Exploring the Possibility of Using CRISPR/Cas9 Technology to Obtain Begonia Plants More Resilient to Fusarium Disease

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Abstract

The Begoniaceae family is a very important pantropical family in ornamental plants, and contains more than 1800 species with 15000 hybrids. The genus *Begonia* has been affected by biotic stress conditions, such as *Fusarium foetens*, which lead to losses in greenhouse and nursery production. Obtaining more-resilient plants is a very important breeding goal in ornamentals. Currently, the achievement of this goal is supported by the new gene-editing technology CRISPR/Cas9, which is the most widely used gene-editing technology because of its precise, accessible, and easy-to-use nature. This study aims to explore an approach to obtain more-resilient begonia plants using the CRISPR/Cas9 system. Two possible disease-resistance-related sequences were first identified based on the limited genetic data about begonia available in databases, but upon further closer examination both of them do not seem to be suited as a target sequence for gene-editing approaches because it was found that knocking out their function is unlikely to result in fungal disease resistance in begonias.

Keywords: *Begonia*, *Fusarium*, CRISPR/Cas9, disease resistance, gene editing

Introduction

There are more than 1800 species in the genus *Begonia* (Tseng et al., 2017), which is one of the largest genera of vascular plants (Neale et al., 2006) and is one of the most species-rich angiosperm genera (Harrison et al., 2016). The family has approximately 15000 hybrids across the world (Tseng et al., 2017) and is a pantropical genus (Goodall-Copestake et al., 2009). Begonias are easily recognizable by their diagnostic characteristics for example asymmetrical leaves, unisexual monoecious flowers, twisted-, papillose stigmas, and dry-, three-winged capsules (Doorenbos et al., 1998) and are popular ornamentals. They can be grown outdoors in pots, baskets, hanging baskets, and garden beds (Hvoslef-Eide and Munster, 2006), or indoors as houseplants (Tian et al., 2012).

Begonia classification is complex and is based on morphological features. Therefore, modern methods such as karyomorphological or cytological studies (Dewitte, 2010) have been useful to allow classification and to distinguish between species (Sandgrind, 2017). According to Haegeman, the genus *Begonia* consists of six groups, which are shown in Table 1 (Haegeman, 1979).

Group	Botanical name	Additional information
Tuberous hybrid begonias (hybrids of different tuberous begonia species from the Andes)	*B.* × *tuberhybrida* Voss	Commonly known as summer-flowering begonias
Loraine begonias (*B. socotrana* × *B. dregei*)	*B.* × *cheimantha*	Everett, a typical winter-flowering begonia, is commonly known as Scandinavian, Norwegian, or Christmas begonia
Elatior begonias (*B. socotrana* × tuberous hybrids)	*B.* × *hiemalis* (Fotsch, 1933)	Commonly known as autumn- or winter-flowering begonias and the Hiemalis begonia
Semperflorens begonias (*B. semperflorens* × *B. schmidtiana*)	*B. semperflorens cultorum*	This type is called 'Semperflorens gracilis' in Europe and sometimes the wax begonia in the USA
Begonias with ornamental foliage are mainly *B. rex* cultivars	*B. rex cultorum*	Also *B. masoniana* (*B.* 'Iron Cross') and hybrids of other Mexican species
Other	–	Those that do not fall into any of the categories above

Table 1: The classification of the genus *Begonia*, (Haegeman, 1979).

The first known article about begonias was published in 1651 by Hernandez. Some begonias have local distribution, but the rest are found in subtropical climates, except Australia. Commercial begonias consist mostly of hybrids created with wild begonias found globally (Sandgrind, 2017). Chromosome counts on some hybrid begonias, as well as genetic diversity and gene flow studies on *B. drege*i and *B. homonya*, are available (Matolweni et al., 2000).

In Europe a specific pattern of localization of begonia nurseries has developed, depending on begonia types. Tuberous begonias are produced mainly in Belgium, Elatior begonias in Germany and the Netherlands, Christmas begonias in Scandinavian countries, and Semperflorens begonias in the UK and southern Europe (Sandgrind, 2017).

B. tuberhybrida is a large and heterogeneous group compared to other begonia groups. The first varieties were obtained by crossing different tuberous begonias. The ease of crossing between begonia species has resulted in diversity and complexity within the group (Hvoslef-Eide and Munster, 2006; Sandgrind, 2017)

According to database research, exact data about begonia production is found in only one paper (Sandgrind, 2017). This paper mentions that approximately 2.75 million commercial begonia plants are grown annually in Norway, and these consist mainly of *Begonia* × *hiemalis*, *Begonia rex*, *Begonia semperflorens*, and *Begonia tuberhybrida* in 2015 (Sandgrind, 2017). The World's begonia production was not found in literature.

Fusarium Disease in Begonias

Farmers can incur economic losses due to soil-borne pathogens in crop fields. Hiemalis begonias (*Begonia* × *hiemalis* Fotsch) have been affected by *Fusarium foetens* as the causal agent of wilt, which has resulted in losses in greenhouse and nursery production. Such losses have been reported in Canada, Germany, Japan, the Netherlands, and the USA (Elmer et al., 2004; Schroers et al., 2004; Sekine et al., 2008; Tian et al., 2010). The first report on *Fusarium foetens* was in Elatior begonias in the Netherlands in 2000 and Germany in 2001(Schroers et al., 2004). In addition, some other reports were made about *Fusarium foetens* in Canada, Japan, and the USA (Tian and Zheng, 2013). *Fusarium* wilt symptoms show up on leaves in two to three weeks, then the plant dies six to eight weeks after infection (Tian and Zheng, 2013) and results in severe losses during greenhouse production of Hiemalis begonias (Elmer et al., 2004; Schroers et al., 2004).

Elatior begonias were affected by new, severe *Fusarium* wilt symptoms in European greenhouses in 2001 (Tschoepe et al., 2007). In the Netherlands, quarantine inspections, especially on plant material for propagation, have been intensified (de Weerdt et al., 2006).

Some different approaches to improve disease resistance have been used, especially mutations and RNA-mediated interference. Virus-induced gene silencing and *Agrobacterium*-mediated insertional mutagenesis are available for genetic improvement to disease resistance (Chen et al., 2015), and wheat, maize, rice, barley, green pea and bean were improved on disease and pest resistance (Kozjak and Meglič, 2012).

Begoniaceae: Mega-Diversity and Important Species

The Begoniaceae family is within the order Cucurbitales, which also contains the Anisophylleaceae, Apodanthaceae, Coriariaceae, Corynocarpaceae, Cucurbitaceae, Datiscaceae, and Tetramelaceae families (Dewitte et al., 2009).

Tuberous and rex begonias are important for commercial cultivars. The main parents of the *B.× tuberhybrida* are *B. boliviensis*, *B. clarkei, B. pearcei*, and *B. veitchii*. Rex begonias are important for the development of the Begonia Rex Cultorum Group, which is cultivated for its beautiful leaves (Dewitte et al., 2009).

Chromosome numbers among begonia species range from 2n=16 in *B. rex* to 2n=156 in *B. acutifolia* (Sarkar 1989; Doorenbos et al.1998; Oginuma and Peng 2002), and small chromosome fragments make them difficult to counting as true chromosomes (Tebbitt, 2005): polyploidy and aneuploidy have occurred frequently within the genus and general basic chromosome numbers are not available (Dewitte et al., 2009). Different basic chromosome numbers of x=6, 7 and 13 were reported by Matsuura and Okuno (1936, 1943), and then Lergo and Haegeman (1971) postulated as x=13 and 14 in Begonia.

One explanation for the wide variation in begonia species is cross-hybridization, which is easy in begonias (Dewitte et al., 2009). Tebbit and Garden reported that some commercial cultivars originate from crossing between species or cultivars (Tebbitt and Garden, 2005).

Gene Editing in Ornamentals

Flower color and plant architecture are the main traits of interest in the ornamentals industry (Shibata, 2008). However, due to the high number of infectious plant diseases that can affect ornamentals, new strategies are necessary for achieving disease resistance (including via inducing disease resistance) in the breeding of ornamental plants. The CRISPR/Cas9 system has become an important technology for targeted genome engineering, as an alternative strategy to traditional breeding, but double-strand repair pathways differ among and cell types (Voytas, 2013; Puchta and Fauser, 2014). For this reason, it is necessary that the feasibility of the CRISPR/Cas9 system is investigated for each plant species (Kishi-Kaboshi et al. 2018).

Use of CRISPR/Cas9 system in ornamentals is still lacking because of relatively small economic importance and limited whole genome information of individual ornamental species. There are only six successfully genome editing reports on ornamental plants published (Table 2) (Kishi-Kaboshi et al. 2018; Andolfo et al., 2016).

Species	Method	Cas9 promoter	sgRNA promoter	Target gene	Reference
Chrysanthemum morifolium	Agrobacterium-mediated transformation	PcUbi	AtU6	CpYGFP	Kishi-Kaboshi et al., 2017
Dendrobium officinale	Agrobacterium-mediated transformation	35S	OsU3	C3H, C4H, 4CL, CCR, IRX	Kui et al., 2017
Imonema nill cv. Violet Japanese morning glory	Agrobacterium-mediated transformation	PcUbi	AtU6	DFR	Watanabe et al., 2017a
Imonema nill cv. AK77	Agrobacterium-mediated transformation	PcUbi	AtU6	CCD4	Watanabe et al., 2017b
Petunia hybrida inbred line Mitchell Diploid	Agrobacterium-mediated transformation	35S	AtU6	PDS	B. Zhang et al., 2016
Petunia hybrida 'Madness' series	Transfection	-	-	NR	Subburaj et al., 2016

CCD4: carotenoid cleavage dioxygenase 4, CCR: cinnamoyl coenzyme A reductase, C3H: coumarate 3-hydroxylase, C4H: cinnamate 4-hydroxylase, DFR: dihydroflavonol-4-reductase, IRX: irregular xylem 5, NR: nitrate reductase, PcUbi: Ubiquitin4-2 promoter from Petroselinum crispum), PDS: phytoene desaturase, 4CL: 4-coumarate:coenzyme A ligase.

Table 2: Retrieved from Kishi-Kaboshi et al., (2018).

Nowadays, gene editing is seen as a new route to the elimination of intrinsic barriers, although some limitations and drawbacks are present (Azadi et al., 2016). For example, Chrysanthemum (*Chrysanthemum morifolium*), as polyploid plant, does not have whole genome information. Therefore, multi-copy transgenes as the targets for genome editing instead of endogenous genes were used by Kishi-Kaboshi et al. (2017). In this way, more than five copies of the target gene were obtained in transgenic Chrysanthemum (Kishi-Kaboshi et al. 2018).

The CRISPR/Cas 9 Gene-Editing System

Available methods can be used to render a gene nonfunctional, and o such as RNA interference to silence genes was reported on rose cultivars (Tanaka and Brugliera, 2013), and this can be used to induce disease resistance in ornamental plants such as begonias, but there are many limitations using RNAi-based technology for pest control, with the effectiveness target gene selection and reliable double-strand RNA (dsRNA) delivery being two of the major challenges (Zhang, 2013). However, irreversible gene disruption and stably altered phenotypes can be achieved via modern direct genome modification (Chandler and Brugliera, 2011; Doudna and Charpentier, 2014).

The CRISPR/Cas9 system has been used for gene editing due to its precise, accessible and easy-to-use method. The system has been repurposed from the type II CRISPR/Cas immunity system in *Streptococcus pyogenes* and can make edits to the genome of almost any organism (Doudna and Charpentier, 2014; Jinek et al., 2012). Therefore, this system can be used to attempt to make modifications in begonia species.

The Cas9 enzyme, as an endonuclease, can introduce double-stranded breaks (DSBs) at sites complementary to a guide sequence in an RNA duplex. This duplex is engineered as a single RNA chimera consisting of CRISPR RNA and trans-activating CRISPR RNA, and is termed single guide RNA (sgRNA). The guide RNA is able to bind to the target sequence at the 3'-5' strand and the Cas9 recognition or PAM site (-NGG-) is on the 5'- 3' strand (Figure 1.) (Doudna & Charpentier, 2014).

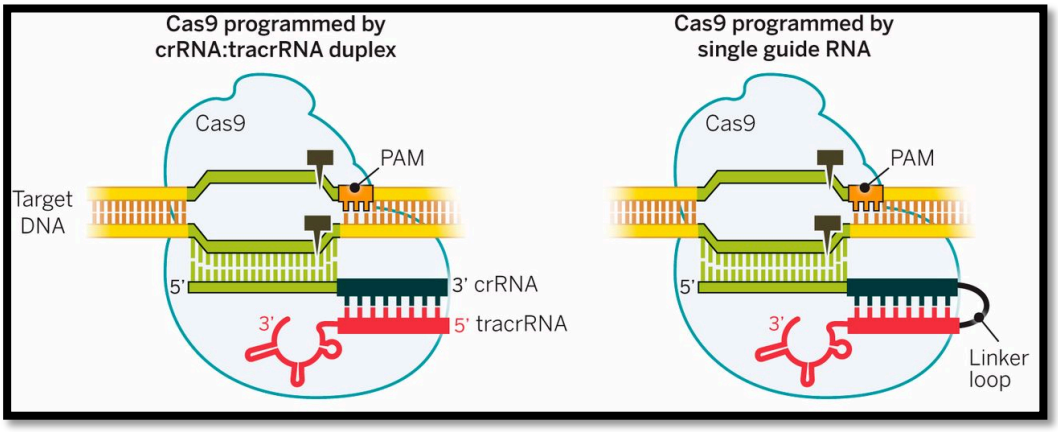

Fig. 1 has derived from (Doudna & Charpentier, 2014)

Protospacer adjacent motifs (PAM), short nucleic acid sequences on the target DNA that are recognised by Cas9, can be targeted to any site upstream by the guide sequence at the 5' side of the sgRNA. A PAM sequence for Cas9 is NGG and this directs Cas9 to induce DSBs directly three base pairs upstream of the PAM (Jinek et al., 2012). In the absence of a DNA template, DSBs can be repaired by non-homologous end joining (Doudna and Charpentier, 2014; Ma et al., 2016) or by homology-directed repair in the presence of the corresponding homologous template (Su et al., 2016). The repair usually results in 1–50 base-pair deletions (indels) or single-base insertions (Doudna and Charpentier, 2014; Ma et al., 2016).

There are other site-specific gene-editing technologies that target specific DNA sequences, such as zinc-finger nucleases (ZFN) and transcription activator-like effector nucleases (TALENs), that are important for engineering. On the other hand, the CRISPR/Cas9 system is enabling faster and cheaper development by designing the sgRNAs only, but still need the gene sequence like ZFN and TALENs as drawback for all site-specific nucleases (Doudna and Charpentier, 2014; Jinek et al., 2012), and possible off-target effects need to be checked for throughout the full DNA sequence of the host organism's genome (Bortesi and Fischer, 2015).

A high specificity (87.3 to 94.3 percent) has been reported in the relatively simple genomes of *Arabidopsis*, rice, tomato, and soybean. Maize, however, which has high levels of repetitive DNA, achieved only 29.5 percent specific targeting. The CRISPR/Cas9 complex can bind with lower-efficiency sequences with one to three mismatches, and expected off-target mutations can be avoided with careful design of the CRISPR/Cas9 tool (Borrelli et al., 2018). Those off-target effects might be eliminated by backcrossing (Barakate and Stephens, 2016; Ma et al., 2016).

The *Agrobacterium*-mediated transformation method has been used for the CRISPR/Cas9 system and is applicable in some monocotyledonous and dicotyledonous plants (Ma et al., 2016).

The CRISPR/Cas9 system's efficiency is still affected by unknown factors. Therefore, selection of more than one sgRNA is recommended (Liang et al., 2016).

Unpredictable regulations around transgenic plants have an effect on plant-breeding efforts as the issue is still unresolved in Europe (Eriksson et al., 2018), although achieving transgene-free breeding is possible via the transient expression of Cas9 and sgRNA(s), or by removing the CRISPR/Cas9 construct via traditional methods such as segregation (Bortesi and Fischer, 2015)., backcross, and genotyping, but those are time consuming and laborious (He., 2018).

Possible Disease-Related Sequence(s): Availability of Disease-Related Genes

Based on literature and gene database research, no sequence data for any begonia species was available in the National Center for Biotechnology Information (NCBI) gene database until 2015, which was also reported by Brown et al. in 2015. However, two disease-related sequences in begonias were reported in the NCBI database in 2016: the first is found in a Begonia hybrid cultivar as the *SGT1* gene, which is a suppressor of the G2 allele of the suppressor of kinetochore protein 1 (SKP1), reported as a disease-resistance gene; the second is in *Begonia rex* clone 2, and was reported as an aspartic acid proteinase inhibitor (API) gene in 2016.

The *SGT1* Gene in Begonia Hybrid Cultivar

The Begonia hybrid cultivar has a 240-base-pair linear mRNA partial cds *SGT1* gene sequence. *SGT1* has an important role at G1-S and G2-M cell-cycle transitions in yeast and functions in disease resistance in both non-host resistance and R-gene-mediated resistance to specific pathogens (Wang et al., 2010) by interacting with various proteins, and functions in protein folding and stability (Meldau et al., 2011a). Moreover, *SGT1* is highly conserved among all eukaryotes and interacts with SKP1/Cullin/F-Box ubiquitin ligase complexes to mediate protein degradation (Wang et al., 2010).

It is also reported that *SGT1* is an essential component affecting the process of cell death during both compatible and incompatible plant–pathogen interactions but defective *SGT1* can cause many phenotypic alterations in plants (Wang et al., 2010). For example, it has been reported that virus-induced gene silencing (VIGS) *SGT1* creates growth defects and diminishes resistance to biotic stresses in *Nicotiana attenuata* (Meldau et al., 2011a).

Plant SGT1 proteins consist of a tetratricopeptide repeat domain (TPR), the CS motif (present in CHP -cysteine and histidine rich domain- and SGT1 proteins), two variable regions (VR1 and VR2), and the SGT1-specific motif (SGS) (Azevedo et al., 2002). The CS and the TPR domains have regional similarities with proteins that interact with heat shock protein 90 (HSP90), and can therefore interact with HSP90 in plants (Botër et al., 2007).

SGT1 has a role in plant resistance to pathogens and herbivores, first reported to confer resistance to *Peronospora parasitica* in an *Arabidopsis thaliana* mutant (Austin et al., 2002), but the underlying mechanism is still not well understood. It is known that after a pathogen attacks, *SGT1* regulates a defense response (Meldau et al., 2011a) via the recognition by plant R-gene products of plant-derived ligands (Dangl and Jones, 2001).

Two scenarios were reported which explain the fundamental function of *SGT1* in pathogen resistance in plants: R-gene levels depend on HSP90 and SGT1 (Meldau et al., 2011a); and SGT1 and HSP90 may function in stabilizing the three-dimensional conformation of nucleotide-binding domain and leucine-rich repeat-containing (NLR) complexes (Shirasu, 2009).

SGT1 is involved in the biosynthesis of Jasmonic acid (JA) (Meldau et al., 2011b), which is an important phytohormone in plant resistance to certain necrotrophic fungi and phytophagous insects. As an example, silencing *SGT1* in *Nicotiana attenuata* causes highly reduced herbivore-feeding-induced and wounding-induced JA levels and compromises defense against the insect herbivore *Manduca sexta* (Meldau et al., 2011b) by affecting the early steps of JA biosynthesis (Gray et al., 2003).

On the other hand, Meldau et al. reported that a double knock-out in *Arabidopsis* mutants is lethal because the *Arabidopsis* genome consists of two copies of *SGT*, namely *SGT1a* and *SGT1b* (Meldau et al., 2011a). Knocking out two *SGT* homologues leads to some growth defects in tomato (Bhattarai et al., 2007), *N. benthamiana* (Peart et al., 2002) and *N. attenuata* (Meldau et al., 2011b). Those growth defects could be explained by an *SGT1* deficiency *because SGT1* seems to have a role in accumulation and signaling of hormones involved in plant development (Meldau et al., 2011a).

One study demonstrated that *SGT1* is required for cell death during the development of disease symptoms in *N. benthamiana* on compatible interaction with fungal pathogens (Wang et al., 2010). Cell death and disease-symptom development during interaction with *Sclerotinia sclotiorum*, a necrotrophic fungal pathogen, were significantly decreased by silencing of *SGT1* on *N. benthamiana* (Wang et al., 2010).

NbSGT1-silenced plants did not display cell death in a study by Wang et al. (2010). This suggests that *SGT1* on *N. benthamiana* might have a general role in disease-associated cell death during plant–fungal interactions (Wang et al., 2010). Moreover, silencing of *SGT1* in barley has been reported and compromised powdery-mildew resistance, revealing its role in R-gene-triggered, Rar1-dependent disease resistance (Azevedo et al., 2002) , Rar1 being a convergence point in the signaling pathway for resistance to this pathogen.

SGT1 seems to be required for resistance in specific interactions between particular plants and pathogens, and acts in limiting pathogen spread.

SGT1 gene was thought as being suitable for inducing *Fusarium* resistance in begonias by knocking it out via CRISPR at the beginning of the thesis, but knocking-out seems to be caused diminishing resistance and lethal on begonias. Still more research is needed to uncover the various roles of SGT1 on modulating plant immunity.

API Gene in *Begonia Rex*

The *Begonia rex* clone 2 *API* gene is described as a 212 base-pair linear piece of DNA with partial cds in the NCBI database. Proteases (proteolytic enzymes) participate in all aspects of plant life, and are involved from seed germination to plant senescence. They are involved in the developmental process and protection from abiotic stress and insects (Rustgi et al., 2018).

Proteases degrade nonfunctional proteins into amino acids and are categorized into classes such as serine, cysteine, threonine, metallo carboxy, and aspartic acid proteases (Hoorn, 2008). Aspartic acid proteases, such as *API*, are the second largest group of plant proteases after serine proteases, and they are related to nitrogen recycling in plants deprived of nutrients (Rustgi et al., 2018).

Protease inhibitors (PIs) are found in plant seeds and tubers (Ryan, 1973). PIs are believed to act as storage proteins and as a defense mechanism. Specific PIs are currently being overexpressed in certain transgenic plants to protect them against phytophagous insects and microorganisms (Habib and Fazili, 2007).

The crucial role of PIs in plants is protection from pest and pathogen attack by inhibiting proteases that are necessary for the growth and development of the invading organism (Vernekar et al., 2001). Thereby, pest and pathogen attack can be controlled based on their target protease specificity (Lawrence and Koundal, 2002). Naturally occurring PIs are essential for regulating the activity of their corresponding proteases and have key regulatory roles in many biological processes (Qi et al., 2005).

Plants have a large variety of PIs, and they are classified based on their target proteases: aspartic acid PIs (pepstatins), cysteine PIs (cystatins), metallo carboxy PIs, and serine PIs (serpins) (Lawrence and Koundal, 2002).

Pepstatin, a powerful inhibitor of aspartic acid proteases, has been shown to inhibit proteolysis by the midgut enzymes of the Colorado potato beetle in *Leptinotarsa decemlineata* (Habib and Fazili, 2007) and thus plays a role in the exogenous defense system of some plants (Oliveira et al., 2003). Also, some examples of PIs active against certain insect species, both in *in vitro* assays against insect gut proteases and in *in vivo* artificial diet bioassays, were described by Leo et al. (Leo et al., 2002).

The *API* gene does not seem to be involved in inhibiting fungal disease in begonias, but is needed for insect resistance. Therefore, the *API* gene might be suitable for inducing insect resistance in begonias, but not for fungal disease.

Delivery System

According to the literature study on the Begoniaceae family, only *Agrobacterium*-mediated transformation has been used successfully up to the present. Therefore, this delivery system may be chosen for the transformation in begonias (Einset and Kopperud, 1995; Kishimoto et al., 2002; Kiyokawa et al., 1996; Ohki et al., 2009).

Successful transformation studies on some begonia species have been reported (Einset and Kopperud, 1995; Kishimoto et al., 2002; Kiyokawa et al., 1996; Ohki et al., 2009). Although none of these transformations of begonia species aimed to increase disease resistance, the protocols can be used for transformation and regeneration of Begonia cultivars.

***Agrobacterium*-Mediated Transformation**

The *Agrobacterium*-mediated transformation method is the most-studied and most-common system for obtaining stable transformation in plants, although it might lead to unintentional gene silencing (Slater et al., 2008).

Agrobacterium tumefaciens leads to tumorous growths, and has a natural ability to move transfer-DNA (T-DNA) into the genome of dicotyledonous plants (Zambryski et al., 1983). The T-DNA region is found on the tumor-inducing (Ti) plasmid of *Agrobacterium tumefaciens* and is bordered by 25-base-pair repeats known as the right border (RB) and the left border (LB). A virulence region (*vir*) is responsible for the transfer of the T-DNA (Bevan, 1984).

Binary vector systems have been applied to the *vir* region (helper plasmid) and the T-DNA plasmid (binary plasmid). The binary plasmid consists of genes encoding antibiotic resistance and origins of replication (ORI), and the T-DNA flanked by the RB and LB, allowing selection and replication in both *Escherichia coli* and *A. tumefaciens* (Hellens et al., 2000).

It is possible to increase bacterial copy number, transformation efficiency, and ease of use by using a binary system. However, undesired non-T-DNA sequences may be transferred. This is an unavoidable, but frequently unobserved and untested, result of transformation. For example, Wenck et al. have reported that entire binary vectors, including backbone sequences and T-DNA sequences, are frequently transferred to *Nicotiana plumbaginifolia* and *Arabidopsis thaliana* cells (Wenck et al., 1997).

Disruption of a target gene Using CRISPR/Cas9: Designing sgRNA and Plasmids

According to Sandgrind's study on *Begonia tuberhybrida*, a particular method of designing sgRNA and plasmids might be adapted to the disruption of a gene on Begonia hybrid cultivar. CRISPR/Cas9 plasmids designed for *Agrobacterium*-mediated transformation and containing *Cas9* codon usage that is optimized for plants may be ordered commercially and used for the disruption of the gene in the cultivar (Sandgrind, 2017). In Sandgrind's experiment, a custom CRISPRPL plasmid was used, which contained: sgRNA; the gene encoding Cas9; the *bar* gene, which conveys glufosinate (a herbicide) resistance, as a selectable marker; two CaMV 35S promoters for expression of the *bar* and *Cas9* genes; the *A. thaliana* AtU6-1 promoter was chosen as sgRNA expression promoter; and the *KanR* gene to convey kanamycin resistance for bacterial selection (Sandgrind, 2017).

Alignment of a target sequence, might be taken from NCBI, may be used to estimate sgRNA specificity by using CRISPR-P 2.0 (Liu et al., 2017), CRISPRdirect (Naito et al., 2015), CRISPR Design (Zhang., 2018), and CRISPOR (Haeussler et al., 2016).

Because of the limited time frame of Sandgrind's thesis work, *B. tuberhybrida* 'Urban Bicolor Pink' transformed and mutated plantlets were not obtained due to their slow growth, but the researcher mentioned that plantlets were growing *in vitro* on selective medium shortly after the thesis was written, by personal communication. Therefore, the same procedure might be used as an approach to disrupt the gene on Begonia hybrid cultivar by paying attention to regeneration time.

As an approach, *Agrobacterium*-mediated transformation may be applied to disrupt a gene via the CRISPR/Cas9 system in Begonia. Sequence availability for the gene in the NCBI database can be used to design the sgRNA target.

Conclusion

The Begoniaceae family is a remarkable family in both size and diversity but there is a limited amount of genetic and sequence data for it, even although many species and hybrids are currently in the market. Therefore, finding new approaches such as CRISPR/Cas9 technology to obtain begonias that are more resilient to pathogens such as *Fusarium* and insects is important.

Two disease-related sequences are available in the NCBI database for begonias: the *SGT1* gene, which is listed as a disease-resistance gene in Begonia hybrid cultivar; the other is the *API* gene on *Begonia rex* clone 2, which encodes an aspartic acid proteinase inhibitor, but this gene is related to insect resistance for plants.

There is only one study in which CRISPR/Cas9 has been used in an attempt to disrupt the enzyme F3'H in *B. tuberhybrida* 'Urban Bicolor Pink' with no available sequence for any begonia species. Starting from this point of view, a target gene might be disrupted using CRISPR/Cas9 technology: with available sequence data, Cas9 and sgRNA are shown to be readily expressed at sufficient levels in Begonia. Also, a method for begonias is available and has been successfully applied. Since begonias are easily hybridized, it is possible to achieve transgene-free plants by removing the CRISPR/Cas9 construct by segregation.

Some points should be taken into consideration: regeneration time might differ between species and slow growth might be seen in begonias.

The *SGT1* was thought as suppressor of the *SKP1* (as disease resistant gene) based on NCBI database. The gene, therefore, was choosen for begonias to induce *Fusarium* resistance by knocking it out via the CRISPR/Cas9 system at the beginning of the thesis study. However, it is realized that the *SGT1* does not seem as a good choice.

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