specific tissues for transplantation (Cai et al., 2016). It also has been used for cancer gene therapy, such as lymphoma, leukemia, melanoma, and lung cancer (Zhan et al., 2019). In addition, CRISPR/Cas9 has been developed to block HIV integration into the host genome by disruption of a critical receptor gene (Kaminski et al., 2016), which provides a new strategy to treat infectious diseases. Moreover, the strategies and technical considerations of the CRISPR/Cas9 application in biological and biomedical science are well expounded in some review papers (Wang et al., 2017; Hsu et al., 2019).

In plant science and agricultural science, the CRISPR/Cas9 system is also becoming a widely utilized technology to modify the genomes of a diverse range of crop plants. In 2013, three independent groups published their studies in Nature Biotechnology, which first established the applications of CRISPR/Cas9-induced plant genome editing system in rice (*Oryza sativa*) and wheat (*Triticum aestivum*) (Shan et al., 2013), *Nicotiana benthamiana* (Nekrasov et al., 2013; Li et al., 2013), and *Arabidopsis thaliana* (Li et al., 2013). Since then, CRISPR/Cas9 has made it easier for plant breeders to control the specific introduction of targeted genome modifications to improve the quality of agricultural crops, which may strengthen sustainable agriculture practices and global food security.

Some reviews illuminated the developmental history and working mechanism of CRISPR/Cas9 mediated genome editing (Ma et al., 2014; Singh et al., 2016; Jiang and Doudna, 2017), others summarized the applications of CRISPR/Cas9 and its variants in plant biology, with major focus on the mechanism of action, system construction strategies, the screening methods used to identify mutants containing edited genes, and the other technical details, and some outlined future perspectives for CRISPR/Cas9, such as the applications in plant synthetic biology, domestication of wild plants, and so on (Bortesi and Fischer, 2015; Bao et al., 2019; Chen et al., 2019; Zhou et al., 2020b). This review does not intend to replace any

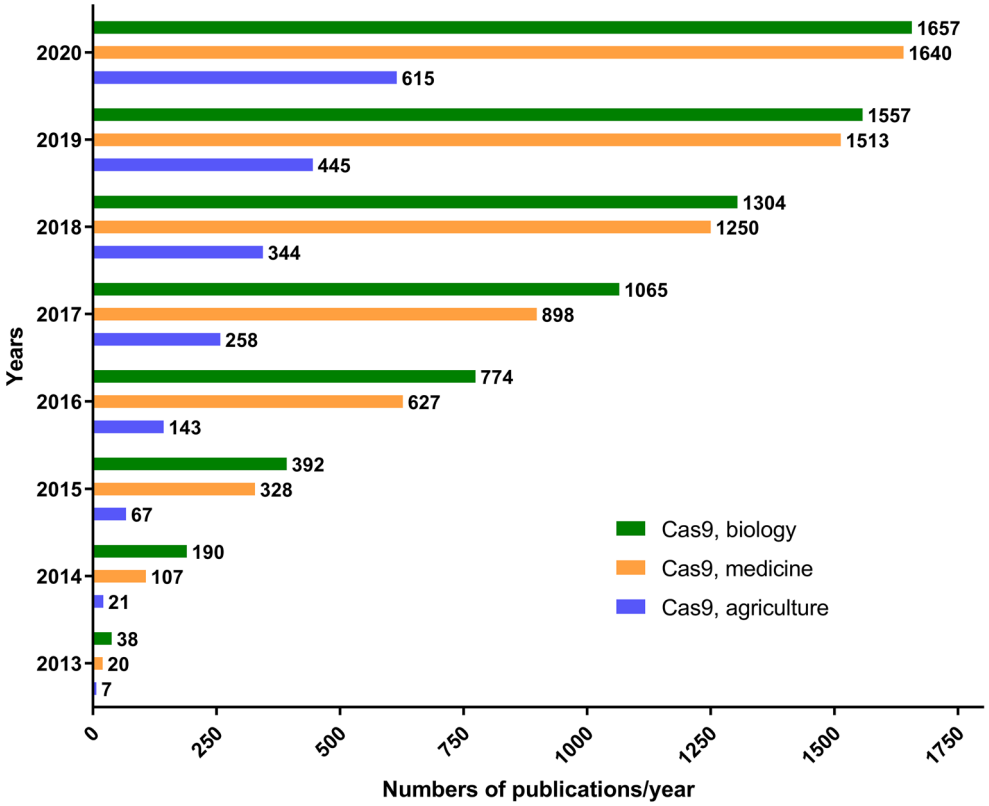

Figure 2. Number of published articles searched by key words "Cas9, biology", "Cas9, medicine" or "Cas9, agriculture" on PubMed.

of the numerous reviews on CRISPR/Cas9 as mentioned above but only to summarize the practical applications of CRISPR/Cas9 and its recent advances in model plants and crops that will be helpful for researchers.

2. Procedure of target plant generation by CRISPR/Cas9 method

Here, we made a customary flowchart to provide direct understanding about the application of CRISPR/Cas9 technology in plant biology according to the previous studies (Gao et al., 2016; Tsutsui and Higashiyama, 2017). As shown in Figure 3, once the sgRNA (single guide RNA) was synthesized, it can be inserted into a linearized vector that contains appropriate label and/or antibiotic resistance, such as green fluorescent protein (GFP) or hygromycin resistance, to form a recombinant vector. In most plant studies, sgRNA is commonly driven by the U3 or U6 promoter, which belongs to the plant RNA polymerase III promoter. Generally, a defined transcription start nucleotide for U3 promoter is "A", while for U6 promoter is "G". As such, the guide sequences in the sgRNA follow the consensus A(N)19 to 22 for the U3 promoter and G(N)19 to 22 for the U6 promoter (Belhaj et al., 2013).

After recombination, the vector can be screened and amplified by positive monoclone bacteria, and transformed into the plant with *Agrobacterium* or other proper delivery methods. Then, the plants will be selected according to the label or antibiotic resistance, and the effect of genome editing will also be validated, before the seeds will be collected for selection of T_1 plants. Next, seeds from the positive T_1 plants will be collected as T_2 seeds, and then those T_2 seeds containing the target gene mutation, but absence of Cas9 gene, will be further used as Cas9 free T_2 seeds to get the T_2 plants. By genotyping T_2 plants, a target mutant T_2 plant will be finally obtained.

3. Applications of CRISPR/Cas9 in various plants

3.1. Applications of CRISPR/Cas9 in model plants

As a model plant, *Arabidopsis thaliana* is one of the plants whose genome was first edited by CRISPR/Cas9 system. In 2013, Li et al. (2013) expressed a plant codon-optimized SpCas9 (pcoCas9) and a sgRNA targeting *AtPDS3* and *AtFLS2* in mesophyll protoplasts, that was freshly isolated from leaf cells, and a mutagenesis frequency of 5.6% and 1.1% was observed, respectively. Then, they designed a cognate sgRNA, targeting both *AtRACK1b* and *AtRACK1c*, and observed mutations in both target genes with a similar mutagenesis frequency of 2.5%–2.7%. Furthermore, they expressed a tandem sgRNAs targeting two juxtaposed targets in *AtPDS3*, and a mutation frequency of 7.7%

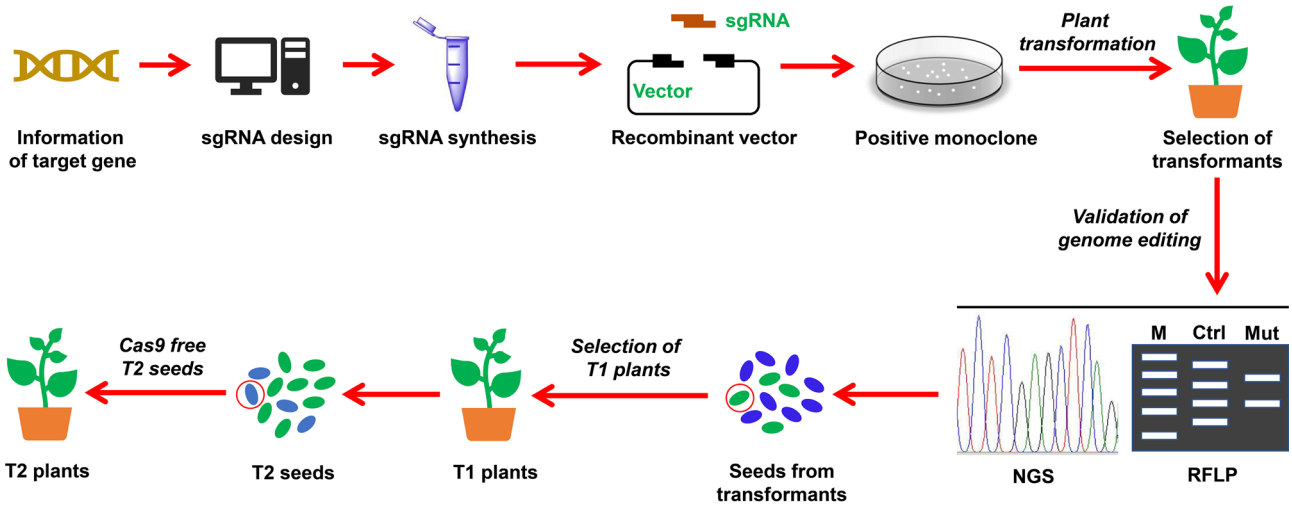

Figure 3. A flow chart of target plant generation by CRISPR/Cas9 method. M, marker; Ctrl, control; Mut, mutant; RFLP, restriction fragment length polymorphism; NGS, next generation sequencing.

was finally observed. This study indicated that CRISPR/Cas9 system cannot only modify single target but also multiple targets in *Arabidopsis thaliana*. In the same year, Mao et al. (2013) constructed the vector for CRISPR/Cas-based gene editing in *Arabidopsis thaliana* targeting *AtCHL1* and *AtCHL2*, in which the designed sgRNAs and optimized SpCas9 were driven by *AtU6* and *AtUBQ* promoters, respectively. Using *Agrobacterium*-mediated transformation, they finally observed variable mutagenesis frequencies, depending upon cassettes, of 50%–89% in single sgRNA expression cassette and a 68%–74% in two sgRNA expression cassettes. Therefore, this research demonstrated that suitable promoter for the CRISPR/Cas9 may generate a higher mutagenesis frequency.

The application of this technology in *Arabidopsis thaliana* is constantly improving. Since the T1 generation, *Arabidopsis thaliana* generated by constitutive overexpression of the CRISPR/Cas9 are usually mosaics. Wang et al. (2015b) developed an egg cell-specific promoter controlled (EPC) CRISPR/Cas9 system, in which the expression of Cas9 is driven by egg cell-specific promoters (*EC1.2* gene), and they obtained a high efficiency of the generation of homozygous or biallelic mutants for multiple target genes in the T1 generation. Miki et al. (2018) also found that CRISPR/Cas9 containing an egg cell and early embryo specific *DD45* gene promoter can improve the frequency of target gene knock-ins and sequence replacements. Results from Feng et al.'s study (2018) showed that expression of the Cas9 under two cell division-specific promoters (*YAO* and *CDC45*) could produce a mutation rate of 80.9%–100% in the T1 generation. Furthermore, *CDC45* promoter can also be used in a multiplex CRISPR/Cas9 system to generate a higher mutation efficiency in the T1 generation that will lead to a higher efficiency of heritable mutations than the original constitutive ubiquitin promoter. Recent research indicates that a plant-specific vector series harboring modified CRISPR/Cas9 system, comprising PAM-altered Cas9 variants and gRNAs, has a higher ability of producing heritable mutations (Yamamoto et al., 2019). To assess and optimize Cas9-mediated gene modification in *Arabidopsis thaliana*, a visual CRISPR/Cas9 system has also been developed in 2017 (Hahn et al., 2017).

Tobacco is another common model plant that was also used to establish the CRISPR/Cas9 mediated genome editing system in plant. In 2013, Li et al. (2013) applicated this system in *Nicotiana benthamiana* by targeting the *NbPDS* gene. They transiently co-expressed Cas9 and sgRNA in *Nicotiana benthamiana* protoplasts and leaves, and found the mutation rate in protoplasts and leaves was 37.7%–38.5% and 4.8%. At the same time, Nekrasov et al. (2013) expressed a GFP-tagged Cas9 and a sgRNA targeting *NbPDS* gene in *Nicotiana benthamiana* leaf tissue, through *Agrobacterium tumefaciens* mediated transient expression assays, but the final mutation efficiency was estimated to be 2.1%. According to these two studies, it is obvious that the mutation rate is higher in the protoplasts than in the leaves, indicating that the cells in a more primitive state are favorable for transformation.

In 2014, a detailed protocol was established for the use of CRISPR/Cas9 system in *Nicotiana benthamiana*, that comprised of designing and constructing dual sgRNAs, transfecting and expressing Cas9/sgRNAs, and evaluating the frequency of targeted genome modifications (Li et al., 2014). The CRISPR/Cas9 system has enabled efficient genome engineering in plant, but the production of transgenics is time consuming and efficient delivery methods remains challenging. To address these concerns,

virus, such as tobacco rattle virus (TRV) and pea early browning virus (PEBV), mediated CRISPR/Cas9 system in *Nicotiana benthamiana* was developed (Ali et al., 2018). Thus, CRISPR/Cas9 technology developed in *Nicotiana benthamiana* has been used as a model for other plants. Mercx et al. (2016) used CRISPR/Cas9 in *Nicotiana tabacum* BY2 cells, which enabled the production of recombinant proteins, and inactivation of proteases involved in protein degradation. Jansing et al. (2019) used CRISPR/Cas9 to delete genes that can affect the activity, potency and immunogenicity of plant-derived proteins to generate knockout lines for the production of efficacious biopharmaceutical glycoproteins. Matsuo and Atsumi (2019) applied this technology to collapse RNA silencing mechanism in *Nicotiana Benthamiana*, which can strengthen the expression of transgene-derived mRNAs to produce high level of recombinant protein. In addition, CRISPR/Cas9 was also used to generate nicotine-free *Nicotiana tabacum* plant (Schachtsiek and Stehle, 2019).

3.2. Applications of CRISPR/Cas9 in tuber crops

Potato is not only one of the most important food crops in the world, but also one of the main crops grown for the production of starch. Thus, enhancing nutritional contents and removal of antinutritional compounds are very necessary in potato cultivation and utilization. Utilizing CRISPR/Cas9 system, mediated by *Agrobacterium* transformation, Wang et al. (2015a) modified the Auxin/indole-3-acetic acid (*StIAA2*) gene to induce the altered Aux/IAA protein expression, and their study indicated that the CRISPR/Cas9 system could generate mutations in stable transgenic potatoes. In a later study, Butler et al. (2015) produced site-specific mutations in the *StALS1* genes, in both diploid and tetraploid potatoes, using the CRISPR/Cas9 system, and those mutations were stably heritable. In 2017, Andersson et al. (2017) successfully mutated the granule-bound starch synthase (*GBSS*) gene that is responsible for the synthesis of amylose. These mutations could change the ratio of amylose and amylopectin to generate high amylopectin potatoes, thus this technology is expected to develop a trait of commercial interest, with applications in food and industrial fields. In terms of reproduction, potatoes are propagated from tubers, because it is an autotetraploid plant. Researchers are expecting to reinvent potatoes as a diploid inbred line, which would accelerate the improvement of potato characteristics, such as tuber quality, and resistance traits. However, for a long time, self-incompatibility, controlled by the S-RNase gene, has impeded the development of inbred lines. In 2018, Ye et al. (2018) first generated self-compatible diploid potatoes by knockout of S-RNase using CRISPR/Cas9 system. In 2019, Enciso-Rodriguez et al. (2019) provided further insight into overcoming self-incompatibility in diploid potatoes, and they deleted three new S-RNase alleles through CRISPR/Cas9 technology to generate stable self-compatible lines. Thus, the CRISPR/Cas9 system opens new avenues for diploid potato breeding. The protocol of genome editing in potato with CRISPR/Cas9 was described by Nadakuduti et al. (2019).

In addition to the potato, CRISPR/Cas9 is now also used in other tuber crops. Wang et al. (2019) edited two starch biosynthetic pathway genes, *GBSSI* and *SBEII*, in sweet potato (*Ipomoea batatas*). They found that the mutation efficiency was 62%–92% and most of the mutations were nucleotide substitutions. In the allopolyploid sweet potato, the *GBSSI*-knockout reduced the amylose percentage, while the *SBEII*-knockout showed the opposite phenotype. Another study also mutated *GBSS* gene in Cassava (*Manihot esculenta Crantz*), which resulted in a reduction of amylose content in root starch (Bull et al., 2018). These studies demonstrate that CRISPR/Cas9 technology is an attractive plant breeding technique for the improvement of starch qualities in tuber crops for food and industrial applications. Besides these applications, CRISPR/Cas9 has been also used to generate crop varieties resistant to plant diseases, such as cassava brown streak disease (CBSD) (Gomez et al., 2019) and African cassava mosaic virus (ACMV) (Rybicki, 2019) in cassava.

3.3. Applications of CRISPR/Cas9 in cereal crops

Hybrid seed technology may lead to remarkable increment in agricultural productivity; however, because of the strong inbreeding habit of bread wheat (*Triticum aestivum*), the development of a commercially viable platform for the production of hybrid wheat has long been challenged. The successful use of the CRISPR/Cas9 gene editing system in bread wheat cells was first reported in 2013 (Upadhyay et al., 2013), in which two genes, inositol oxygenase (*inox*) and phytoene desaturase (*pds*), were targeted, thus opening doors for genes editing in allohexaploid crop species using CRISPR/Cas9. From then on, studies have focused on the issue of production of hybrid wheat. *Ms45* (male sterile 45) gene encodes a strictosidine synthase-like enzyme and is normally required for male fertility in wheat. Singh et al. (2018) mutated this gene using CRISPR/Cas9 and found that the mutation of *Ms45* is necessary to abort pollen development and contributes to male fertility. On the other hand, Okada et al. (2019) used the CRISPR/Cas9 system to generate mutations in wheat male fertility gene *Ms1*, which resulted in complete male sterility. These studies indicated the utility of the CRISPR/Cas9 for the rapid generation of male sterility in commercial wheat cultivars. In addition, CRISPR/Cas9 was also applied to study the basic biological questions of wheat. For instance, Liu et al. (2020) found that CRISPR/Cas9 mediated editing of wheat *TaQ* genes, one of the AP2-like transcription factors, alters spike morphogenesis and grain threshability, which contributes to the understanding of the underlying

mechanisms of AP2-like family genes in regulating wheat floral organs and inflorescence development.

Since 2013, the method of CRISPR/Cas9 application in wheat has been used to improve traits of wheat, such as low-gluten (Sánchez-León et al., 2018) and resistance against wheat dwarf virus (Kis et al., 2019). The detailed protocol of genome editing in bread wheat using CRISPR/Cas9 was introduced by Liang et al. (2018). Another transiently expressing CRISPR/Cas9 system that can result in efficient genome editing with significantly reduced transgene integration and produce homozygous wheat mutants in the T0 generation was established by Zhang et al. (2016b). In 2017, Liang et al. (2017) reported that when CRISPR/Cas9 ribonucleoproteins (RNPs), instead of the CRISPR/Cas9 DNA were introduced into bread wheat, the mutant plants obtained not only were completely transgene free but also showed no off-target mutations, thus CRISPR/Cas9 RNPs may represent a promising method for clean and specific genome editing.

Rice (*Oryza sativa*) is another widespread plant used in CRISPR/Cas9 studies. In 2013, Shan et al. (2013) reported that CRISPR/Cas9 was suitable to modify target genes (*OsPDS*, *OsBADH2*, *Os02g23823* and *OsMPK2*) in rice. Their results demonstrated CRISPR/Cas9 can, in principle, target any sequences, such as 5′-A-N(20)-GG-3′ in rice. Later on, this technology was developed and has been used for different types of gene editing, such as gene replacements and insertions (Li et al., 2016), precise base editing (Hua et al., 2018), and been applied in rice breeding (Li et al., 2019b). On the basis of these technological advances, a series of achievements have been made in the application of Cas9 in rice. Huang et al. (2020) generated novel *Waxy* (*Wx*) alleles by editing the *Wx* promoter, which not only improved the grain quality for eating and cooking but also demonstrated that the targeted modification of gene core promoters is a reliable method for the fine adjustment of the expression of target genes. Zafar et al. (2020) generated resistance against bacterial blight in Super Basmati rice by editing the promoter of the *OsSWEET14* gene through the CRISPR/Cas9 method. Dong et al. (2020) engineered a rice that can synthesize carotenoid through targeted carotenoid cassette insertion using CRISPR/Cas9. Tang et al. (2021) deleted the exonic nucleotide at the exon-intron junction of rice *OsBADH2* gene by CRISPR/Cas9, which led to an exon skipping during pre-mRNA splicing and caused a shifting in the reading frame and a downstream premature termination codon. By investigating the phenotypic characteristics of the mutant plants, they concluded that CRISPR/Cas9-mediated exon skipping could promote the improvement of important traits in rice and other plants. Recently, Zhou et al. (2021) successfully deleted multiple circle RNA loci (*Os02circ25329*, *Os06circ02797*, *Os03circ00204*, and *Os05circ02465*) in rice using CRISPR/Cas9 technology, demonstrating the application of CRISPR/Cas9 for functional study of noncoding RNAs in plants.

CRISPR/Cas9 has also been used to perform sequence specific genome editing in other widely cultivated food crops, such as maize (*Zea mays*), and sorghum (*Sorghum bicolor*). In 2014, targeting the *ZmIPK* gene in maize, Liang et al. (2014) first reported that the CRISPR/Cas9 system can also be used for genome modification in maize. In 2013, Jiang et al. (2013b) successfully applied the CRISPR/Cas9 system in sorghum. As the application system was well established, the CRISPR/Cas9 was used to generate changes that can be introduced directly into commercial genotypes, such as haploid induction (Zhong et al., 2019) and improvement of grain yield (Shi et al., 2017) in maize, and improving protein quality and digestibility (Li et al., 2018) in sorghum. Moreover, a recent study demonstrated that the continuing activity of Cas9/sgRNA could be passed from the T0 generation to the T1 generation, which results in more site-specific mutations in T1 generation in sorghum (Char et al., 2020).

3.4. Applications of CRISPR/Cas9 in legume crops

Soybean (*Glycine max*) is a major protein and oil crop grown worldwide and is used extensively in the food industry and industrial applications. In 2015, three researchers successively reported the application of Cas9 mediated genome editing in Soybean (Jacobs et al., 2015; Li et al., 2015; Sun et al., 2015), which demonstrated that the CRISPR/Cas9 is a rapid, highly efficient and specific genome editing tool in soybean. After that, many studies edited various genes of soybean to create good traits using this method. Do et al. (2019) mutated the fatty acid desaturase 2 (*GmFAD2*) gene that converts the monounsaturated oleic acid (C18:1) to the polyunsaturated linoleic acid (C18:2) in soybean, and they found that this mutation showed dramatic increases in oleic acid content (up to 80%), which is expected to make the soybean oil more suitable for cooking, baking and frying. Cai et al. (2020) found that modification of *GmFT2a* and *GmFT5a* contributes for expanding the regional adaptability of soybean, such as late flowering and adaption to high latitude. Besides these studies, CRISPR/Cas9 was also used to improve the quantity and quality of storage proteins in soybean seeds by creating of mutagenesis of seed storage protein genes (Li et al., 2019a). The detailed protocol for applying CRISPR/Cas9 technology in soybean is summarized by Liu et al. (2019) and Bao et al. (2020).

Cowpea (*Vigna unguiculata*) is another legume crop that is widely cultivated across the world. Because of its good tolerance to low rainfall, low fertilization requirements, as well as its high nutrition and health benefits, cowpea is more popular in many semiarid countries. However, lack of efficient tools for gene editing in Cowpea makes

the research of gene inactivation dramatically hampered. In 2019, Ji et al. (2019) established the system of genome editing using CRISPR/Cas9 in Cowpea, which may accelerate the functional genomics analyses of many important agronomical traits in this legume crop.

3.5. Applications of CRISPR/Cas9 in forage crops

Due to its high feed value and high yield productivity, alfalfa (*Medicago sativa*) isknown as the queen of forage crops and is the most important forage crops in the world. The improvement of alfalfa yield and quality is an urgent need for the development of the feed industry, the animal husbandry, and the dairy industry. However, compared to the main cereal crops, such as rice, wheat, and maize, the study of functional genomics in alfalfa is still limited, because of the complex autotetraploid genome and the limited molecular genetic modification tools, which also restrict the development of molecular breeding technology in forage crop.

In 2015, Michno et al. (2015) mutated target genes in somatic cells of *Medicago truncatula*, close relative of alfalfa, by root hair transformation, suggesting the application potential of CRISPR/Cas9 in forage crops. In 2017, Meng et al. (2017) developed an efficient CRISPR/Cas9 system by targeting *MtPDS* gene and obtained monoallelic and biallelic homozygous mutants, which firstly reported the CRISPR/Cas9-based stable transformation in forage legumes. The full method of CRISPR/Cas9 mediated gene editing in *Medicago truncatula* was described by Curtin (2018). However, *Medicago truncatula* is a leguminous plant with diploid genetics and small genomes, thus the application of the CRISPR/Cas9 in a multiplex genome, such as autotetraploid genome of alfalfa will ultimately lead to major advances in the improvement of forage crops. In 2018, Gao et al. (2018) successfully applied CRISPR/Cas9 technique to mutate squamosa promoter binding protein like 9 (*SPL9*) gene in alfalfa, opening the way to apply this technology in alfalfa breeding. Recently, some studies further improved the CRISPR/Cas9-based genome editing system in alfalfa, such as Wolabu et al. (2020) established a highly efficient multiplex gRNA-CRISPR/Cas9 genome editing system which could generate homozygous mutants with a complete knockout of the four allelic copies in the T0 generation, while Chen et al. (2020) presented a CRISPR/Cas9-based transgene-free genome editing strategy, hence, all these improvements may accelerate research and molecular breeding of this important forage crop.

3.6. Applications of CRISPR/Cas9 in fruits

Apple (*Malus x domestica*) is one of the major fruit crops planted in the world. Conventionally, the apple is bred by clonal propagation, which makes traditional cultivars still dominant and limits the speed of introduction of new hybrid varieties in the market. Genome editing appeared as a powerful tool in crop breeding to accelerate the improvement of quality of apple. In 2016, Nishitani et al. (2016) reported an induction of a targeted mutation of phytoene desaturase (*PDS*) gene in the apple using the CRISPR/Cas9 system, demonstrated that this technology can be practically applied to modify the apple genome. After that, CRISPR/Cas9 was also used to improve good characters in apple, for example *DIPM-1*, *DIPM-2*, and *DIPM-4* in the apple were targeted to increase resistance to fire blight disease (Malnoy et al., 2016) and *MdCNGC2* was targeted to increase resistance to *Botryosphaeria dothidea* (Zhou et al., 2020a). The detailed method of application of CRISPR/Cas9 in apple was described by Osakabe et al. (2018).

Besides apple, the CRISPR/Cas9 was also applied in many other fruits, such as grape (*Vitis vinifera*) (Malnoy et al., 2016; Ren et al., 2016), pear (*Pyrus spp*) (Charrier et al., 2019), strawberry (*Fragaria vesca*) (Zhou et al., 2018), watermelon (*Citrullus lanatus*) (Tian et al., 2017), and Citrus (*Citrus reticulata*) (Jia et al., 2017).

3.7. Applications of CRISPR/Cas9 in vegetables

Carrot (*Daucus carota*) is an important vegetable crop for the consumption of its root, which contains not only carotenoids, vitamins and dietary fiber, but also minerals and antioxidants. As these components are reported to possess benefits to human health, it is an urgent subject to study the genetic factors of growth development and phytochemical accumulation of carrot. The release of carrot genome information as well as the application of CRISPR/Cas9 for precise genome editing in many other plant species, make it possible for researchers to study the above subject.

In 2018, Klimek-Chodacka et al. (2018) first established an efficient CRISPR/Cas9-based genome editing system in carrot cells by targeting the carrot flavanone-3-hydroxylase (*F3H*) gene. Although this study brings the hope to apply the CRISPR/Cas9 in this important vegetable crop, it did not achieve a whole gene editing carrot plant. In 2019, Xu et al. (2019) designed four sgRNA expression cassettes, targeting four sites of *DcPDS* and *DcMYB113-like* genes and driven by four different promoters individually, and assembled them in a single CRISPR/Cas9 vector. Through *Agrobacterium*-mediated genetic transformation, they finally obtained stable gene editing carrot plants, suggesting that CRISPR/Cas9-mediated multiple targeted mutagenesis in carrot plants is feasible.

Until now, in addition to the carrot, the CRISPR/Cas9 has been applied to at least the following vegetables, including tomato (*Solanum lycopersicum*) (Shimatani et al., 2017), *Brassica oleracea* (Lawrenson et al., 2019), and cucumber (*Cucumis sativus*) (Hu et al., 2017).

3.8. Applications of CRISPR/Cas9 in ornamental plants

Theoretically, CRISPR/Cas9 can be used to edit genomes in each plant species of interest. However, its use in

ornamental plants has not been well developed not only because of commercial factors, for example the economic importance of individual ornamental plants generally is relatively small, but also the lack of whole genome information in most ornamentals.

Petunia (*Petunia hybrida*) is one of the most popular bedding plant species in the world. It represents the first ornamental plant in which the CRISPR/Cas9 technology was applied by targeting the petunia nitrate reductase (*NR*) gene locus (Subburaj et al., 2016) and phytoene desaturase (*PDS*) gene (Zhang et al., 2016a) in 2016, demonstrating the potential of CRISPR/Cas9-mediated gene editing in ornamental plant. *Dendrobium officinale* is a special orchid plant that has a highly ornamental value. However, the slow-growth of *Dendrobium officinale* has hindered traditional breeding techniques. In 2017, Kui et al. (2017) reported the application of the CRISPR/Cas9 system in *Dendrobium officinale*, in which five genes (*C3H*, *C4H*, *4CL*, *CCR*, and *IRX*) involved in the lignocellulose biosynthesis pathway were edited.

Besides the ornamental plants mentioned above, another ornamental plant in which the CRISPR/Cas9 system was applied so far is chrysanthemum (*Chrysanthemum morifolium*). However, due to the lack of whole genome information, in 2017, Kishi-Kaboshi et al. (2017) constructed transgenic chrysanthemum plants expressing the yellowish-green fluorescent protein gene from *Chiridius poppei* (*CpYGFP*) and then targeted *CpYGFP* for gene editing. Even so, this study first established CRISPR/Cas9-mediated gene editing system in chrysanthemum and opens the way to gene modification in chrysanthemum once its whole genome information is available.

3.9. Applications of CRISPR/Cas9 in other plants

In addition to the plants mentioned above, there are some other plants in which the CRISPR/Cas9-mediated gene editing system was established. Such as, in 2015, Fan et al. (2015) described the genome editing via the CRISPR/Cas9 system by targeting phytoene desaturase gene 8 (*PtoPDS*) gene in poplars (*Populus trichocarpa*), demonstrating that CRISPR/Cas9 can be used to create knockout mutations in woody plants. In 2017, Jiang et al. (2017) disrupted fatty acid desaturase 2 (*FAD2*) gene in *Camelina sativa* by CRISPR/Cas9, which resulted in an increase of oleic acid content from 16% to over 50% of the fatty acid composition. In 2018, Okuzaki et al. (2018) also edited *FAD2* gene in *Brassica napus* with CRISPR/Cas9 and produced desirable mutant plants with a significant increase in the content of oleic acid. In 2019, using the CRISPR/Cas9 method, Janga et al. (2019) showed that the *GoPGF* (synonym CGF3) gene plays a critical role in the formation of glands in the cotton (*Gossypium hirsutum*). In 2020, Shao et al. (2020) reported that modification of *MaGA20ox2* gene in Banana (*Musa spp.*) using CRISPR/Cas9 generated semidwarf mutants, which may contribute to reduce the severe damages caused by typhoons and storms.

4. Conclusion

In conclusion, the application of the CRISPR/Cas9 system in plants could introduce mutations in single gene and multiple genes simultaneously, and create conditional alleles. Since the CRISPR/Cas9 technology was established, its strategies and methods of gene constructs were developed constantly, which makes the CRISPR/Cas9 as a powerful tool in genome editing in plants (Table 1) and some of these gene-edited plants/crops are already on their way to commercial use (Table 2). Compared with ZFNs and TALENs, as a genome editing technique CRISPR/Cas9 has many advantages, such as design simplicity, higher engineering feasibility, multiplex genome editing, high efficiency, and low cost. In addition to the function of genomic sequence editing, CRISPR/Cas9 also has been adapted for transcriptional activation (CRISPRa) or inhibition (CRISPRi) at endogenous genomic loci. A nuclease-deficient Cas9 (dCas9) fused with an activation domain can facilitate the transcriptional activation, while a dCas9 fused with a repression domain can inhibit gene expression (Gentzel et al., 2020). Actually, the CRISPR/Cas system is constantly evolving, besides Cas9, other endonucleases were also discovered, such as Cpf1 (also known as Cas12a), Cas13, and Cas14 (Manghwar et al., 2019).

However, deficiencies and concerns are always present. First, CRISPR/Cas9 technology suffers from the off-target effects and the limitation of transformation methods, which are the major disadvantages to be improved in the future. For the off-target effects, a researching direction is to develop the CRISPR/Cas9 ribonucleoproteins or even the CRISPR/Cpf1. For the transformation methods, the biolistic transformation is preferred over the commonly used *Agrobacterium*-mediated transformation and protoplast transformation. Second, although the CRISPR/Cas9 system is used in many plants, these plants are mainly model plants and crop plants, its applications in ornamental plants and woody plants are still limited. Third, from a scientific standpoint, plants obtained through CRISPR/Cas9-mediated gene editing may not bring higher health risks or environmental hazards, but legal implication of CRISPR/Cas9 technology is still questionable. Therefore, there is a need to solve these problems.

Nevertheless, there are reasons to believe that with the increasingly technical maturity, CRISPR/Cas9 will have a

Table 1. Summary of the representative applications of CRISPR/Cas9 technology in plant genome editing. N/A: not applicable.

Species	Target gene	Cas9 promoter	sgRNA promoter	Transformation method	Characteristic	Reference
Arabidopsis thaliana	AtPDS3, AtFLS2, AtRACK1b and AtRACK1c	35S promoter	U6 promoter	*Agrobacterium*-mediated transformation	First report in Arabidopsis thaliana	(Li et al., 2013)
Tobacco	NbPDS	35S promoter	U6 promoter	*Agrobacterium*-mediated transformation	First report in Tobacco	(Li et al., 2013; Nekrasov et al., 2013)
Potato	StIAA2	CaMVE 35S promoter	StU6 promoter	*Agrobacterium*-mediated transformation	First report in Potato	(Wang et al., 2015a)
Wheat	TaMLO	CaMVE 35S promoter	Wheat U6 promoter	*Agrobacterium*-mediated transformation	First report in wheat	(Shan et al., 2013)
	Inox and pds	CaMVE 35S promoter	CaMVE35S promoter	*Agrobacterium*-mediated transformation	Application of CRISPR/Cas9 in genome editing in very large genomes (17 Gb) of wheat	(Upadhyay et al., 2013)
Rice	OsBADH2, Os02g23823, and OsMPK2	CaMVE 35S promoter	Rice U3 promoter	*Agrobacterium*-mediated transformation	First report in rice	(Shan et al., 2013)
	Xa13	CaMVE 35S promoter	U3 and U6 promoter	N/A	Editing the promoter region of target gene to produce transgene-free rice	(Li et al., 2020)
Maize	ZmIPK	CaMVE 35S promoter	Maize U3 promoter	*Agrobacterium*-mediated transformation	First report in maize	(Liang et al., 2014)
	LIG1, Ms26, Ms45, and ALS2	Maize Ubiquitin-1 promoter	Maize U6 promoter	Particle bombardment	First report of DNA-free genome editing in maize	(Svitashev et al., 2016)
Sorghum	N/A	Maize ubiquitin 1 promoter	Maize U6 promoter	*Agrobacterium*-mediated transformation	First testing of the Cas9 and sgRNA gene activity in sorghum	(Jiang et al., 2013b)
	SbFT and SbGA2ox5	Maize ubiquitin 1 promoter	Rice U6 promoter	*Agrobacterium*-mediated transformation	The expression of CRISPR/Cas9 continuously induced new mutations in T1 generation	(Char et al., 2020)
Soybean	GFP, Glyma07g14530, and DDM1	2×35S promoter	*Medicago truncatula* U6.6 promoter	Biolistic transformation	First report in soybean	(Jacobs et al., 2015)
Cowpea	VuSYMRK	2×35S promoter	U6 promoter	*Agrobacteri*um-mediated transformation	First report in cowpea	(Ji et al., 2019)
M. truncatula	A GUS transgene	2×35S promoter	Arabidopsis U6 promoter	*Agrobacterium*-mediated transformation	First report in *M. truncatula*	(Michno et al., 2015)
Alfalfa	MsPDS and MsPALM1	CaMV 35S promoter	MtU6 promoter	*Agrobacterium*-mediated transformation	An efficient transgene-free genome editing for alfalfa	(Chen et al., 2020)
Apple	PDS	2×35S promoter	U6 promoter	*Agrobacterium* method	First report in Apple	(Nishitani et al., 2016)

Table 1. (Continued).

Grape	IdnDH	35S promoter	U6 promoter	*Agrobacterium*-mediated transformation	Determined the CRISPR/Cas9 is a useful tool in grape	(Ren et al., 2016)
Pear	MdPDS and MdTFL1.1	PcUbi4-2 promoter	MdU3 and MdU6 promoters	*Agrobacterium*-mediated transformation	First report in pear	(Charrier et al., 2019)
Strawberry	TAA1 and ARF8	AtUBQ10 promoter	Strawberry U6 promoter	*Agrobacterium*-mediated transformation	First report in strawberry	(Zhou et al., 2018)
Watermelon	ClPDS	2×35S promoter	U6 promoter	*Agrobacterium*-mediated transformation	First report in watermelon	(Tian et al., 2017)
Citrus	CsLOB1	CaMVE35S promoter	CaMVE35S promoter	*Agrobacterium*-mediated transformation	First report in citrus	(Jia et al., 2017)
Carrot	F3H	2×35S CaMV promoter	AtU3 promoter	*Agrobacterium*-mediated transformation	First report in carrot	(Klimek-Chodacka et al., 2018)
Tomato	SlAGO7	35S promoter	AtU6 promoter	*Agrobacterium*-mediated transformation	First report in tomato	(Brooks et al., 2014)
Brassica oleracea	FRI and PDS	N/A	N/A	PEG-mediated transformation	DNA-free genome editing of the genus *Brassica*.	(Murovec et al., 2018)
Cucumber	eIF4E	35S promoter	AtU6 promoter	*Agrobacterium*-mediated transformation	First report in cucumber	(Chandrasekaran et al., 2016)
Petunia	PhPDS	35S promoter	AtU6 promoter	*Agrobacterium*-mediated transformation	First report in petunia	(Zhang et al., 2016a)
Dendrobium officinale	C3H, C4H, 4CL, CCR, and IRX	35S promoter	OsU3 promoter	*Agrobacterium*-mediated transformation	First report in *Dendrobium officinale*	(Kui et al., 2017)
Chrysanthemum	CpYGFP	Ubiquitin promoter	U6 promoter	*Agrobacterium*-mediated transformation	First report in chrysanthemum	(Kishi-Kaboshi et al., 2017)
Poplars	PtoPDS	35S promoter	AtU3b, AtU3d, AtU6-1 and AtU6-29 promoters	*Agrobacterium*-mediated transformation	First report in poplars	(Fan et al., 2015)
Camelina sativa	FAD2	CaMV 35S promoter	U6 promoter	*Agrobacterium*-mediated transformation	First report in *Camelina sativa*	(Jiang et al., 2017)
Brassica napus	BnaA.ALC.a, BnaC.ALC.a	PcUbi4-2 promoter	AtU6-26 promoter	*Agrobacterium*-mediated transformation	First report in *Brassica napus*	(Braatz et al., 2017)
Cotton	DsRed2 and GhCLA1	GhU6.9	GhU6.9	*Agrobacterium*-mediated transformation	First report in cotton	(Wang et al., 2018)
Banana	RAS-PDS1 and RAS-PDS2	CaMV 35S promoter	Rice U3 promoter	*Agrobacterium*-mediated transformation	First report in banana	(Kaur et al., 2018)

Table 2. Commercially available crops that have been edited by CRISPR technology.[1]

Plants/crops	Tools of gene editing	Purpose of gene editing	Company	Location
Corn, soybeans, wheat, and tomato	CRISPR/Cas9	Increase yield	Syngenta	Basel, Switzerland
Camelina	CRISPR/Cas9	Higher oil content	Yield10 Bioscience	Woburn, MA, USA
Banana	CRISPR	Disease resistance	Tropic Biosciences	Norwich, UK
Corn and soybeans	CRISPR/Cas9	Increase productivity, disease resistance	Pairwise	Durham, NC, USA
Corn	CRISPR/Cas9	Altered starch composition	Corteva	Wilmington, DE, USA

[1] TheScientist (2021). The name of resource is Companies Use CRISPR to Improve Crops. Website https://www.the-scientist.com [1, Feb, 2019].

wide range of application in basic and applied science, for example in the plant molecular genetic breeding field. The application of CRISPR/Cas9 in various plants will hold significant promise for plant biologists and breeders to create plants with valuable new agronomic, nutritional and novel traits, which will be beneficial to not only farmers but also consumers.

Contribution of authors

Y.Z.Z. conceived and wrote the original draft. Q.Q.L. collected the data that shown in the Table 1. M.W.Y. and A.E.C. summarized the data of published articles. H.Y.W provided advice and supervision, and finally revised the manuscript.

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