

REVIEW

Overview of CRISPR/Cas9: a chronicle of the CRISPR system and application to ornamental crops

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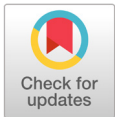
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Abstract

Since its first demonstration as a practical genome editing tool in the early 2010s, the use of clustered regularly interspaced short palindromic repeat (CRISPR) along with the endonuclease Cas9 (CRISPR/Cas9) has become an essential choice for generating targeted mutations. Due to its relative simplicity and cost-effectiveness compared to other molecular scissors, i.e., zinc finger nuclease (ZFN) and transcription activator-like effector nuclease (TALEN), the CRISPR/Cas9 system has been shown to have a massive influence on genetic studies regardless of the biological kingdom. Although the system is in the process of being established, numerous protocols have already been released for the system and there have been various topics of CRISPR related papers published each year in ever-increasing manner. Here, we will briefly introduce CRISPR/Cas9 system and discuss the variants of the CRISPR system. Also, their applications to crop improvement will be dealt with mainly ornamental crops among horticultural crops other than *Arabidopsis* as a model plant. Finally, some issues on the barriers restraining the use of CRISPR system on floricultural crops, the prospect of CRISPR system as a DNA-free genome editing tool with efficient facilitators and finally, the future perspectives on the CRISPR system will be described.

Keywords: base editing, CRISPR/Cas9, dead Cas9 (dCas9), double strand break (DSB), ornamental crops



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Introduction

The CRISPR/Cas system is derived from a bacterial adaptive immune system acquired by prokaryotic species when a viral infection occurs (Jansen et al., 2002; Mojica et al., 2005; Pourcel et al., 2005). An infected host cell retains a 21 - 37 nucleotide foreign DNA fragment from invaders and coalesces the fragment into its CRISPR locus (Mojica et al., 2005; Pourcel et al., 2005). When invaders with the corresponding protospacer (Barrangou et al., 2007) infect the host cells, pre-CRISPR RNAs (pre-crRNAs) are transcribed along with trans-activating CRISPR RNAs (tracrRNAs) that are partially complementary to the crRNA (Brouns et al., 2008; Marraffini and Sontheimer, 2008). Together, crRNAs and tracrRNAs form an RNA duplex that acts as a guide RNA (gRNA) and the

association of Cas9 nuclease completes the development of the crRNA-Cas9 complex (Hale et al., 2008; Hale et al., 2009). Directed by the gRNA, Cas9 recognizes target sequences directly upstream of protospacer adjacent motif (PAM) (3'-NGG) and silences invading exogenous nucleic acids by generating a double-strand break (DSB) 3 bp upstream of PAM (Mojica et al., 2009). Using DNA repair mechanisms such as non-homologous end joining (NHEJ) and homology direct repair (HDR) in the presence of template DNA, DSBs can be mended (Babu et al., 2011). NHEJ occurs more often than HDR and its error-prone property of repair introduces insertion or deletion mutations (InDel mutations) at the target site of the gene of interest (Lieber, 2010) (Fig. 1).

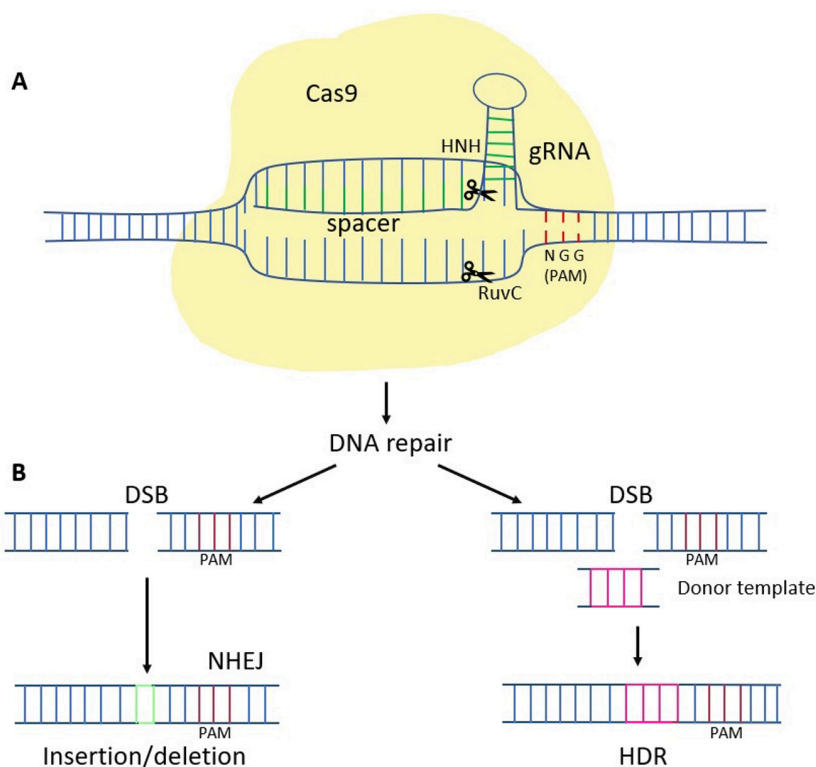


Fig. 1. Scheme of the clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) system and two prominent DNA repair mechanisms after the double-strand break (DSB) generated by Cas9 endonuclease activity. (A) Structure of the Cas9 + single guide RNA (sgRNA) complex. (B) DSBs can be repaired either by non-homologous end joining or homology directed repair in the presence of the donor template. HNH, histidine-asparagine-histidine endonuclease; RuvC, RuvC endonuclease; NHEJ, non-homologous end joining; HDR, homology-directed repair; PAM, protospacer-adjacent motif.

In 2012, Doudna and her research fellows first proposed the use of the CRISPR/Cas9 system as a genome editing tool (Jinek et al., 2012), and since then, this revolutionary work has been demonstrated to be practical and powerful over site-directed mutagenesis by numerous research groups (Friedland et al., 2013; Ran et al., 2013). Other variants based on the CRISPR system are also designed to target specific sites of the gene by utilizing Cas9 as a vehicle that delivers companion nucleases and expression regulators to control the viability and expression of the target gene. The CRISPR/Cas9 system is widely used in the genome editing of kingdom plantae (Belhaj et al., 2015; Bortesi and Fischer, 2015) and the use of the CRISPR/Cas9 system may enable researchers to evade genetically modified organisms-related regulations depending on delivery methods, such as direct delivery of purified Cas9 and sgRNA (ribonucleoprotein, RNP) through polyethylene glycol (PEG) and

electroporation (Woo et al., 2015). As the PEG-mediated genome edited *Petunia hybrida* produced by the research group of Geung-Joo Lee has been approved as a non-genetically modified organism by the ISAAA (2020), the CRISPR/Cas9 system could potentially shorten the process of breeding, under conditions that optimize protocols for regeneration of the target species from the protoplasts readily prepared.

Despite the immense potential of the CRISPR/Cas system, publications on the application of the tool to floricultural crops are rather scarce owing to limited availability of genetic information and polyploidy of ornamental species (Braatz et al., 2017; Kishi-Kaboshi et al., 2018). Especially in ornamental cultivars, color patterns, shapes, longevity of flowering organs, and fragrance are the most demanded and pronounced traits (Noman et al., 2017; Manzoor et al., 2019) to consider targeting because these species are grown in gardens and indoors for their decorative value (Saint-Oyant et al., 2018). The qualitative changes in these features are often related to polyploidy (Manzoor et al., 2019). To overcome these deterring factors, abundant genetic data of diverse species must be built and enhancement of accuracy and efficacy of genome editing tools is essential.

In this paper, we briefly introduce CRISPR/Cas derived genome editing techniques, their application in ornamental species exhibiting decorative features, and future prospects of employing editing tools in floricultural crops.

CRISPR/Cas9 variants

Cas12a (CPF1)

Cas12a is a subtype of the Cas protein and the name is initially originated from the bacterial species *Prevotella* sp. and *Francisella novicida* (Cpf1) (Zetsche et al., 2015). Cas12a can be alternatively used after T-rich PAM (5'-NTT) (Zetsche et al., 2015) followed by target sequences. Compared to Cas9, Cas12a can generate DSB 20 nucleotides downstream of the PAM with sticky ends that leave a few nucleotides overhanging its end (Zetsche et al., 2015). Minimum requirements for degrading the target gene make Cas12 shorter in sequences and simpler in structure than Cas9. Cas12a only requires the crRNA and RuvC domain to cleave the non-target strand, while the Nuc domain residing in RuvC cleaves the target strand to generate DSBs (Zetsche et al., 2015; Zetsche et al., 2017) (Fig. 2). In contrast, Cas9 requires an additional HNH domain to cleave the target strand, thereby inducing the DSB (Deltcheva et al., 2011; Chylinski et al., 2013). Cas12a is also more likely to induce mutations at the target site, whereas close proximity of the Cas9 cleavage site to PAM can eventually lead to deletion of PAM, further eliminating the possibility for Cas9 to re-target the specified site (Zetsche et al., 2015). Cas12a has been shown to exhibit on-target effects similar to those of Cas9, while the off-target effects are less than those of Cas9 (Kim et al., 2016; Kleinstiver et al., 2016).

Versatile use of catalytically impaired Cas9

Cas9 has two distinct cleavage domains to generate a DSB. One is HNH for target strand cleavage and the other is RuvC for non-target strand cleavage (Deltcheva et al., 2011; Chylinski et al., 2013). Mutations in these key domains impair the endonuclease activity of Cas9 (dCas9); however, dCas9 remains capable of binding to the target DNA sequences (Fujita and Fujii, 2013; Qi et al., 2013). The mobility of dCas9 has drawn the attention of researchers, and various applications have been recognized.

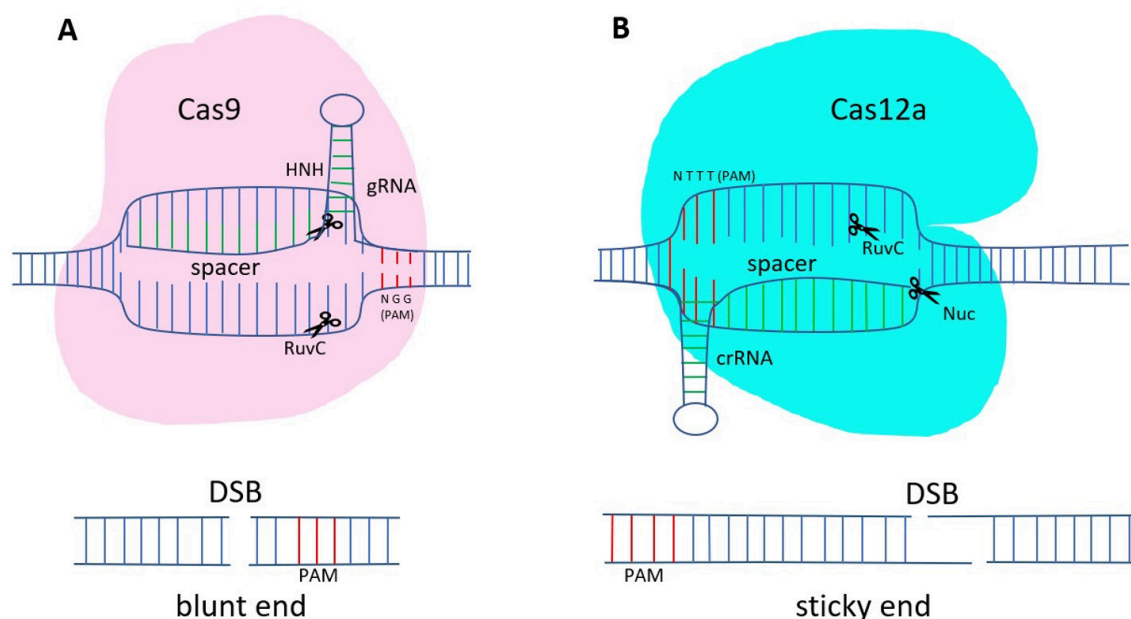


Fig. 2. Differences between CRISPR-associated protein 9 (Cas9) and CRISPR-associated protein 12a. (A) Typical Cas9 from *Streptococcus pyogenes* generates a blunt-ended double-strand break (DSB) 3 bp upstream of the protospacer-adjacent motif (PAM). HNH, histidine-asparagine-histidine endonuclease; RuvC, RuvC endonuclease; gRNA, guide RNA; crRNA, CRISPR RNA; Nuc, nuclease.

dCas9-FokI

The employment of catalytically inactive dCas9 with *FokI* nuclease has been proposed to reduce off-target editing of Cas9 (Guilinger et al., 2014; Hara et al., 2015). dCas9 fused to *FokI* detects the target site using gRNA, and the cleavage event is conducted by *FokI* (Fig. 3). A dCas9-*FokI* monomer alone cannot generate DSB, and therefore, requires another set of dCas9-*FokI* targeting nearby sequences. dCas9-*FokI* can be applied to target repetitive DNA (Guilinger et al., 2014; Hara et al., 2015; Wyvekens et al., 2015).

Base editing

Base editing is devised to induce a point mutation at the target site of DNA or RNA, thereby altering a base to another desired base without generating DSBs (Rees and Liu, 2018; Kim et al., 2019). Together with various base editors, this approach also employs dCas9 or Cas9 nickase (Cas9n), which is only capable of introducing a single strand break because of the mutation in one or the other cleavage domain. The conversion of bases is limited to that of C to T, G to A, A to G, and T to C because of the lack of proper known deaminases (Komor et al., 2016; Nishida et al., 2016; Gaudelli et al., 2017; Vogel et al., 2018). Because detrimental mutations largely take the point mutation into account (Landrum et al., 2014; Landrum et al., 2016), considerable efforts have been made to seek means for reverting the mutation to its original state.

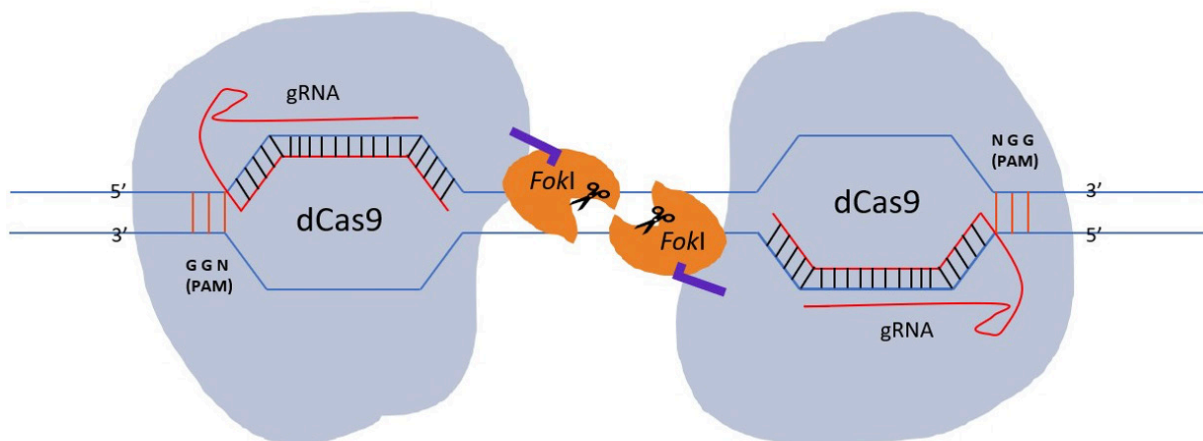


Fig. 3. Scheme of dead Cas9-FokI (dCas9-FokI). Two FokI enzymes attached by dCas9 replace the catalytic activity of Cas9. The system requires two molecules of dCas9 each bound to the flanking strand of the target region to generate a double-strand break with enhanced precision. gRNA, guide RNA; PAM, protospacer-adjacent motif.

CRISPRi

Utilization of dCas9 is also able to suppress gene expression (CRISPRi) (Qi et al., 2013). Knockdown of a gene of interest is achieved by the binding activity of dCas9 to the promoter of the gene and base pairing of gRNA complementary to the non-template strand, consequently resulting in transcriptional repression of the gene by RNA polymerase blockage (Larson et al., 2013; Qi et al., 2013). Initially, CRISPRi comprised dCas9 and chimeric gRNA comprised guiding sequences, a Cas9-binding hairpin, and transcription terminator attained from *Streptococcus pyogenes* (Larson et al., 2013; Qi et al., 2013) (Fig. 4). Attempts to widen the range of CRISPRi applications have made use of repressing elements such as KRAB (Gilbert et al., 2013) and MeCP2 (Xiong et al., 2019). In plants, Piatek et al. (2015) demonstrated that dCas9 alone and dCas9-SRDX efficiently downregulated *PDS* in *Nicotiana benthamiana*.

Although one may associate RNA interference (RNAi) with CRISPRi, the CRISPRi complex shows high specificity for the target site and exceptionally low rates of off-target effects and is able to modulate repression of multiple targets, whereas the use of RNAi can lead to unintended downregulation of genes with similar sequences (Smith et al., 2017).

CRISPRa

Using a similar approach as that of CRISPRi, attaching effector domains with upregulating properties to dCas9 can activate the expression of the target gene (CRISPRa) (Gilbert et al., 2013). Transcriptional activators such as VP64 (Maeder et al., 2013; Nihongaki et al., 2015) and p65 (Nihongaki et al., 2015) are commonly used for CRISPRa, and fusion of supplementary activator domains increases its activating proficiency (Fig. 4). A framework for applying CRISPRa in plantae employed EDLL and TAD-AD transcription factors to upregulate the *NbPDS* gene (Piatek et al., 2015).

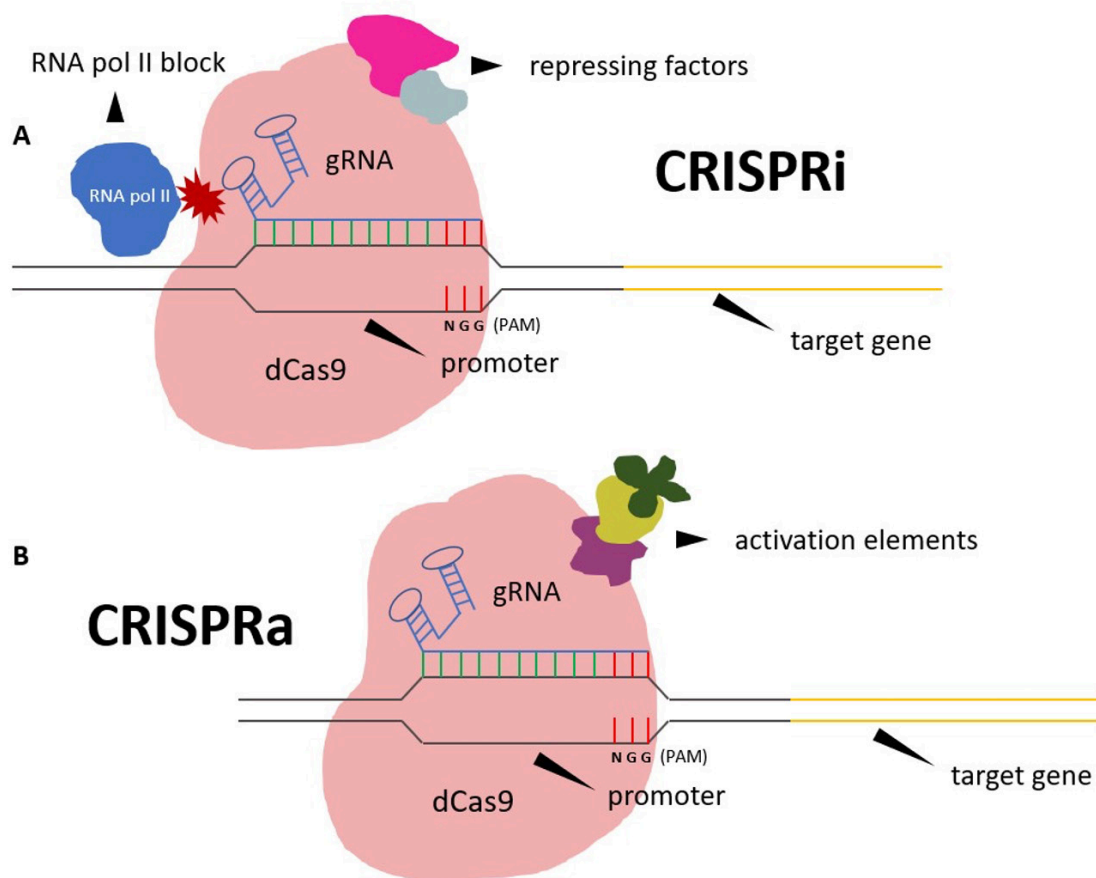


Fig. 4. Composition of CRISPR interference (CRISPRi) and CRISPR activation (CRISPRa). (A) Repressing factors down-regulate the target gene expression by blocking RNA polymerase II activity. (B) Attachment of activation elements upregulates the target gene expression by amplified promoter activity. gRNA, guide RNA; dCas9, dead Cas9; PAM, protospacer-adjacent motif.

Prime editing

Prime editing is the latest development in the CRISPR/Cas chronicle. Its intricacy and precision in editing undesired bases facilitates all 12 possible bases to base conversions, overcoming the limitations of existing base editors (Anzalone et al., 2019). In combination with Cas9 H840A (nCAs9 with deactivated HNH domain) and its C-terminal fused with modified Moloney murine leukemia virus reverse transcriptase (M-MLV-RT) and prime editing guide RNA [pegRNA comprising the primer-binding site, reverse transcriptase (RT) template with edit sequences, and sgRNAs], the prime editing system works to reshape the target site (Anzalone et al., 2019) (Fig. 5).

Precision editing via the prime editor is achieved by the following steps: nCas9 binds to the target site under the direction of sgRNA and nicks the non-target stand. The nicked strand hybridizes to the primer-binding site and reverse transcription extends the edited strand by making use of the polymerized strand as a primer. The 3' flap containing the edit and the unedited 5' flap undergo flap equilibration, resulting in edited DNA by subsequent activities of structure-specific endonucleases and 5' exonucleases (Anzalone et al., 2019; Marzec et al., 2020).

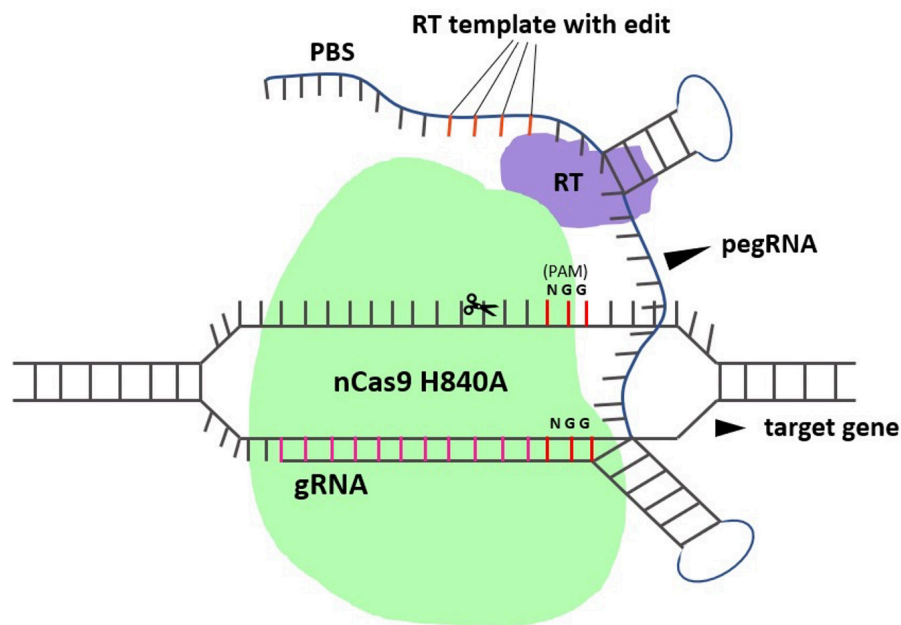


Fig. 5. Structure of prime editor 3. Cas9 nickase (H840A Cas9) generates a single-strand break on the non-target strand and the nicked strand directly polymerizes with a short-edited strand produced by reverse transcriptase attached on Cas9 nickase. The modified sequences of the reverse transcriptase (RT) template are part of prime editing guide RNA (pegRNA). PBS, primer binding site; PAM, protospacer-adjacent motif.

The initial research produced generations of the prime editor revised up to prime editor 3 (Anzalone et al., 2019). Lin et al. (2020) instantly applied the system in rice and wheat for validation, and plant prime editor 3 has been proven to be adoptable in higher plants (Li et al., 2020).

CRISPR Knock-in

In the presence of a donor template, the DSB can be mended by HDR (Liang et al., 1998; Lieber, 2010). Operation of HDR with a manipulated donor template containing viable sequences allows foreign DNA fragments to “Knock-in” (Zheng et al., 2014) while repeated generation of the DSB on the target sequences by Cas9 routinely “Knock-outs” the gene through NHEJ (Wu et al., 2013). Employing CRISPR knock-in permits the performance of a wide range of genetic engineering from base substitution to removal of unwanted sequences for gene replacement and insertion (Schaeffer and Nakata, 2015), depending on the length of the modified sequences within homology arms (Gilles et al., 2015; Yu et al., 2017; Roth et al., 2018) and the purpose of the experiment. An ideal donor template consists of a 5' homology arm (left arm) and 3' homology arm (right arm) flanking replacing sequences, and each arm must share identical sequences to the host gene for homology recognition (Zheng et al., 2014) (Fig. 6). For the nested gene to properly function in the host gene, a promoter needs to be accompanied at the end of the left arm (Li et al., 2016). Double strand DNA (dsDNA) and single strand DNA (ssDNA) can be used as the donor template; however, recent studies have shown that the use of ssDNAs as templates manifested higher on-target integration rates and lower cytotoxicity than those with dsDNA donors (Roth et al., 2018; Li et al., 2019). Successful operation of the CRISPR knock-in has been demonstrated in rice (Zhang et al., 2016; Xu et al., 2020b) and tomato (Yu et al., 2017).

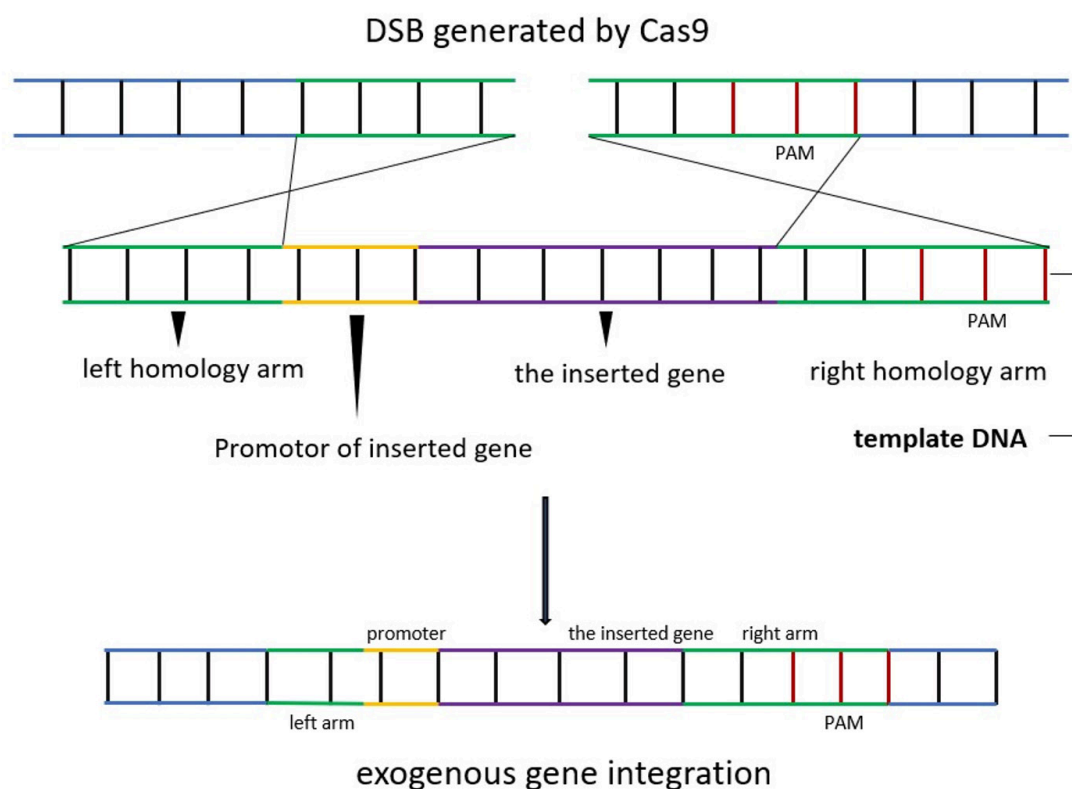


Fig. 6. Gene replacement through homology direct repair (HDR). A functional gene can be inserted to double-strand breaks (DSBs) in the presence of homology arms flanking each end of the target region. Cas9, CRISPR-associated protein 9; PAM, protospacer-adjacent motif.

Application of CRISPR/Cas for floricultural crops

Initial studies of CRISPR/Cas9-mediated transformation in ornamental species mainly targeted genes related to chlorophyll synthesis and nitrogen accumulation, such as phytoene desaturase (Yan et al., 2019; Zhu et al., 2019) and nitrate reductase (Subburaj et al., 2016) to induce a conspicuous phenotype, albinism and yellowing, as the transformed plant material would immediately show a sign of the mutations. Subburaj et al. (2016) applied PEG-mediated delivery of the RNP complex to produce mutant petunia protoplasts free of external DNA (Table 1).

Later studies focused on modification of flower colors and successfully altered flower colors by targeting genes involved in anthocyanin biosynthesis (Watanabe et al., 2017; Nishihara et al., 2018; Watanabe et al., 2018; Yu et al., 2020). Among these experiments, Watanabe et al. (2017) had a distinct approach to increase carotenoid content in petals of Japanese morning glory (*Ipomoea nil*) by targeting *carotenoid cleavage dioxygenase 4 (CCD4)*, which is known to reduce carotenoid accumulation. A DNA-free approach was applied to obtain a complete modification of flower color by simultaneous targeting of both F3HA and F3HB loci via CRISPR/Cas9 ribonucleoproteins in petunia (Yu et al., 2020).

Table 1. Application of CRISPR/Cas9 on ornamental species and delivery methods of vectors.

Species	Delivery method	Material	Cas9 promoter	sgRNA promoter	Target gene	Final produce	Phenotype	Reference
<i>Chrysanthemum morifolium</i> 'Set-Marin'	Agrobacterium	Young leaves	<i>PcUbi</i>	<i>AtU6</i>	<i>GpYGFP</i>	Plant	Decreased fluorescence	Kishi-Kaboshi et al., 2017
<i>Crocus sativus</i>	Agrobacterium	Corms	CaMV 35S	-	-	Plant	-	Chib et al., 2020
<i>Dendrobium officinale</i>	Agrobacterium	Protocorms	CaMV 35S	OsU3	<i>C3HC4H, 4CL, CCR, IRX</i>	Plant	-	Kui et al., 2017
<i>Fortunella hindsii</i>	Agrobacterium	Seedlings	CaMV 35S	AtU6	<i>FhpDS</i>	Plant	Albino leaves	Zhu et al., 2019
<i>Ipomoea nil</i> 'AK77'	Agrobacterium	Embryos	<i>PcUbi</i>	AtU6g	<i>InCCD4</i>	Plant	Altered flower color	Watanabe et al., 2018
<i>Ipomoea nil</i> 'AK77'	Agrobacterium	Embryos	<i>PcUbi</i>	AtU6g	<i>InEPHI</i>	Plant	Extended flower longevity	Shibuya et al., 2018
<i>Ipomoea nil</i> 'Violet'	Agrobacterium	Embryos	<i>PcUbi</i>	AtU6g	<i>InDFR</i>	Plant	Altered flower color	Watanabe et al., 2017
<i>Lilium longiflorum</i> 'White Heaven'	Agrobacterium	Seedling scales	Ubi	OsU3	<i>LIPDS</i>	Plant	Albino leaves	Yan et al., 2019
<i>Lilium purnilum</i>	Agrobacterium	Embryos	Ubi	OsU3	<i>LpPDS</i>	Plant	Albino leaves	Yan et al., 2019
<i>Petunia hybrida</i> 'Madness Midnight'	PEG	Protoplasts	-	-	<i>PhNR</i>	Protoplasts	-	Subburaj et al., 2016
<i>Petunia hybrida</i> 'Madness Midnight'	PEG	Protoplasts	-	-	<i>PhF3H</i>	Protoplasts	Flower color-	Yu et al., 2020
<i>Petunia hybrida</i> 'Mirage Rose'	Agrobacterium	Young leaves	CaMV 35S	AtU6	<i>PhACO1</i>	plant	Extended flower longevity	Xu et al., 2020a
<i>Petunia hybrida</i> 'Mitchell'	Agrobacterium	Young leaves	CaMV 35S	AtU6	<i>PhPDS</i>	plant	Albino leaves	Zhang et al., 2016
<i>Petunia inflata</i>	Agrobacterium	Young leaves	CaMV 35S	AtU6	<i>PiSSK1</i>	plant	Altered compatibility of pollens	Sun and Kao, 2018
<i>Torenia fourieri</i> 'Crown Violet'	Agrobacterium	Young leaves	CaMV 35S	AtU6	<i>TTF3H</i>	plant	Altered flower color	Nishihara et al., 2018

Recently, the primary focus of genome editing in ornamental species has moved to extending the vase life of reproductive organs (Shibuya et al., 2018; Xu et al., 2020a). Shibuya et al. (2018) identified a Nac transcription factor *Ephemeral 1*, whose expression is predominant in petals of Japanese morning glory. Knockout of *InEPHI* resulted in delayed petal senescence in Japanese morning glory. In 2019, prolonged flower longevity was observed in petunia by modulating ethylene biosynthesis (Xu et al., 2020a).

Despite the ever-increasing fame of CRISPR/Cas9, publications regarding the improvement in ornamental value in floricultural crops are barely noticeable owing to the factors described in chapter 3.

Factors impeding the application of CRISPR/Cas9 for ornamental species

Diversity could be said to be a key attribute of ornamental species (Clark et al., 2003). Numerous ornamental species are introduced to the floriculture industry each year (Adkins et al., 2003). Because thorough considerations must be made to determine the target gene and an elucidated biosynthetic pathway is required prior to genome editing, the diversity of floricultural crops further complicates the application of the CRISPR/Cas9 system. Currently, the majority of genome editing work in plantae is focused on model plants and horticultural crops (Karkute et al., 2017) (Fig. 7) because of easier access to genetic information and facilitated protocols for cultivation than those in floricultural crops. The most frequently used plant species for CRISPR-mediated genome editing since 2013 is Asian rice (*Oryza sativa*, 217 publications). Thale cress (*Arabidopsis thaliana*, 104), tomato (*Solanum lycopersicum*, 89), common wheat (*Triticum aestivum*, 66), soybean (*Glycine max*, 36), potato (*Solanum tuberosum*, 32), and maize (*Zea mays*, 28) are next in descending order. Adequate genetic studies for each species need to be conducted to promote the use of the CRISPR system for floricultural crops.

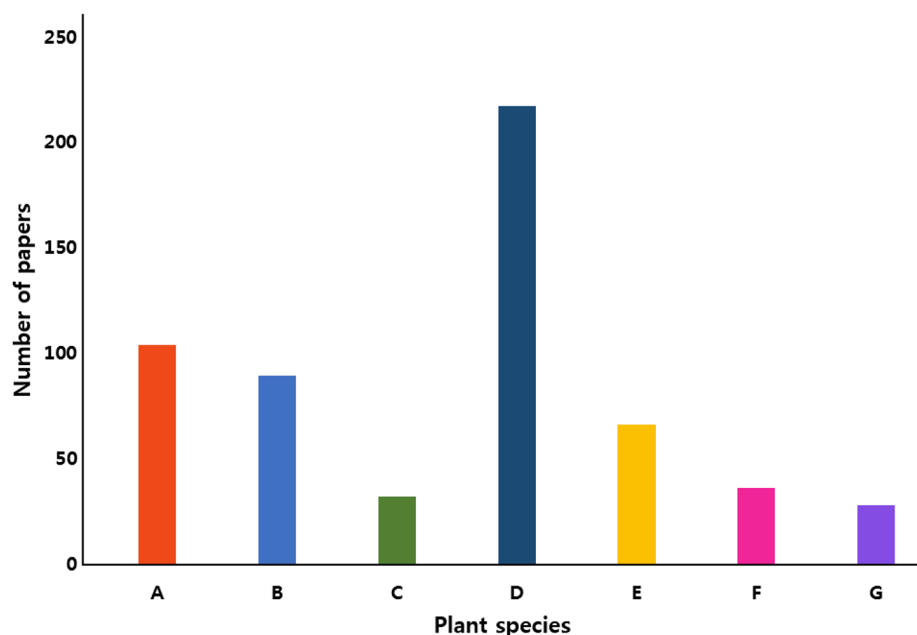


Fig. 7. Cumulative numbers of publications for plant species used for clustered regularly interspaced palindromic repeats (CRISPR)-mediated genome editing of various plant species. (A) *Arabidopsis thaliana*, (B) *Solanum lycopersicum*, (C) *Solanum tuberosum*, (D) *Oryza sativa*, (E) *Triticum aestivum*, (F) *Glycine max*, (G) *Zea mays*.

In general, ploidy of plants is another factor affecting the stability and efficiency of genome editing (Kishi-Kaboshi et al., 2017). As extra sets of chromosomes can manipulate cell size, color depth, and other features leading to morphological changes, polyploidy is common in ornamental cultivars (Manzoor et al., 2019). Complete knockout of multiple alleles of polyploid species becomes increasingly challenging as the number of chromosome sets increases (Yu et al., 2020). A few alleles may continue to function while simultaneous gene silencing is in process in transgenic plants, for which *Agrobacterium* has been used as the transformation mediator (Kishi-Kaboshi et al., 2017). Direct delivery of sgRNA and purified recombinant Cas9 by chemical and physical methods still faces the same dilemma (Andersson et al., 2017).

Selection of diploid cultivars as plant material may avoid the aforementioned difficulty in a limited manner; however, the use of experimental subjects with more than two sets of chromosomes is often inevitable because an ideal plant material would possess commercially favorable traits in addition to the gene of interest aimed for modification. Moreover, there are ample possibilities that genome-edited plant material is directly introduced to the horticulture industry; therefore, we suggest that researchers operate genome editing tools on competitive strains of plant material. As seen in the knock-out experiment of *InCCD4* in Japanese morning glory (Watanabe et al., 2017), mutant lines showed a 20-fold increase in carotenoid accumulation relative to the wild type, but yellow coloration was not prominent owing to genetic limitations of the plant material used in the experiment. The results of the experiment implied the importance of the genetic capacity that the subject holds. Making use of haploid plants from pollen culture and microspore embryogenesis may increase the efficiency of site-directed mutagenesis in polyploid plant material (Wang et al., 2019a). The ploidy can be restored by chemical treatment such as the use of colchicine after evaluation of genome editing of haploid plant subjects (Saint-Oyant et al., 2018).

Tolerance of polyploidy and the mode of reproduction, wherein somatic mutations can be inherited through generations by differentiation of reproductive organs from multiple shoot meristems, enable plants to have higher mutation rates (Schoen and Schultz, 2019) in contrast to animals. Intra-specific and intra-cultivar variations in DNA can often be detected (Wang et al., 2019b), thus making it necessary for researchers to precisely sequence the target gene from their plant material. This issue results in more complicated operations to generate target-specific mutations in plant cells than in animals.

DNA-free genome editing

RNP in combination with gRNA and Cas9 can be directly introduced into competent protoplasts (Yu et al., 2020). Delivery of naked DNA material through the chemical action of PEG (Junker et al., 1987; Maas and Werr, 1989) and physical mechanisms of electroporation (Fromm et al., 1985; Fromm et al., 1987) is particularly important as introduced RNP molecules generate mutations similar to natural mutations in the target region and degenerate soon after the transfection, resulting in mutant protoplasts without any exogenous DNA material remaining. In addition, the two methods are most commonly used for transfection.

PEG-mediated transfection

PEG comprises repetitive ethylene glycol polymers. PEG is known to be innocuous to cells and is hydrophilic (Lee and Kim, 2005), making it an ideal reagent to help increase the solubility of proteins and genetic material by bioconjugation. Different PEGs with varying molecular weights are commercially available, and the broad range of use of PEG in biology is attributed to its low-cost, accessibility, and fair transfection efficiencies (Lee and Kim, 2005).

The conjugates from covalent binding of biomolecules with PEG have positive charges and interact with the negatively charged cell membranes of the protoplasts, resulting in uptake of the biomolecules into the cytoplasm through endocytosis (Wagner et al., 1991; Zalipsky and Harris, 1997). Woo et al. (2015) successfully introduced RNPs to protoplasts of *Arabidopsis thaliana*, tobacco, lettuce, and rice by PEG-mediated transfection in 2015, and this approach has also been adopted in petunia (Subburaj et al., 2016; Yu et al., 2020), apples, and grapes (Osakabe et al., 2018).

Electroporation

Electroporation is a process in which exogenous biomolecules are introduced to the cytoplasm through cell membranes that are temporarily permeabilized using electric pulses (Neuman, 1982). Cells in mixture with biomolecules are exposed to an intense electric field, and the lipid bilayer is disrupted to undergo electric breakdown. Increased conductance of cell membranes leads to transient pore formation, and external biomolecules can be transmitted to the cells (Neuman, 1982; Tsong, 1989; Tieleman, 2004; Potter and Heller, 2018). Although transfection by electroporation can be applied to any cell type, no published article in terms of delivery of RNP by electroporation in higher plants has been reported to date.

Future aspects of genome editing in ornamental species

Possession of attractive colors is one of the most important factors affecting customers' purchase decisions in the floriculture industry (Behe et al., 1999). Chen et al. (2017) isolated an *R2R3* transcription factor from grape hyacinth, namely *MaAN2*. Transformation and overexpression of *MaAN2* in tobacco upregulated genes involved in anthocyanin biosynthesis and produced transgenic tobacco portraying visible dark coloration.

Vase life is another major factor that attracts customers (Rihn et al., 2014). Ethylene is a plant hormone associated with various developmental processes in plants (Burg and Burg, 1965). Elevated levels of exogenous and endogenous ethylene accelerate senescence by causing ethylene responses (Picton et al., 1993). Liu et al. (2016) silenced putative ethylene signaling downstream components *EIL1* and *EIL2* in petunia by the virus-induced gene silencing method. Their monitoring revealed that *EIL* genes are functionally redundant and down-regulation of the two genes influences the longevity of petunia flowers.

We expect that replacing one of the *EIL* genes (Solano et al., 1998; Liu et al., 2016) with *MaAN2* (Chen et al., 2017) by CRISPR knock-in and enhancement of pigmentation by CRISPRa-facilitated upregulation of the transgene would simultaneously improve two categories of aesthetic quality in mutant lines: beauty and consistency of the beauty per se. The resulting plants would produce flowers with deep color and extended longevity, with negligible after-effects. Failure in ethylene perception often triggers severe maldevelopment in plants (Shibuya et al., 2004). However, silencing of an *EIL* gene did not display noticeable phenotypic changes other than extended flower longevity in transfected plants, indicating functional redundancy of *EIL* genes (Tieman et al., 2001; Liu et al., 2016).

Irrespective of the preference of patrons and the ease of detection, natural alteration of colors in plants is commonly observed (Sparrow and Sparrow, 1976). Because metabolic pathways related to the production of pigments incorporate diverse metabolites catalyzed by numerous enzymes, the chances of mutations are high in enzymes involved in complicated biosynthesis, such as anthocyanin and carotenoid synthesis (Clegg and Durbin, 2000; Tanaka et al., 2010). Flower phenotypes with pale colors or albinism often result from very small changes in base sequences (Clegg and Durbin, 2000; Nishihara et al., 2011) in the form of point mutations. The function of the mutated gene can be modified by CRISPR/Cas9 derived techniques and base editing, prime editing, and CRISPR knock-in through HDR are good candidates (Yu et al., 2020).

The emergence of the CRISPR/Cas system has replaced many existing genetic engineering techniques. Fresh approaches using the CRISPR system are being actively and continuously proposed and instantly applied, although validation of their safety, stability, and feasibility is ongoing. New advances and future discoveries of the system are expected to hasten plant breeding, and it will be up to society to assess its superior performance among other genome-editing tools.

Conclusion

The mainstream genome engineering community apparently favors the CRISPR/Cas system. In this review, we introduced several genome editing techniques derived from the CRISPR/Cas system within the scope of our knowledge and application of these tools in floricultural crops. We anticipate that our work will expand the reader's imagination and understanding in terms of genome editing mediated by the CRISPR/Cas system. The development of genetic engineering has enabled mankind to decipher biological phenomena related to our well-being. The CRISPR/Cas system will pave the way toward new discoveries and applications much further than ever achieved through other technologies. We confidently assert that researchers working on breeding ornamental species will take advantage of the use of the CRISPR/Cas system.

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References

- Adkins JA, Werner DJ, Ranney TG. 2003. Prospects for genetically modified ornamental plants. *Proceedings of The Southern Nursery Association* 48:502-504.
- Andersson M, Turesson H, Nicolai A, Fält AS, Samuelsson M, Hofvander P. 2017. Efficient targeted multiallelic mutagenesis in tetraploid potato (*Solanum tuberosum*) by transient CRISPR-Cas9 expression in protoplasts. *Plant Cell Reports* 36:117-128.
- Anzalone AV, Randolph PB, Davis JR, Sousa AA, Koblan LW, Levy JM, Chen PJ, Wilson C, Newby GA, Raguram A, Liu DR. 2019. Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature* 576:149-157.

- Babu M, Beloglazova N, Flick R, Graham C, Skarina T, Nocek B, Gagarinova A, Pogoutse O, Brown G, Binkowski A, Phanse S. 2011. A dual function of the CRISPR–Cas system in bacterial antiviral immunity and DNA repair. *Molecular Microbiology* 79:484-502.
- Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, Romero DA, Horvath P. 2007. CRISPR provides acquired resistance against viruses in prokaryotes. *Science* 315:1709-1712.
- Behe B, Nelson R, Barton S, Hall C, Safley CD, Turner S. 1999. Consumer preferences for geranium flower color, leaf variegation, and price. *HortScience* 34:740-742.
- Belhaj K, Chaparro-Garcia A, Kamoun S, Patron NJ, Nekrasov V. 2015. Editing plant genomes with CRISPR/Cas9. *Current Opinion in Biotechnology* 32:76-84.
- Bortesi L, Fischer R. 2015. The CRISPR/Cas9 system for plant genome editing and beyond. *Biotechnology Advances* 33:41-52.
- Braatz J, Harloff HJ, Mascher M, Stein N, Himmelbach A, Jung C. 2017. CRISPR-Cas9 targeted mutagenesis leads to simultaneous modification of different homoeologous gene copies in polyploid oilseed rape (*Brassica napus*). *Plant Physiology* 174:935-942.
- Brouns SJ, Jore MM, Lundgren M, Westra ER, Slijkhuis RJ, Snijders AP, Dickman MJ, Makarova KS, Koonin EV, Van Der Oost J. 2008. Small CRISPR RNAs guide antiviral defense in prokaryotes. *Science* 321:960-964.
- Burg SP, Burg EA. 1965. Ethylene action and the ripening of fruits: Ethylene influences the growth and development of plants and is the hormone which initiates fruit ripening. *Science* 148:1190-1196.
- Chen K, Liu H, Lou Q, Liu Y. 2017. Ectopic expression of the grape hyacinth (*Muscari armeniacum*) R2R3-MYB transcription factor gene, *MaAN2*, induces anthocyanin accumulation in tobacco. *Frontiers in Plant Science* 8:965.
- Chib S, Thangaraj A, Kaul S, Dhar MK, Kaul T. 2020. Development of a system for efficient callus production, somatic embryogenesis and gene editing using CRISPR/Cas9 in Saffron (*Crocus sativus* L.). *Plant Methods* 16:1-10.
- Chylinski K, Anaïs LR, Charpentier E. 2013. The tracrRNA and Cas9 families of type II CRISPR-Cas immunity systems. *RNA Biology* 10:726-737.
- Clark DG, Loucas H, Shibuya K, Underwood B, Barry K, Jandrew J. 2003. Biotechnology of floriculture crops—scientific questions and real world answers. In *Plant Biotechnology 2002 and Beyond*. pp. 337-342. Springer, Dordrecht, Netherlands.
- Clegg MT, Durbin ML. 2000. Flower color variation: A model for the experimental study of evolution. *Proceedings of the National Academy of Sciences* 97:7016-7023.
- Deltcheva E, Chylinski K, Sharma CM, Gonzales K, Chao Y, Pirzada ZA, Eckert MR, Vogel J, Charpentier E. 2011. CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature* 471:602-607.
- Friedland AE, Tzur YB, Esvelt KM, Colaiácovo MP, Church GM, Calarco JA. 2013. Heritable genome editing in *C. elegans* via a CRISPR-Cas9 system. *Nature Methods* 10:741-743.
- Fromm M, Callis J, Taylor LP, Walbot V. 1987. [21] Electroporation of DNA and RNA into plant protoplasts. In *Methods in Enzymology*. Vol. 153. pp. 351-366. Academic Press, Cambridge, USA.
- Fromm M, Taylor LP, Walbot V. 1985. Expression of genes transferred into monocot and dicot plant cells by electroporation. *Proceedings of The National Academy of Sciences* 82:5824-5828.
- Fujita T, Fujii H. 2013. Efficient isolation of specific genomic regions and identification of associated proteins by engineered DNA-binding molecule-mediated chromatin immunoprecipitation (enChIP) using CRISPR. *Biochemical and Biophysical Research Communications* 439:132-136.
- Gaudelli NM, Komor AC, Rees HA, Packer MS, Badran AH, Bryson DI, Liu DR. 2017. Programmable base editing of A•T to G•C in genomic DNA without DNA cleavage. *Nature* 551:464-471.
- Gilbert LA, Larson MH, Morsut L, Liu Z, Brar GA, Torres SE, Stern-Ginossar N, Brandman O, Whitehead EH, Doudna JA, Lim WA. 2013. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell* 154:442-451.
- Gilles AF, Schinko JB, Averof M. 2015. Efficient CRISPR-mediated gene targeting and transgene replacement in the beetle *Tribolium castaneum*. *Development* 142:2832-2839.

- Guilinger JP, Thompson DB, Liu DR. 2014. Fusion of catalytically inactive Cas9 to FokI nuclease improves the specificity of genome modification. *Nature Biotechnology* 32:577.
- Hale C, Kleppe K, Tern RM, Terns MP. 2008. Prokaryotic silencing (psi) RNAs in *Pyrococcus furiosus*. *Rna* 14:2572-2579.
- Hale CR, Zhao P, Olson S, Duff MO, Graveley BR, Wells L, Terns RM, Terns MP. 2009. RNA-guided RNA cleavage by a CRISPR RNA-Cas protein complex. *Cell* 139:945-956.
- Hara S, Tamano M, Yamashita S, Kato T, Saito T, Sakuma T, Yamamoto T, Inui M, Takada S. 2015. Generation of mutant mice via the CRISPR/Cas9 system using FokI-dCas9. *Scientific Reports* 5:11221.
- ISAAA (International Service for the Acquisition of Agri-biotech Applications). 2020. South Korea's first genome edited petunia approved in the U.S. Accessed in www.isaaa.org on 16 October 2020.
- Jansen R, Embden JDV, Gaastra W, Schouls LM. 2002. Identification of genes that are associated with DNA repeats in prokaryotes. *Molecular Microbiology* 43:1565-1575.
- Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337:816-821.
- Junker B, Zimny J, Lührs R, Lörz H. 1987. Transient expression of chimaeric genes in dividing and non-dividing cereal protoplasts after PEG-induced DNA uptake. *Plant Cell Reports* 6:329-332.
- Karkute SG, Singh AK, Gupta OP, Singh PM, Singh B. 2017. CRISPR/Cas9 mediated genome engineering for improvement of horticultural crops. *Frontiers in Plant Science* 8:1635.
- Kim DS, Kim JE, Hur JK, Been KW, Yoon SH, Kim JS. 2016. Genome-wide analysis reveals specificities of Cpf1 endonucleases in human cells. *Nature Biotechnology* 34:863-868.
- Kim R, Song JE, Ga EJ, Min MK, Lee JY, Lim SH, Kim BG. 2019. Current status of CRISPR/Cas9 base editor technologies and their applications in crop precision breeding. *Korean Journal of Agricultural Science* 46:885-895. [in Korean]
- Kishi-Kaboshi M, Aida R, Sasaki K. 2017. Generation of gene-edited *Chrysanthemum morifolium* using multicopy transgenes as targets and markers. *Plant and Cell Physiology* 58:216-226.
- Kishi-Kaboshi M, Aida R, Sasaki K. 2018. Genome engineering in ornamental plants: Current status and future prospects. *Plant Physiology and Biochemistry* 131:47-52.
- Kleinstiver BP, Tsai SQ, Prew MS, Nguyen NT, Welch MM, Lopez JM, McCaw ZR, Aryee MJ, Joung JK. 2016. Genome-wide specificity profiles of CRISPR-Cas Cpf1 nucleases in human cells. *BioRxiv* 2016:057802.
- Komor AC, Kim YB, Packer MS, Zuris JA, Liu DR. 2016. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature* 533:420-424.
- Kui L, Chen H, Zhang W, He S, Xiong Z, Zhang Y, Yan L, Zhong C, He F, Chen J, Zeng P. 2017. Building a genetic manipulation tool box for orchid biology: Identification of constitutive promoters and application of CRISPR/Cas9 in the orchid, *Dendrobium officinale*. *Frontiers in Plant Science* 7:2036.
- Landrum MJ, Lee JM, Benson M, Brow G, Chao C, Chitipiralla S, Gu B, Hart J, Hoffman D, Hoover J, Jang W. 2016. ClinVar: Public archive of interpretations of clinically relevant variants. *Nucleic Acids Research* 44:862-868.
- Landrum MJ, Lee JM, Riley GR, Jang W, Rubinstein WS, Church DM, Maglott DR. 2014. ClinVar: Public archive of relationships among sequence variation and human phenotype. *Nucleic Acids Research* 42:980-985.
- Larson MH, Gilbert LA, Wang X, Lim WA, Weissman JS, Qi LS. 2013. CRISPR interference (CRISPRi) for sequence-specific control of gene expression. *Nature Protocols* 8:2180-2196.
- Lee M, Kim SW. 2005. Polyethylene glycol-conjugated copolymers for plasmid DNA delivery. *Pharmaceutical Research* 22:1-10.
- Liang F, Han M, Romanienko PJ, Jasin M. 1998. Homology-directed repair is a major double-strand break repair pathway in mammalian cells. *Proceedings of The National Academy of Sciences* 95:5172-5177.
- Lieber MR. 2010. The mechanism of double-strand DNA break repair by the non-homologous DNA end-joining pathway. *Annual Review of Biochemistry* 79:181-211.
- Li H, Beckman KA, Pessino V, Huang B, Weissman JS, Leonetti MD. 2019. Design and specificity of long ssDNA donors for CRISPR-based knock-in. *BioRxiv* 2019:178905.

- Li H, Li J, Chen J, Yan L, Xia L. 2020. Precise modifications of both exogenous and endogenous genes in rice by prime editing. *Molecular Plant* 13:671-674.
- Li J, Meng X, Zong Y, Chen K, Zhang H, Liu J, Li J, Gao C. 2016. Gene replacements and insertions in rice by intron targeting using CRISPR-Cas9. *Nature Plants* 2:1-6.
- Lin Q, Zong Y, Xue C, Wang S, Jin S, Zhu Z, Wang Y, Anzalone AV, Raguram A, Doman JL, Liu DR. 2020. Prime genome editing in rice and wheat. *Nature Biotechnology* 38:582-585.
- Liu F, Hu L, Cai Y, Lin H, Liu J, Yu Y. 2016. Molecular characterization and functional analysis of two petunia PhEILs. *Frontiers in Plant Science* 7:1606.
- Maas C, Werr W. 1989. Mechanism and optimized conditions for PEG mediated DNA transfection into plant protoplasts. *Plant Cell Reports* 8:148-151.
- Maeder ML, Linder SJ, Cascio VM, Fu Y, Ho QH, Joung JK. 2013. CRISPR RNA-guided activation of endogenous human genes. *Nature Methods* 10:977-979.
- Manzoor A, Ahmad T, Bashir MA, Hafiz IA, Silvestri C. 2019. Studies on colchicine induced chromosome doubling for enhancement of quality traits in ornamental plants. *Plants* 8:194.
- Marraffini LA, Sontheimer EJ. 2008. CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. *Science* 322:1843-1845.
- Marzec M, Braszewska-Zalewska A, Hensel G. 2020. Prime editing: A new way for genome editing. *Trends in Cell Biology* 30:257-259.
- Mojica FJ, Díez-Villaseñor C, García-Martínez J, Almendros C. 2009. Short motif sequences determine the targets of the prokaryotic CRISPR defence system. *Microbiology* 155:733-740.
- Mojica FJ, García-Martínez J, Soria E. 2005. Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. *Journal of Molecular Evolution* 60:174-182.
- Neuman E. 1982. Gene transfer into mouse lymphoma cells by electroporation in high electric field. *The European Molecular Biology Organization Journal* 1:841-845.
- Nihongaki Y, Yamamoto S, Kawano F, Suzuki H, Sato M. 2015. CRISPR-Cas9-based photoactivatable transcription system. *Chemistry & Biology* 22:169-174.
- Nishida K, Arazoe T, Yachie N, Banno S, Kakimoto M, Tabata M, Mochizuki M, Miyabe A, Araki M, Hara KY, Shimatani Z. 2016. Targeted nucleotide editing using hybrid prokaryotic and vertebrate adaptive immune systems. *Science* 353:aaf8729.
- Nishihara M, Higuchi A, Watanabe A, Tasaki K. 2018. Application of the CRISPR/Cas9 system for modification of flower color in *Torenia fournieri*. *BMC Plant Biology* 18:1-9.
- Nishihara M, Hikage T, Yamada E, Nakatsuka T. 2011. A single-base substitution suppresses flower color mutation caused by a novel miniature inverted-repeat transposable element in gentian. *Molecular Genetics and Genomics* 286:371-382.
- Noman A, Aqeel M, Deng J, Khalid N, Sanaullah T, Shuilin H. 2017. Biotechnological advancements for improving floral attributes in ornamental plants. *Frontiers in Plant Science* 8:530.
- Osakabe Y, Liang Z, Ren C, Nishitani C, Osakabe K, Wada M, Komori S, Malnoy M, Velasco R, Poli M, Jung MH. 2018. CRISPR-Cas9-mediated genome editing in apple and grapevine. *Nature Protocols* 13:2844-2863.
- Piatek A, Ali Z, Baazim H, Li L, Abulfaraj A, Al-Shareef S, Aouida M, Mahfouz MM. 2015. RNA - guided transcriptional regulation in planta via synthetic dCas9 - based transcription factors. *Plant Biotechnology Journal* 13:578-589.
- Picton S, Barton SL, Bouzayen M, Hamilton AJ, Grierson D. 1993. Altered fruit ripening and leaf senescence in tomatoes expressing an antisense ethylene - forming enzyme transgene. *The Plant Journal* 3:469-481.
- Potter H, Heller R. 2018. Transfection by electroporation. *Current Protocols in Molecular Biology* 121:9-3.
- Pourcel C, Salvignol G, Vergnaud G. 2005. CRISPR elements in *Yersinia pestis* acquire new repeats by preferential uptake of bacteriophage DNA and provide additional tools for evolutionary studies. *Microbiology* 151:653-663.
- Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP, Lim WA. 2013. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* 152:1173-1183.

- Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. 2013. Genome engineering using the CRISPR-Cas9 system. *Nature Protocols* 8:2281-2308.
- Rees HA, Liu DR. 2018. Base editing: Precision chemistry on the genome and transcriptome of living cells. *Nature Reviews Genetics* 19:770-788.
- Rihn AL, Yue C, Hall C, Behe BK. 2014. Consumer preferences for longevity information and guarantees on cut flower arrangements. *HortScience* 49:769-778.
- Roth TL, Puig-Saus C, Yu R, Shifrut E, Carnevale J, Li PJ, Hiatt J, Saco J, Krystofinski P, Li H, Tobin V. 2018. Reprogramming human T cell function and specificity with non-viral genome targeting. *Nature* 559:405-409.
- Saint-Oyant LH, Ruttink T, Hamama L, Kirov I, Lakhwani D, Zhou NN, Bourke PM, Daccord N, Leus L, Schulz D, Van de Geest H. 2018. A high-quality genome sequence of *Rosa chinensis* to elucidate ornamental traits. *Nature Plants* 4:473-484.
- Schaeffer SM, Nakata PA. 2015. CRISPR/Cas9-mediated genome editing and gene replacement in plants: Transitioning from lab to field. *Plant Science* 240:130-142.
- Schoen DJ, Schultz ST. 2019. Somatic mutation and evolution in plants. *Annual Review of Ecology, Evolution and Systematics* 50:49-73.
- Shibuya K, Barry KG, Ciardi JA, Loucas HM, Underwood BA, Nourizadeh S, Ecker JR, Klee HJ, Clark DG. 2004. The central role of PhEIN2 in ethylene responses throughout plant development in petunia. *Plant Physiology* 136:2900-2912.
- Shibuya K, Watanabe K, Ono M. 2018. CRISPR/Cas9-mediated mutagenesis of the *EPHEMERAL1* locus that regulates petal senescence in Japanese morning glory. *Plant Physiology and Biochemistry* 131:53-57.
- Smith I, Greenside PG, Natoli T, Lahr DL, Wadden D, Tirosch I, Narayan R, Root DE, Golub TR, Subramanian A, Doench JG. 2017. Evaluation of RNAi and CRISPR technologies by large-scale gene expression profiling in the Connectivity Map. *PLoS Biology* 15:e2003213.
- Solano R, Stepanova A, Chao Q, Ecker JR. 1998. Nuclear events in ethylene signaling: A transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. *Genes & Development* 12:3703-3714.
- Sparrow AH, Sparrow RC. 1976. Spontaneous somatic mutation frequencies for flower color in several *Tradescantia* species and hybrids. *Environmental and Experimental Botany* 16:23-43.
- Subburaj S, Chung SJ, Lee CG, Ryu SM, Kim DH, Kim JS, Bae SS, Lee GJ. 2016. Site-directed mutagenesis in *Petunia × hybrida* protoplast system using direct delivery of purified recombinant Cas9 ribonucleoproteins. *Plant Cell Reports* 35:1535-1544.
- Sun L, Kao TH. 2018. CRISPR/Cas9-mediated knockout of PiSSK1 reveals essential role of S-locus F-box protein-containing SCF complexes in recognition of non-self S-RNases during cross-compatible pollination in self-incompatible *Petunia inflata*. *Plant Reproduction* 31:129-143.
- Tanaka A, Shikazono N, Hase Y. 2010. Studies on biological effects of ion beams on lethality, molecular nature of mutation, mutation rate, and spectrum of mutation phenotype for mutation breeding in higher plants. *Journal of Radiation Research* 51:223-233.
- Tieleman DP. 2004. The molecular basis of electroporation. *BMC Biochemistry* 5:10.
- Tieman DM, Ciardi JA, Taylor MG, Klee HJ. 2001. Members of the tomato *LeEIL* (EIN3 - like) gene family are functionally redundant and regulate ethylene responses throughout plant development. *The Plant Journal* 26:47-58.
- Tsong TY. 1989. Electroporation of cell membranes. In *Electroporation and Electrofusion in Cell Biology*. pp. 149-163. Springer, Boston, MA, USA.
- Vogel P, Moschref M, Li Q, Merkle T, Selvasaravanan KD, Li JB, Stafforst T. 2018. Efficient and precise editing of endogenous transcripts with SNAP-tagged ADARs. *Nature Methods* 15:535-538.
- Wagner E, Cotten M, Foisner R, Birnstiel ML. 1991. Transferrin-polycation-DNA complexes: The effect of polycations on the structure of the complex and DNA delivery to cells. *Proceedings of the National Academy of Sciences* 88:4255-4259.
- Wang B, Zhu L, Zhao B, Zhao Y, Xie Y, Zheng Z, Li Y, Sun J, Wang H. 2019a. Development of a haploid-inducer mediated genome editing system for accelerating maize breeding. *Molecular Plant* 12:597-602.

- Wang L, Ji Y, Hu Y, Hu H, Jia X, Jiang M, Zhang X, Zhao L, Zhang Y, Jia Y, Qin C. 2019b. The architecture of intra-organism mutation rate variation in plants. *PLoS Biology* 17:e3000191.
- Watanabe K, Kobayashi A, Endo M, Sage-Ono K, Toki S, Ono M. 2017. CRISPR/Cas9-mediated mutagenesis of the dihydroflavonol-4-reductase-B (DFR-B) locus in the Japanese morning glory *Ipomoea (Pharbitis) nil*. *Scientific Reports* 7:1-9.
- Watanabe K, Oda-Yamamizo C, Sage-Ono K, Ohmiya A, Ono M. 2018. Alteration of flower colour in *Ipomoea nil* through CRISPR/Cas9-mediated mutagenesis of carotenoid cleavage dioxygenase 4. *Transgenic Research* 27:25-38.
- Woo JW, Kim JE, Kwon SI, Corvalán C, Cho SW, Kim HR, Kim SG, Kim ST, Choe SH, Kim JS. 2015. DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins. *Nature Biotechnology* 33:1162-1164.
- Wu Y, Liang D, Wang Y, Bai M, Tang W, Bao S, Yan Z, Li D, Li J. 2013. Correction of a genetic disease in mouse via use of CRISPR-Cas9. *Cell Stem Cell* 13:659-662.
- Wyvekens N, Topkar VV, Khayter C, Joung JK, Tsai SQ. 2015. Dimeric CRISPR RNA-guided FokI-dCas9 nucleases directed by truncated gRNAs for highly specific genome editing. *Human Gene Therapy* 26:425-431.
- Xiong K, Marquart KF, la Cour Karottki KJ, Li S, Shamie I, Lee JS, Gerling S, Yeo NC, Chavez A, Lee GM, Lewis NE. 2019. Reduced apoptosis in Chinese hamster ovary cells via optimized CRISPR interference. *Biotechnology and Bioengineering* 116:1813-1819.
- Xu J, Kang BC, Naing AH, Bae SJ, Kim JS, Kim HR, Kim CK. 2020a. CRISPR/Cas9-mediated editing of 1-aminocyclopropane-1-carboxylate oxidase1 enhances *Petunia* flower longevity. *Plant Biotechnology Journal* 18:287-297.
- Xu Y, Wang F, Chen Z, Wang J, Li WQ, Fan F, Tao Y, Zhao L, Zhong W, Zhu QH, Yang J. 2020b. Intron-targeted gene insertion in rice using CRISPR/Cas9: A case study of the *Pi-ta* gene. *The Crop Journal* 8:424-431.
- Yan R, Wang Z, Ren Y, Li H, Liu N, Sun H. 2019. Establishment of efficient genetic transformation systems and application of CRISPR/Cas9 genome editing technology in *Lilium pumilum* DC. Fisch. and *Lilium longiflorum* 'White Heaven'. *International Journal of Molecular Sciences* 20:2920.
- Yu J, Tu L, Subburaj S, Bae S, Lee GJ. 2020. Simultaneous targeting of duplicated genes in *Petunia* protoplasts for flower color modification via CRISPR-Cas9 ribonucleoproteins. *Plant Cell Reports* (in print). doi.org/10.1007/s00299-020-02593-1
- Yu QH, Wang B, Li N, Tang Y, Yang S, Yang T, Xu J, Guo C, Yan P, Wang Q, Asmutola P. 2017. CRISPR/Cas9-induced targeted mutagenesis and gene replacement to generate long-shelf life tomato lines. *Scientific Reports* 7:1-9.
- Zalipsky S, Harris JM. 1997. In introduction to chemistry and biological applications of poly (ethylene glycol). p. 1-13. ACS publications, Washington D.C., USA.
- Zetsche B, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, Essletzbichler P, Volz SE, Joung J, Van Der Oost J, Regev A, Koonin EV. 2015. Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell* 163:759-771.
- Zetsche B, Heidenreich M, Mohanraju P, Fedorova I, Kneppers J, DeGennaro EM, Winblad N, Choudhury SR, Abudayyeh OO, Gootenberg JS, Wu WY. 2017. Multiplex gene editing by CRISPR-Cpf1 using a single crRNA array. *Nature Biotechnology* 35:31-34.
- Zhang B, Yang X, Yang C, Li M, Guo Y. 2016. Exploiting the CRISPR/Cas9 system for targeted genome mutagenesis in *petunia*. *Scientific Reports* 6:20315.
- Zheng Q, Cai X, Tan MH, Schaffert S, Arnold CP, Gong X, Chen CZ, Huang S. 2014. Precise gene deletion and replacement using the CRISPR/Cas9 system in human cells. *Biotechniques* 57:115-124.
- Zhu C, Zheng X, Huang Y, Ye J, Chen P, Zhang C, Zhao F, Xie Z, Zhang S, Wang N, Li H. 2019. Genome sequencing and CRISPR/Cas9 gene editing of an early flowering Mini - Citrus (*Fortunella hindsii*). *Plant Biotechnology Journal* 17:2199-2210.