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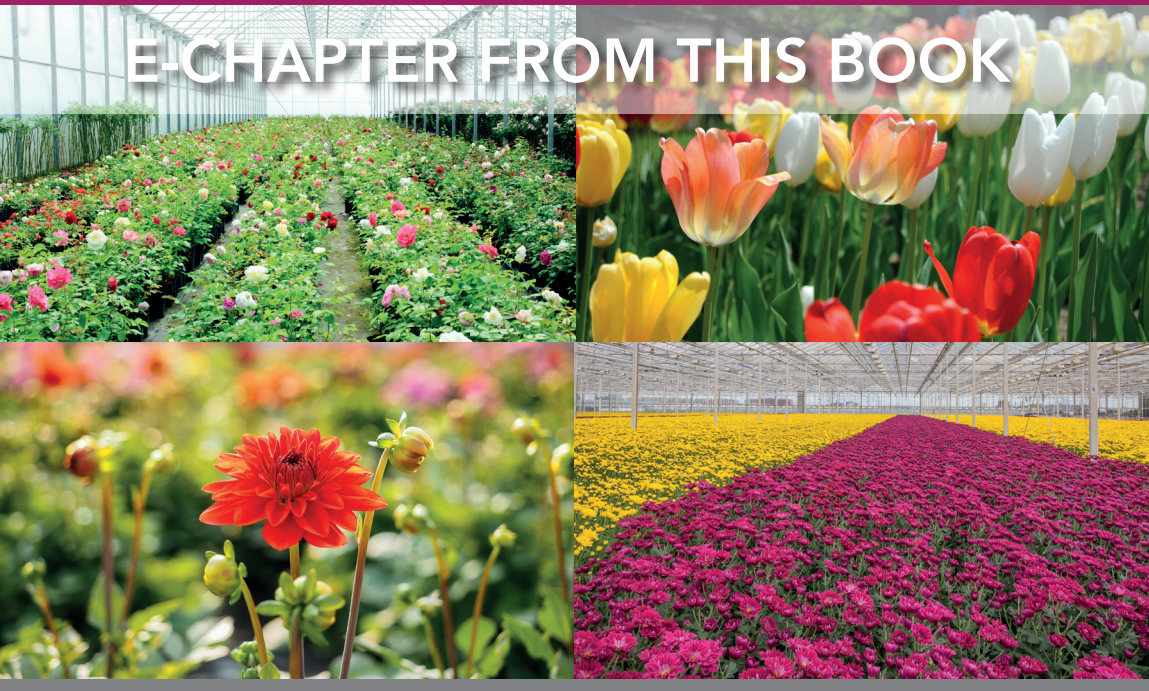

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The use of gene-editing techniques in breeding improved ornamentals

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1 Introduction

The ornamental plant market is of global economic significance with Europe being the major player where the turnover from the Netherlands reached €4.7 billion in 2017 (FloraHolland, 2018). There is a continued quest for cultivars with new and improved characteristics, for example, petal/foliage colors, enhanced scent, improved plant architecture, biotic/abiotic stresses and extended postharvest life, for example, tolerance to the phytohormone ethylene (Azadi et al., 2016). Although, the transfer of new traits is achievable with conventional and mutational breeding it encounters restrictions. For example, hybridization barriers prevent natural introgression of traits from distantly related species (Kuligowska et al., 2016; Shibata, 2008; Teixeira da Silva et al., 2011). A way to circumvent this issue has been obtained in the form of genetic engineering through guided mutations, which is synergistically driven by the development of genome-sequencing initiatives of ornamental plants (as reviewed by Azadi et al., 2016).

Novel-targeted delivery of new traits had an impressive development after the clustered regularly interspaced short palindromic repeats (CRISPR) technique advent, revolutionizing the molecular breeding possibilities. Since the first publication exploiting the CRISPR system for genome editing in 2013 (as reviewed by Eş et al., 2019), the number of published articles has steadily grown

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reaching over 17 000 in less than 7 years (Pubmed query: CRISPR, Updated on 17 February 2020). This chapter first describes the background for the genome-editing technology, with specific emphasis on the CRISPR technology followed by its applicability in plants. Subsequently, the use in horticulture is depicted through a case study targeting the potted plant *Campanula*. In this example, an experiment was carried out in the attempt to produce plants less sensitive to ethylene. A step-by-step genetic-editing method will be outlined. Finally, the ornamental perspective uses and regulations are described.

1.1 Genome-editing background

The aim to more precisely control DNA modifications has led to the development of genome-editing technologies. Precise genome editing refers to the use of engineered sequence specific nucleases (SSN) to modify targeted-genome sequences (Gaj et al., 2013; Kemp et al., 2017). The central idea behind these technologies is creating double stranded breaks (DSB) in genomic DNA and induce cellular repair by either of two different mechanisms: homology directed repair (HDR) or non-homologous end-joining (NHEJ) (Fig. 1) (Xiong et al., 2015).

The advantage of NHEJ is its error-prone feature, thus the DNA lesions will be repaired imperfectly, generating insertions or deletions (indels) within the target sequence (Wyman and Kanaar, 2006). Indels can generate a frameshift mutation, disrupting important functional domains, causing, for example, a gene knockout (Zhou et al., 2014).

On the other hand, the HDR pathway mediates insertion of specific sequences in the targeted locus by using a homologous donor template, of either endogenous or exogenous origin (Gratz et al., 2014; Liang et al., 2017).

The idea of using engineered nuclease systems, such as CRISPR, was preceded by other systems like zinc-finger nucleases (ZFNs) and later, transcription activator-like effector nucleases (TALENs). These systems overcame some of the limitations of approaches like homologous recombination and RNA interference (Boettcher and McManus, 2015; Gaj et al., 2013; Kim and Kim, 2014). Through the use of tailored DNA-binding domains fused to nucleases, the system is capable of recognizing specific DNA sequences and introduces DSBs.

However, the valuable target precision facilitated by the ZFN and TALEN methodologies is hampered by their elaborate and time-consuming procedures. The size of the delivered cassette can negatively affect the cloning and delivery steps, where the typical TALEN cDNA is approximately 3 kb whereas the ZFN cDNA is approximately 1 kb. Furthermore, both systems require dimerization, which increases the final effective size while limited by the transfer capacity of effective adenovirus delivery systems, that is, < 5 kb (Ellis et al., 2013; Gupta

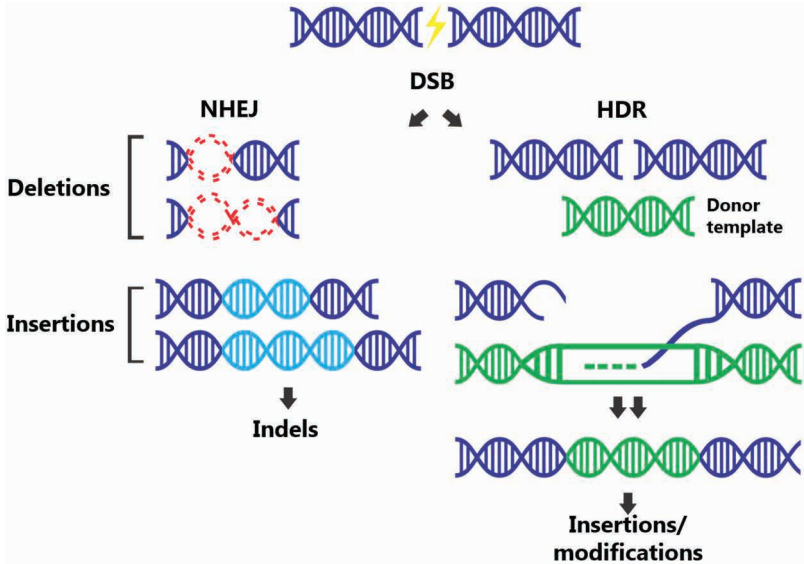

Figure 1 The double-stranded break (DSB) repair mechanisms, that is, non-homologous end joining (NHEJ) or homology-directed repair (HDR). The result changes depending on the donor template presence; when absent, the NHEJ pathway will prevail generating small insertions or deletions (indels). While in its presence, the donor template with extended homology arms, leads to introduction of single or multiple inserts to correct or replace existing gene sections (HDR).

and Musunuru, 2014). Moreover, a high risk of sequence rearrangement and truncation in TALEN systems are associated with the repetitive DNA-binding domains (Holkers et al., 2013) which require extensive transcriptional and translational optimizations considering, for example, codon usage or promoter design (Liu et al., 2015).

The precise genome-editing field was revolutionized by the appearance of CRISPR-associated protein 9 (Cas9) technology (Cong et al., 2013). This technology was modified based on archaea and bacteria defense mechanism first hypothesized by Mojica et al. (2005) and proposed by Makarova et al. (2006). The identification of gene regions with small direct repeats in prokaryotes, that is, CRISPRs, has been known since 1987 (Ishino et al., 1987) and it is shared among multiple species (Mojica et al., 2000). These repetitive regions are interspaced by non-repetitive regions where in the upstream region nuclease homology is always found (i.e. *CRISPR-associated* (*CAS*) genes) (Jansen et al., 2002). In addition, the non-repetitive regions are derived from bacteriophages and are dissimilar even between closely related species (Makarova et al., 2006). Thus, leading to the adaptive immunity hypothesis involving CRISPR regions and nucleases, where foreign bacteriophage DNA, that is, fragment/spacer, is

integrated into the prokaryote genome, preventing a future attack by the same phage strain (Barrangou et al., 2007).

The spacer sequences are complementary to previously encountered viral DNA (Marraffini and Sontheimer, 2008) and are located upstream of a conserved protospacer adjacent motif (PAM) (Sternberg et al., 2014). In a next viral infection event, bacterial transcription of a CRISPR RNA (crRNA) library matches the invading DNA and a variety of defense systems can result in DNA inactivation. The studies of defense system type II in prokaryotes paved the way to the precise genome editing possibilities. In defense type II, the crRNA and the trans-activating RNA (tracrRNA) form the duplex at the target spacer plus PAM leading to dsDNA cleavage by the Cas9 protein (Fig. 2) (Sorek et al., 2013). It was early noticed that by exchanging the crRNA complementarity, the whole complex could precisely edit different sequences. Moreover, the type II is suited for use in genome editing because it has a single protein performing the targeting and cutting (Garneau et al., 2010).

A system optimization toward a simpler programmable RNA-guided DNA endonuclease later arose with the crRNA and tracrRNA fusion into a single-guide RNA (sgRNA), rather than two (Gasiunas et al., 2012).

1.2 Precise genome editing in plants

So far, most research utilizing the CRISPR/Cas9 system has been conducted in prokaryote and mammalian cells, however many researchers within the

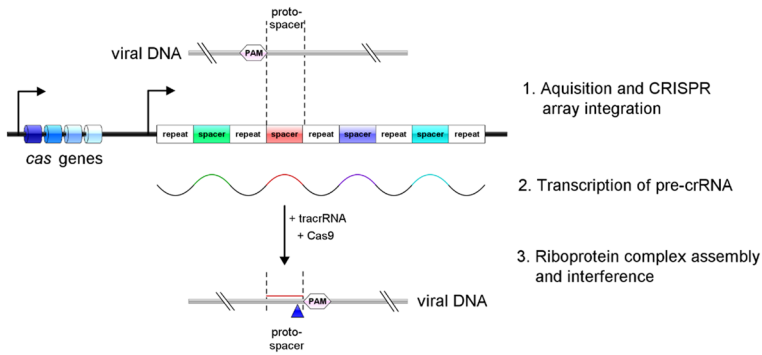

Figure 2 Steps of the CRISPR/Cas9 adaptive immune system in prokaryotes. (1) During bacteriophage attack, the foreign DNA is injected into the cell and small pieces of direct repeats are integrated in the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) loci. (2) Upon subsequent invasion, the bacterial cells transcribe and process crRNAs that form riboprotein complexes with the transactivating RNA and the Cas-proteins. (3) These complexes enable the direct targeting by Cas nucleases toward viral DNA inducing its cleavage (blue triangle) 3 nucleotide upstream of the protospacer adjacent motif (PAM) site.

field of plant research have recognized the potential for the technique. Some approaches focus on the application of the system, that is, the 'dead' Cas9 (dCas9) where the nuclease activity is not present, but the targeted aspects of the system continues to be present (Eid et al., 2018). This can lead to applications like reversible gene silencing in *Staphylococcus aureus* (Zhao et al., 2017), replication fork arrest for DNA replication studied (Whinn et al., 2019) or base editing as it does not require DSB. In addition, transcriptional activator or repressor can be fused to dCas9 (Hille and Charpentier, 2016) whereas other studies are focusing on optimizing or expanding the functionalities of the method. The CRISPR/Cas9 system comprises multiple important elements, many of which can be constantly optimized to increase the efficiency of the system.

Due to the fact that both the protein (Cas9) and the RNA (sgRNA) are part of the expression cassette, two separate promoters are needed to ensure accurate timing of expression of either *Cas9* or sgRNA. The expression of *Cas9* is often controlled by the constitutive promoter *Cauliflower Mosaic Virus 35S* (*35S*). However, some studies have experimented with alternative promoters to obtain tissue specific expression of both *Cas9* and sgRNA (Table 1). Examples such as the *ICU2p* and *EC1.2* promoters with activity in frequently dividing cells, that is, callus and meristems, have been shown to increase efficiency and result in fewer chimeric T1 plants (Hyun et al., 2015; Wang et al., 2015). Previous studies have successfully utilized the RNA polymerase III promoters, *AtU6* and *AtU6-26* to transcribe sgRNAs in various plants, for example, *Nicotiana benthamiana* and *Solanum lycopersicum*, showing that these promoters can facilitate transcription in other dicots. The CRISPR/Cas9 system has also been modified to employ multiple sgRNAs in a single vector – a technique that often results in higher efficiency and enables the testing of multiple sgRNAs at once (Li et al., 2013; Ma et al., 2015). This could save time as the efficiencies of sgRNAs are not entirely predictable.

Precise DNA insertion and replacement provide new possibilities in plant breeding. Higher plants are often referred to as being recalcitrant to homologous recombination (HR) (Eckardt, 2006; Hanin and Paszkowski, 2003). The nickase Cas9 proteins in which one of the two catalytic domains, HNH or RuvC, have been inactivated by deletions allow single-stranded cleavage as opposed to the DSB of the wild type Cas9 (Trevino and Zhang, 2014). It has been shown that nickases may be used to enhance DNA insertions through HDR and decrease off-target effects (Fauser et al., 2014). Furthermore, recent studies have reported the successful use of gemini viruses' vectors, a technique that has shown to increase the efficiency of HR-mediated insertions in plants. The rationale behind usage of gemini viruses as vectors is their capability to infect many host species across numerous families while requiring a single protein (RepA for master viruses) for replication initiation in the host cell

Table 1 Examples of studies using the novel precise genome-editing tool CRISPR/Cas9 in ornamental plants

Plant species	Cas9 promoter	sgRNA promoter	Delivery method	Traits/targets	References
Chrysanthemum morifolium	*PcUbi*	*AtU6-26*	*A. tumefaciens*	Decreased fluorescence	Kishi-Kaboshi et al. (2017)
Dendrobium officinale	C3H, C4H, 4CL, CCR, IRX	*OsU3*	*A. tumefaciens*	Lignocellulose biosynthesis pathway	Kui et al. (2017)
Ipomoea nil	*pDE*	*AtU6*	*A. tumefaciens*	Decreased petal senescence	Shibuya et al. (2018)
Ipomoea nil 'Violet'	*PcUbi*	*AtU6*	*A. tumefaciens*	Flower color change	Watanabe et al. (2017)
Ipomoea nil 'AK77'	*PcUbi*	*AtU6*	*A. tumefaciens*	Flower color change	Watanabe et al. (2018)
Petunia hybrida Mitchell diploid	*35S*	*AtU6*	*A. tumefaciens*	Albino	Zhang et al. (2016)
Petunia hybrida 'Madness' series	-	-	Transfection	-	Subburaj et al. (2016)
Torenia fournieri	35S	*AtU6-26*	*A. tumefaciens*	Flower color modification	Nishihara et al. (2018)
Trapogon spp.	*AtUBQ10*	*AtU6-1, AtU6-26, AtU6-29*	*A. tumefaciens*	Polyploidy studies	Shan et al. (2018)

Key elements have been highlighted, such as promoters and method of delivery.

(Baltes et al., 2014). In addition, its replication mechanism involves HR and interferes with the host cell cycle, reverting it to the S-phase, which is ideal for HR (Richter et al., 2016). Moreover, geminiviruses display high-replication efficiency producing replicons at elevated rates, consequently increasing SSNs while improving targeting efficiency (Čermák et al., 2015; Hanley-Bowdoin et al., 2013). Moreover, the delivery methods vary from particle bombardment to *Agrobacterium*-mediated methods (Table 1). Optimal strategies for delivery and transformation are highly dependent on plant species and may present an obstacle when working with non-model plant species.

2 Applications in horticulture: a case study on Campanula

The first reports for the CRISPR genome-editing technique mainly covered model plants, for example, *Arabidopsis thaliana* and *N. benthamiana*, but recent articles include major agricultural crops, for example, *Brassica oleracea* var. *capitata* (Ma et al., 2019), *Glycine max* (Bao et al., 2019), *Oryza sativa* (Pathak et al., 2019) among others and several ornamental species (Table 1). The development and potential of precise genome editing is progressing at a rapid pace and the applicability of the system to new plant species follows.

For the potted plant and cut flower industry, flower longevity is pivotal and of great importance for the customer appeal and satisfaction (Ferrante et al., 2015). Exposure to the phytohormone ethylene leads to senescence symptoms like leaf, bud and flower drop in many plants which significantly impair the plant product. Hence, ethylene insensitivity is a desirable trait to target in breeding of ornamental plants. Because of this, ethylene biosynthesis and the signal transduction pathway are of great interest for researchers working with ornamentals. Previous studies have shed light on the correlation of mutations in genes associated with the ethylene signal transduction pathway and ethylene insensitivity. This has inspired various studies in which genetic transformation has been utilized to introduce ethylene insensitivity in plants. In Europe, the use of transgenic plants may reduce the commercial market potential for such cultivars. Precise genome editing may provide a new and in some cases, less controversial way of modifying plant traits in existing cultivars. Ethylene signaling is involved in a wide array of biological functions and completely interrupting ethylene signaling may therefore not be fortuitous for plant breeders, as it will have consequences for other plant functions (Little et al., 2009). Consequently, it is important to consider the implications of targeting various ethylene-associated genes. This section will elaborate on scientific literature within this topic and propose strategic targets for precise gene knockout in *Campanula* mediated by CRISPR/Cas9.

To inhibit senescence and increase longevity of ornamental plants, producers often apply chemical inhibitors such as 1-methylcyclopropene

(1-MCP), which acts as a competitive ligand and prevents ethylene signal transduction. However, increased awareness of conscientious use of chemicals has incited research into alternative solutions (Lütken et al., 2012). Ethylene signal transduction is perceived by ER-membrane embedded receptors that act as negative regulators of ethylene responses in the absence of ethylene (Fig. 3) (Hua and Meyerowitz, 1998). When ethylene is perceived, the receptors disassociate from the Constitutive Triple Response factor 1 (CTR1) (Fig. 3). CTR1 is a ser/thr kinase repressor that constitutively represses ethylene insensitive 2 (EIN2) by targeting it for proteolytic degradation (Huang et al., 2003). When

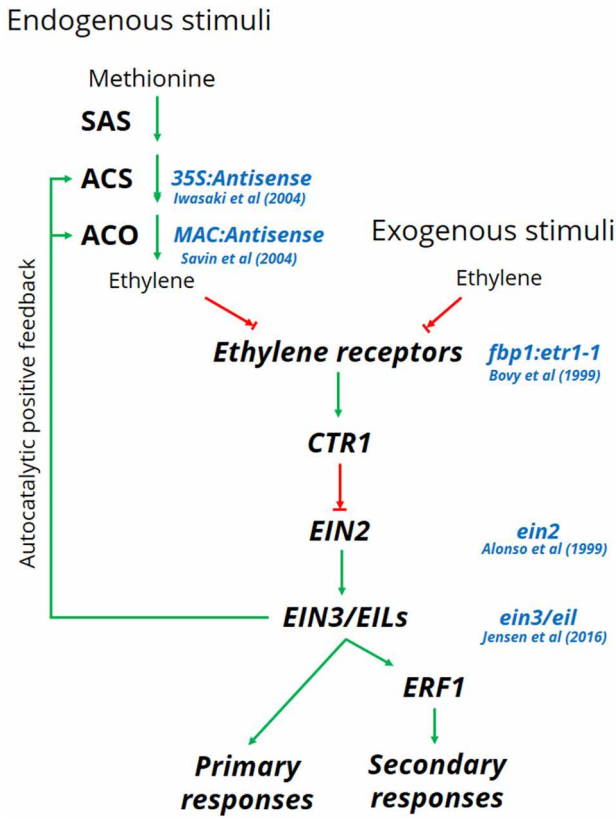

Figure 3 Ethylene synthesis and signaling targets for molecular modification. Key components of the ethylene biosynthesis and simplified signaling pathways are shown in black. Positive and negative regulations are shown in green and red, respectively. Molecular modification strategies are shown in blue. SAS: S-adenosylmethionine synthase; ACS: aminocyclopropane carboxylate synthase; *35S*: constitutive promoter; ACO: aminocyclopropane carboxylic acid oxidase; *MAC*: constitutive promoter; *fbp1*: flower specific promoter; CTR1: constitutive triple response 1; *EIN2*: *ethylene insensitive 2*; *EIN3*: *ethylene insensitive 3* / EILs: EIN3-like proteins; ERF1: ethylene response factor 1.

ethylene is perceived, the C-terminal end of EIN2 is cleaved and translocated to the nucleus where it serves as a positive regulator of ethylene responses. This is likely achieved by stabilization of transcription factors such as EIN3/EILs, by inhibiting EIN3 binding F-box 1 and 2 (EBF1/2) mediated degradation (An et al., 2010). The EIN3 and EILs transcription factors initiate downstream ethylene responses (Chao et al., 1997; Yamasaki et al., 2005) (Fig. 3). Previous studies have utilized transgenic approaches to alter ethylene perception, leading to decreased ethylene sensitivity in various ornamental plants, for example, *Campanula* and *Kalanchoë* (Fig. 3) (Sanikhani et al., 2008; Sriskandarajah et al., 2007). However, these studies rely on transgenic expression of missense-mutated ethylene response 1 (*etr1*) ethylene receptors from *A. thaliana* making them fall within the scope of GMO legislation (Lütken et al., 2012).

2.1 Targets for precise genome editing

Based on the scientific literature discussed in the previous section there are multiple candidate targets for precise genome editing that may result in decreased ethylene sensitivity. However, because ethylene signaling is involved in many important developmental functions it is important to consider the consequence of altering ethylene signaling. The optimal target for genetic modification should result in reduced ethylene-induced flower senescence to prevent bud and flower drop, while having minimal effect on other ethylene-induced responses.

It can be hypothesized that interference in later steps is preferable to early steps as this may result in fewer undesired pleiotropic effects. RNA interference of ACS and ACO genes have previously shown success in reducing endogenous ethylene production and extending vase life of *D. caryophyllus* cut flowers from regenerated lines harboring the ACS sense and ACS/ACO antisense constructs (Iwazaki et al., 2004; Savin et al., 1995). One of the problems with that approach was that it interferes early in the pathway and may result in lack of proper ethylene signaling in other biological functions, for example, low yield in regeneration after transformation. Another issue is that it does not inhibit exogenous stimuli.

Ethylene insensitivity may also be achieved by overexpression of receptors or by knockout of *EIN2*, these approaches have some drawbacks. Constitutive overexpression may have a greater chance of having pleiotropic effects as ethylene signaling is interfered equally throughout all developmental stages and downstream responses. Previous studies have therefore used a flower specific promoter (*FBP1*) from petunia (Bovy et al., 1999) (Fig. 3). By using a *fbp1::etr1-1* mutant, Winkelmann et al. (2016), showed that the use of a tissue specific promoter was effective in elevating gene expression in the perianth of *Burrageara* orchids. In comparison to untransformed plants, the vase life was

extended 7 days and the bud drop initiation was delayed up to 19 days in the transgenic plants. This is however not a suitable target for targeted knockout by precise genome editing since single mutations in ethylene receptors often results in little to no phenotype (Hua and Meyerowitz, 1998). Mutagenic knockout of EIN2 may be even more troublesome as it resulted in complete ethylene insensitivity in *A. thaliana* (Alonso et al., 1999) (Fig. 3). Downstream transcription factors may also have multiple response genes related to abiotic stresses and be involved in various developmental stages (reviewed by Debbarma et al., 2019). However, results from the recent study by Jensen et al. (2016) correlated a natural mutation in *EIL2* in the commercially available *C. medium* with reduced ethylene-induced senescence. This is a very interesting target for precise genome editing as it suggests that a mutation in orthologous genes in related species may result in similar phenotypes.

The study by Jensen et al. (2016) correlated an ethylene-insensitive phenotype in *C. medium* with a 7 bp frameshift mutation in the *Cmeil2* gene, sharing homology to EIN3/EIL transcription factors (Figs. 4 and 5). In that study,

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CfEIL1_KX058427.1       CTCGCTTTTATCAGCTTTGATGCAGCATTGTGACCCTCCTCAGAGAAGGTTCCCTCTTGA   474
CmEIL1_KX058429.1       CTCGCTTTTATCAGCTTTGATGCAGCATTGTGACCCTCCTCAGAGAAGGTTCCCTCTTGA   474
CpEIL1b_KX058426.1      CTCACTTTTATCAGCTCTGATGCAGCATTGTGACCCTCCGCAAAGGCGGTTCCCTCTAGA   474
CpEIL1a_KX058425.1      CTCACTTTTATCAGCTCTGATGCAGCATTGTGACCCTCCTCAGAGACGGTTCCCTCTAGA   477
CfEIL2_KX058428.1       TTCGTTATTATCTGCTCTCATGCAGCATTGTGATCCTCCCCAGAGGCGTTTCCCTCTGGA   480
CmEIL2_KX058430.1       TTTGTTATTATCTGCTCTC-------ATTGTGATCCTCTACAGAGGCGTTTCCCTCTCGA   473
                         *   * ***** *** *        ******* ****  ** **  * ******** **
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Figure 4 Nucleotide sequence alignment of *Campanula EIL* sequences. Sequence alignment shows the targeted view showing the alignment of *ethylene insensitive 3-like (EIL)* sequences surrounding the 7 bp deletion in *Cmeil2*. Abbreviations: *C. medium* (Cm)*, C. formanekiana* (Cf), *C. portenschlagiana* (Cp) and asterisk (*) indicates alignment consensus. A 7 bp deletion in *Cmeil2* results in loss of gene function. The alignment was produced in Clustal Ω (McWilliam et al., 2013) and the NCBI accession numbers are listed on the figure.

Figure 5 Translated amino acid sequence alignment of AtEIN3 and EIL sequences from *Campanula* species. Partial EIL nucleotide sequences were translated into amino acid sequences and aligned to the annotated EIN3 sequence from *Arabidopsis thaliana*. The red-shaded region represents the proline-rich region, and the black box shows the basic domain III both of which are thought to be part of the DNA-binding domain of the transcription factors. The asterisk marks an important Lys-245 to Asn mutation found in ein3-3 which impairs EIN3 function. The red-highlighted sequence shows the nonsense sequence following a 7 bp deletion found in the nucleotide sequence of *C. medium eil2*. Alignment was made using Clustal Omega and Jalview. Sequences were derived from (Chao et al., 1997; Jensen et al., 2016).

senescence was assessed in response to exposure to ethylene in *C. formanekiana* and *C. medium*. RT-PCR and RT-qPCR revealed that *Cmeil2* was transcribed 100-fold less than *CfEIL2*. The mutation in *Cmeil2* introduced a premature stop codon resulting in a truncated mRNA sequence. *CfEIL* sequences were constitutively expressed in the four-flower developmental stages investigated, whereas *Cmeil2* was only found in very low amounts relative to *CfEIL2* transcripts. In the wild type scenario, an ethylene response would result in stabilization of the transcription factors, thereby eliciting ethylene responses.

However, in the case of *Cmeil2* this will not happen as the protein function has likely been lost (Fig. 4). This would explain the ethylene-insensitive phenotype of *C. medium*. Jensen et al. (2016) also isolated two ~600 bp CpEIL sequences from *C. portenschlagiana* which were named *CpEIL1a* and *b* as they share highest similarity to *CfEIL1* and *CmEIL1* (Fig. 4). These sequences differ by the presence of a *DdeI* restriction site in the *CpEIL1a*.

2.2 Approaches for defining targets in Campanula portenschlagiana

The *Cmeil2* mutation presents a beacon for developing *Campanula* plants with reduced ethylene sensitivity. Unfortunately, *C. medium* is not an important horticultural ornamental plant product, however, *C. portenschlagiana* is a key ornamental plant, with more than 20 million potted plants produced annually in Denmark (Floradania, 2015) This section outlines a strategy describing the overall steps (Table 2) that we have pursued so far for targeting silencing of *EIL1A* and *B* in *C. portenschlagiana*.

2.2.1 Designing gRNAs

Designing several gRNAs (the sequence specificity is conferred by a ~20 bp) for each target gene is needed to direct the nuclease activity for the respective

Table 2 General steps for genome editing of plants

Step	Task	Outcome
1	Designing gRNAs	Target-specific sequences
2	Creating an expression cassette	Gene construct
3	*Agrobacterium tumefaciens* transformation with destination vector	Delivery system
4	Plant transformation, selection using antibiotic marker and regeneration	Putatively edited plants
5	Molecular characterization	Edited plant
6	Outcrossing	Marker and *Cas9* free plants

genomic target to be modified. In the current case study, six gRNAs were designed to introduce a frameshift mutation in the putative *EIL1A* and *B* sequences from *C. portenschlagiana* (Fig. 6a). They were designed using the BroadInstitute online sgRNA designer tool (Doench et al., 2016), where a list based on the algorithm scoring system was generated. The gRNA sequences were selected by both high algorithm score and when disrupting a restriction enzyme (RE) site. The gRNA spacer sequences were appended with an initial G if not present before, as this is required for RNA polymerase III transcription. The gRNAs are shown in Fig. 6b in terms of the activating key component in the CRISPR/Cas9 genome-editing system. Once designed, the sense and antisense (reverse complemented) gRNAs were ordered as small nucleotides (approximately 20 bp plus restriction site sequences). The oligonucleotide pairs were annealed in a thermocycler by ramping the equimolar mixture to 25°C from 95°C at 5°C min^{-1} to create the dsDNA oligonucleotides.

2.2.2 Creating an expression cassette

In the current study, the cloning procedure was divided into two steps, first the dsDNA oligonucleotides were cloned into the entry vector pENTR-CRISPR (Li et al., 2013), creating the expression cassette. Subsequently, *Escherichia*

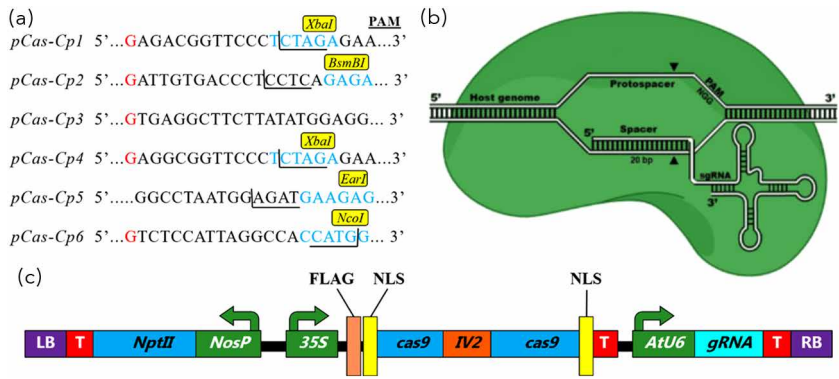

Figure 6 Schematic overview of the CRISPR/Cas9 system design. (a) The six different guide RNA (gRNA) designs used in this case study, illustrated by their DNA targets. Red G's represent appended guanines, blue sequences represents restriction enzyme (RE) recognition sites, black lines show RE cut sites. Yellow boxes show RE enzymes for each sequence. (b) Overview of the CRISPR/Cas9 genome-editing system. The system is activated and directed by the sgRNA. The system introduces blunt DSBs in the targeted DNA sequence (black triangles, Kemp et al., 2017). (c) Schematic overview of the expression cassette design: Kanamycin selection in plants by *NosP::NptII*; *35S::Cas9* with a potato *IV2* intron and flanked by FLAG-tag and nuclear localization signals (NLS); *AtU6*::gRNA; the expression cassette is flanked by left and right border sequences (LB and RB).

coli was transformed and positive clones were selected by ampicillin. The transcription of *SpCas9* from *Streptococcus pyogenes* is plant codon optimized and controlled by the constitutive *35S* promoter whereas the gRNA is controlled by the polymerase III promoter, *AtU6*, from *A. thaliana* (Fig. 6c). *SpCas9* has been appended with nuclear localization signals at both terminal ends and a FLAG-tag at the 5′ end of the *SpCas9*. A potato intron, *IV2*, has been inserted in the *SpCas9* sequence to inhibit adverse effects of the *SpCas9* in bacterial hosts during cloning (Li et al., 2013).

Secondly, the expression cassette was transferred into a binary destination vector (in this case *pK2GW7*) (Karimi et al., 2002). The reaction is driven by recombination and followed the Invitrogen™ Gateway™ LR-Clonase™ II (Thermo Fischer Scientific Inc.) instructions. Subsequently, *E. coli* was transformed and positive clones were selected by spectinomycin. The destination vector confers kanamycin resistance in plants via the *neomycin phosphotransferase II* (*NptII)* gene as selective marker and contains the left and right boarders (LB/ RB) necessary for *Agrobacterium*-mediated transformation (Fig. 6c). To confirm the destination vector *pENTR-CRISPR:pK2GW7* construction, a double RE assay with *EcoRI* and *HindIII* was made. Successful expression vector constructs should exhibit two bands, that is, 900 and 500 bp.

2.2.3 Agrobacterium tumefaciens transformation with destination vector

A. tumefaciens strain GV2850 was transformed according to standard protocols with three sequenced-confirmed plasmids *pENTR-CRISPR:pK2GW7* of each gDNA target from the previous step and positive colonies selected with 50 mg L^{-1} rifampicin / 75 mg L^{-1} spectinomycin.

2.2.4 Plant transformation and regeneration

The *A. tumefaciens* harboring the respective *pENTR-CRISPR:pK2GW7* of each gDNA target was cultured in liquid media using the same abovementioned antibiotics. Petioles of *C. portenschlagiana* derived from axenic culture were inoculated and transformed according to Hegelund et al. (2017) with minor modifications.

2.2.5 Molecular analysis

The first molecular analyses were initiated in young shoots. Firstly, PCR was conducted with primers targeting *Cas9* in order to identify transformed tissue (Fig. 7a). Similarly, PCR with primers targeting the endogenous *Actin* gene were used to verify presence of plant DNA (Fig. 7a). Subsequently, PCR was

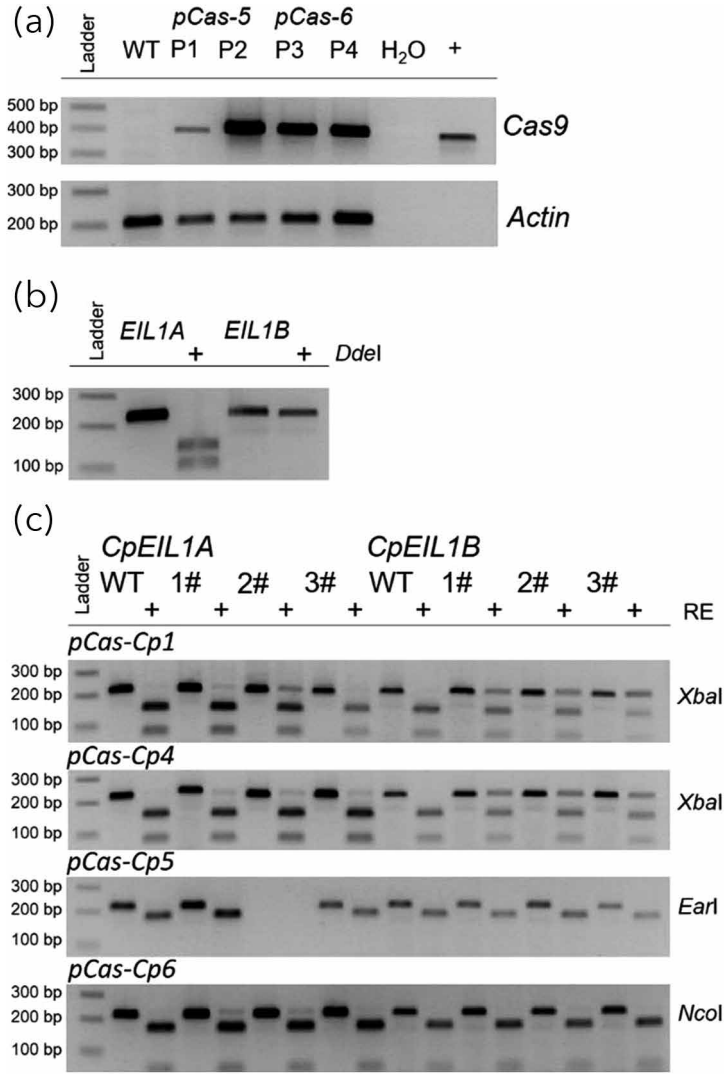

Figure 7 Molecular analysis of transformed plants and tissue. (a) PCR amplification of *Cas9* and *Actin* to test for the presence of expression cassette in four green regenerated shoots (P1-4). WT and water were used as negative controls, *pENTR-CRISPR* was used as positive control. (b) Specific primers for the CRISPR/Cas9-targeted *EIL1A* and *B* sequences were designed. To verify specificity amplicons from WT DNA template was amplified with both primer sets and digested with *Dde*I, which only has a recognition site in *EIL1A*. Digested samples were run next to undigested samples in equal concentrations. (c) *EIL1A* and *B* amplicons were amplified from three representative calli transformations, with four different gRNA designs (*pCas-Cp1-4*). Amplicons were digested with enzymes predicted to be disrupted from CRISPR/Cas9-mediated mutations. Resistant bands indicate CRISPR/Cas9 activity. Restriction digests and undigested (+) amplicons were analyzed in equal concentration. WT amplicons were used to check for complete digestion.

conducted using primers flanking the gRNA. These amplicons were digested with RE with cutting sites predicted to be disrupted from the CRISPR/Cas9 mediated mutations. Hence, resistant bands indicate CRISPR/Cas9 activity (Fig. 7b). Restriction digests (in this case with *Dde*I) were analyzed next to undigested amplicons in equal concentration. Simultaneously, WT amplified amplicons were used to check for complete digestion. In the case study, *EIL1A* and *B* PCR amplicons from four regenerated plants and from 12 calli samples representing in total four separate gRNA designs, *pCas*-[*Cp1, Cp4, Cp5 and Cp6*] were analyzed by RE digest, to indicate RE motif-disrupting indels (Vouillot et al., 2015). *EIL1* amplicons from regenerated plants showed no RE-resistant bands, suggesting that no CRISPR/Cas9 mediated indels have been produced (data not shown). Also, no indels were observed in calli tissue from transformants of *pCas-Cp5* and *pCas-Cp6*. However, calli tissue transformed with *pCas-Cp1*and *pCas-Cp4* showed RE-resistant bands in *EIL1A* and *B* amplicons (Fig. 7c). These results indicate CRISPR/Cas9-mediated indel mutations in targeted C*pEIL1* sequences in calli transformed with *pCas-Cp1+4*. Following regeneration of plantlets, indels should be reconfirmed by RE digests and verified by sequencing.

2.2.6 Outcrossing

Once a genome-edited plant has been obtained, the next step to consider would be the generation of a *Cas9* and marker-free plant. This is achievable by outcrossing of both the antibiotic resistance genes as well as the transgenic *Cas9* gene (Lawrenson et al., 2015; Zhou et al., 2015).

3 Prospective uses and regulation of ornamental plants

The potential benefits of novel transgenic ornamental plants may provide faster implemented diversity in floral and foliage color, architecture and tolerance to stresses (Wolt et al., 2016). However, its applicability is highly dependent on how it is perceived legally, but there is generally no consensus whether the genome-edited plants should be assessed according to the current GMO legislation (Sprink et al., 2016). In the United States, the USDA-APHIS provides a regulatory omission to genetically engineered plants under the regulations at 7 CFR part 340 (USA, 2018). Hilscher et al. (2017) showed an updated list of genome-edited crops approved, including maize with high amylopectin content, white button mushroom with no browning among others.

In the European Union, similar decisions were firstly made and released by Germany, confirming that gRNAs are not recombinant DNA, and the Swedish Board of Agriculture, stated in 2015 the equivalency of CRISPR/Cas9 to mutagenesis as no foreign DNA would remain in plants. However, the Court

of Justice of the European Union ruled against the initial understandings of the breakthrough technique and decided all products CRISPR-derived should fall under the 2001 Directive (European Union, 2001, 2018).

In Brazil, the National Biosafety Technical Commission (CNTBio) approved Normative 16 (Brazil, 2018) indicating a case-by-case assessment and if no transgene is inserted, the crop will not be subjected to GMO regulation. Similar regulations were previously approved by Argentina in 2015 (Orroño and Vesprini, 2018) and Chile in 2017 (Metje-Sprink et al., 2019) where the central factor is the lack of recombinant DNA in the final product for the non-GMO classification. Further policy alignment in the Latin America region has been signaled by Paraguay and Uruguay, these countries have intention to coordinate efforts in expanding Argentina, Brazil and Chile's case-by-case and genome-editing friendly legislation (CAS, 2018).

4 Conclusion

The recent and fast developing CRISPR/Cas9 technique is capable of precise genome editing in plants. The system key factor is specificity and it is defined by a 20 bp long sequence, that is, sgRNA, upstream a PAM sequence, which will guide the Cas9 protein to the DNA target creating a DSB. The DNA damage will be repaired by either NHEJ or HDR. The first can be used to generate knockout mutants and develop further knowledge on specific and combined gene functions, but the later has also the great potential of inserting genes through the HDR repair mechanism. The method has been applied in several agricultural plants and its potential in ornamental species is emerging. Improved tolerance toward the phytohormone ethylene is a key target in breeding as hormones greatly impact the quality of many ornamental plants. A mutation present in *Campanula*, leading to decreased ethylene sensitivity can serve as a platform for CRISPR/Cas9-mediated longevity of ornamental plants.

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6 Where to look for further information

In terms of CRISPR future research it is clear that enormous advancements are made by the day, therefore it is pivotal to maintain yourself updated and a simple way of doing it is to create citation or topic alerts in well-established research databases, for example Scopus, Science Direct, Web of Knowledge and Google Scholar. The experimental workflow will vary depending on the ultimate goal, for instance, if you detected a gene mutation that resulted in a phenotype that is beneficial, the experiment will pursue a CRISPR construct targeting the reproduction of a similar mutation (Table 2). Another strategy is to induce indels in a group of genes to understand the regulatory loop by generating knockout mutants. Additionally, the off-targets prediction tools vastly improved since the advent of gene editing by CRISPR, but always check for recent updates or novel prediction algorithms. Please consult the list of references, there are further readings to complement this book chapter.

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