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Breeding of *Begonia tuberhybrida* using modern biotechnology

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For Sondre.

Sjur Sandgrind

Oslo, 13th August 2017

Abstract

Tuberous begonias (*Begonia tuberhybrida*) are popular ornamental bedding plants, of which approximately 300 000 units are being produced in Norway each year, and novel cultivars are a Norwegian export article. Blue and purple flowers are absent throughout the vast and diverse *Begonia* genus due to a lack of the plant pigment delphinidin and its derivates.

To achieve a blue-flowered *B. tuberhybrida*, the gene encoding the enzyme flavonoid 3',5'-hydroxylase (F3'5'H), which catalyse the biosynthesis of delphinidin precursors, was introduced using *Agrobacterium*-mediated transformation.

A total of 1 692 *B. tuberhybrida* 'Urban Bicolor Pink' explants were treated, and 616 putatively transformed plantlets were obtained, out of which 163 plants survived and flowered. Stable integration of the transgene was verified in 18 out of 28 putatively transformed plants selected for PCR screening.

However, no significant shift in flower colour could be observed in any of the 163 plants that flowered, but a slight shift towards blue could be measured with a colorimeter when sap from sepals and petals was extracted from two transformants and compared to sap from the wild type.

Because competition for substrate between the enzyme flavonoid 3'-hydroxylase (F3'H) and F3'5'H has been shown to inhibit maximum biosynthesis of delphinidin in other species, CRISPR/Cas9 was used in an attempt to disrupt *F3'H* in *B. tuberhybrida* 'Urban Bicolor Pink'. Because *F3'H* has not been sequenced in *B. tuberhybrida*, *F3'H* sequences from *Cucumis melo*, *Cucumis sativus*, and *Arabidopsis thaliana*, all of which belong to the same clade (Rosids) as *B. tuberhybrida*, were aligned to find possible target sites for CRISPR/Cas9 likely conserved in *B. tuberhybrida*. CRISPR/Cas9 was also used in an attempt to disrupt *F3'H* in the sequenced model plant *A. thaliana* as a proof of concept.

No 'Urban Bicolor Pink' plants gene edited with CRISPR/Cas9 could be regenerated and analysed during the time frame of this thesis, but several putatively transformed plantlets were growing *in vitro* at the time of conclusion. Five *A. thaliana* plants randomly selected for PCR screening were verified as transformed with the CRISPR/Cas9 constructs. However, no predicted mutations could be detected when performing enzyme mismatch cleavage assays or Sanger sequencing, adding to the growing body of research showing that *A. thaliana* is much more difficult to gene edit with CRISPR/Cas9 than other species.

Sammendrag

Knollbegonia (*Begonia tuberhybrida*) er populære prydplanter i blomsterbed. Omtrent 300 000 *B. tuberhybrida* planter produseres årlig i Norge, og nye kultivarer er en norsk eksportartikkel. Blå og lilla blomster finnes ikke i det omfattende og mangfoldige *Begonia* genuset på grunn av mangel på plantepigmentet delphinidin og dens derivater.

For å oppnå en *B. tuberhybrida* med blå blomster, så ble genet som koder for enzymet flavonoid 3',5'-hydroxylase (F3'5'H), som katalyserer biosyntesen av delphinidin-forløpere, introdusert ved å bruke *Agrobacterium*-mediert transformasjon.

Totalt ble 1 692 *B. tuberhybrida* 'Urban Bicolor Pink' blad- og bladstilk-biter behandlet, og 616 formodentlig transformerte småplanter ble regenerert. Av disse 616 småplantene, så var det 163 planter som overlevde og blomstret. Stabil integrering av transgenet ble verifisert i 18 av 28 formodentlig transformerte planter utvalgt til PCR screening.

Ingen signifikant endring i blomsterfarge mot blå kunne observeres, men en svak endring mot blått kunne måles med en fargemåler når plantesaft fra begerblad og kronblad ble ekstrahert fra to transformanter og sammenlignet med plantesaft ekstrahert fra villtypen.

Fordi konkurranse for substrat mellom enzymet flavonoid 3'-hydroxylase (F3'H) og F3'5'H har vist seg å inhibere maksimal biosyntese av delphinidin i andre arter, så ble CRISPR/Cas9 brukt i et forsøk på å slå av *F3'H* i *B. tuberhybrida* 'Urban Bicolor Pink'. Fordi *F3'H* ikke har blitt sekvensert i *B. tuberhybrida*, så ble *F3'H* sekvenser fra *Cucumis melo*, *Cucumis sativus* og *Arabidopsis thaliana*, alle medlemmer av den samme kladen (Rosidae) som *B. tuberhybrida*, sidestilt og sammenlignet for å finne mulige målsekvenser for CRISPR/Cas9 trolig konservert i *B. tuberhybrida*. CRISPR/Cas9 ble også brukt i et forsøk på å skru av *F3'H* i *A. thaliana* for å bevise at systemet fungerer.

Ingen 'Urban Bicolor Pink'-planter genredigert med CRISPR/Cas9 kunne bli regenerert og analysert innenfor tidsrammen for denne avhandlingen, men flere formodentlig transformerte småplanter vokste *in vitro* ved avhandlingens sluttpunkt. Fem *A. thaliana*-planter tilfeldig utvalgt til PCR screening ble verifisert som transformert med CRISPR/Cas9-konstruktene, men ingen predikerte mutasjoner kunne detekteres når enzym mismatch cleavage analyser og Sanger-sekvensering ble utført. Dette stiller seg inn i rekken av forskning som viser at *A. thaliana* er mye vanskeligere å genredigere med CRISPR/Cas9 enn andre arter.

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1 Introduction

1.1 About *Begonia*

Begonia is one of largest genera of vascular plants (Frodin 2004) consisting of 1 835 currently accepted species (Hughes et al. 2015) (updated 24.03.17), in addition to around 15 000 hybrids (Wagner 1999). Begonias are popular ornamental plants, commonly grown as pot, basket, hanging, and bedding plants. They readily hybridize within the genus and most of the commercially grown begonias today are hybrids (Hvoslef-Eide & Munster 2006).

Haegeman (1979) divides the cultivated begonias into six groups, where the tuberous begonias (*Begonia tuberhybrida*) make up their own large and heterogeneous group. The cultivated tuberous begonias are hybrids of tuberous begonia species originating from the Andes, but the history is shrouded in mystery as the first commercial cultivar of tuberous begonia, named *Begonia* 'Sedenii', first exhibited in 1869 and used in most subsequent crosses, was a hybrid between *Begonia boliviensis* and an unnamed species (Haegeman 1979). The history is made even more elusive by the fact that there are no scientific articles on molecular classification, and the botanical parents of crosses are often kept a secret by the commercial breeders (Hvoslef-Eide & Munster 2006). The flower morphology in *B. tuberhybrida* cultivars is very diverse, with single coloured and bicoloured flowers in a wide range of colours (Haegeman 1979; Hvoslef-Eide & Munster 2006), but blue or purple flowers, which are normally caused by delphinidin-based anthocyanins, have not been found in either wild or hybrid species of begonia (Kiyokawa et al. 2001).

Approximately 2.75 million *Begonia* plants, mainly *Begonia x hiemalis, Begonia rex, Begonia semperflorens*, and *Begonia tuberhybrida*, were grown in commercial facilities in Norway in 2015. *B. tuberhybrida* made up about 10.8% (298 000 units) of the total number of *Begonia* plants produced. The amount of *B. tuberhybrida* plants grown commercially has been consistent during the past two decades, with a slight reduction of approximately 14 000 units being reported compared to 1999 levels (S Bøckman pers. comm. 25 April 2017).

The commercial value of ornamental plants is often directly related to the colour(s) they display and colour is hence an important goal for breeders. Cultivars exhibiting novel coloration are much sought after on the international flower market and such novel cultivars of *B. tuberhybrida* are a Norwegian export article (Bergh & Bjelland 2014). *B. tuberhybrida* with blue flowers would be an exciting addition of potential commercial interest, but because blue and purple flowers are lacking throughout the vast *Begonia* genus it has not been possible to introduce blue colour to a new

cultivar by cross-breeding (Kiyokawa et al. 2001). However, genetic engineering and genome editing creates possibilities to breed cultivars accumulating non-native pigments in flower tissues by over expressing transgenes or altering existing pigment biosynthesis pathways. Several such transgenic plants exhibiting uniquely coloured flowers have been engineered during the past decades (Nishihara & Nakatsuka 2011). Blue and purple pigment (delphinidin-based anthocyanins) has been introduced into important ornamental species not normally accumulating blue or purple pigment such as *Chrysanthemum x morifolum* (Brugliera et al. 2013; Noda et al. 2013), *Dianthus cryophyllus* (Tanaka et al. 1998), and *Rosa hybrida* (Katsumoto et al. 2007). This has been achieved by introducing transgenes, often in addition to downregulating competing pathways (Tanaka & Brugliera 2013).

The only transgenic plants that are approved for sale in Norway are cut flowers from five purple cultivars of *Dianthus cryophyllus* which have been engineered to accumulate delphinidin-based anthocyanins (Eriksson et al. 2017 (In Press.)). Consumers in Norway are very sceptic of genetically modified food products (Bugge & Bartmann 2017; Hess et al. 2016), but it has been put forward that genetically modified ornamentals which cannot survive in the Norwegian environment, such as a transgenic or gene edited *B. tuberhybrida* cultivar, could act as a door opener for other genetically modified products (Hvoslef-Eide & Munster 2006).

Successful transformation of several *Begonia* species' have been reported; *Begonia x cheimantha* (Einset & Kopperud 1995) (revealed by Hvoslef-Eide and Munster (2006) as mistakenly named *Begonia x hiemalis*), *B. tuberhybrida* (Kiyokawa et al. 1996), *Begonia x hiemalis* (Kishimoto et al. 2002), and *Begonia* Rex (Ohki et al. 2009). Although none of the aforementioned transformations of *Begonia* spp. targeted pigment synthesis, they have established transformation and regeneration protocols that can be used to alter the pigment composition of *B. tuberhybrida*.

1.2 Regulation of flower colour development

The major groups of pigments which cause flower colour are flavonoids, carotenoids, and betalains. Anthocyanins are water-soluble vacuolar pigments, the most common type of flavonoids, and the basis for nearly all pink, red, orange, scarlet, purple, blue, and blue-black flower colours (Davies 2009). Flower colour is mainly determined by the structure, type, and concentration of the anthocyanins, generally colourless (or weakly coloured) flavones and flavonols (co-pigments), and the vacuolar pH in flower tissues. Other factors also have substantial effect on flower colour, such as other pigments, metal ions, carbohydrates, tissue structure, plant hormones, and physical and

chemical factors such as temperature, light, water availability, soil acidity, and mineral nutrients (Davies 2009; Fukui et al. 2003; Tanaka & Brugliera 2013; Zhao & Tao 2015).

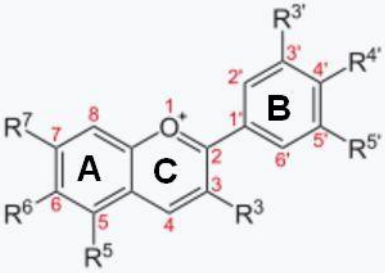
Basic structure	Anthocyanidin	R_3'	R_4'	R_5'	R_3	R_5	R_6	R_7
	Aurantinidin	−H	−OH	−H	−OH	−OH	−OH	−OH
	Cyanidin	−OH	−OH	−H	−OH	−OH	−H	−OH
	Delphinidin	−OH	−OH	−OH	−OH	−OH	−H	−OH
	Europinidin	$-OCH_3$	−OH	−OH	−OH	$-OCH_3$	−H	−OH
	Pelargonidin	−H	−OH	−H	−OH	−OH	−H	−OH
	Malvidin	$-OCH_3$	−OH	$-OCH_3$	−OH	−OH	−H	−OH
	Peonidin	$-OCH_3$	−OH	−H	−OH	−OH	−H	−OH
	Petunidin	−OH	−OH	$-OCH_3$	−OH	−OH	−H	−OH
	Rosinidin	$-OCH_3$	−OH	−H	−OH	−OH	−H	$-OCH_3$

Fig. 1. *The basic structure of anthocyanidin and the substitution pattern at numbered positions of selected anthocyanidins. After NEUROtiker (2008).*

The various anthocyanins are formed by glycosylation of anthocyanidin, the chromophore of anthocyanin. The three main anthocyanidins, pelargonidin, cyanidin, and delphinidin, can be distinguished by their colour range which is determined by the hydroxylation pattern of the B-ring in the shared C6-C3-C6 (ring A, C, and B, respectively) carbon skeleton. Delphinidin-based anthocyanins cause purple and blue colouration and are hydroxylated in the 3’ and 5’ positions of the B-ring, as shown in Fig. 1. As shown in Fig. 2, the enzyme flavonoid 3’,5’-hydroxylase (F3’5’H) catalyse the hydroxylation of dihydrokaempferol (DHK) to form dihydromyricetin (DHM), a necessary precursor to form delphinidin (Davies 2009; Tanaka et al. 2008; Tanaka et al. 2009; Zhao & Tao 2015). The absence of delphinidin in begonia (Kiyokawa et al. 2001) is hypothesized to be because of no F3’5’H activity, as *F3’5’H* is rare amongst higher plants (Seitz et al. 2015).

A possible approach to create blue flowers is to shift the synthesis towards delphinidin precursors by introducing the gene encoding F3’5’H together with a strong promoter such as Cauliflower mosaic virus 35S (CaMV 35S). However, the efficiency of the *F3'5'H* transgenes from different sources varies when inserted into other species under different promoters, and it is often also necessary to downregulate competing pathways to produce significant changes in flower colouration (Brugliera et al. 2013; Noda et al. 2013; Tanaka & Brugliera 2013; Zhao & Tao 2015). Moreover, maximum biosynthesis of delphinidin can be inhibited by competition for substrate (DHK) from flavonoid 3’-hydroxylase (F3’H), which catalyse the conversion of DHK to dihydroquercetin (DHQ), and dihydroflavonol 4-reductase (DFR), which catalyse the conversion of DHK, DHQ, and DHM to the leucoanthocyanidin precursors for pelargonidin, cyanidin, and delphinidin, respectively. Furthermore, DFR can have a preference for DHQ or DHK instead of DHM, effectively inhibiting delphinidin synthesis (Brugliera et al. 2013; Davies 2009; Tanaka & Brugliera 2013).

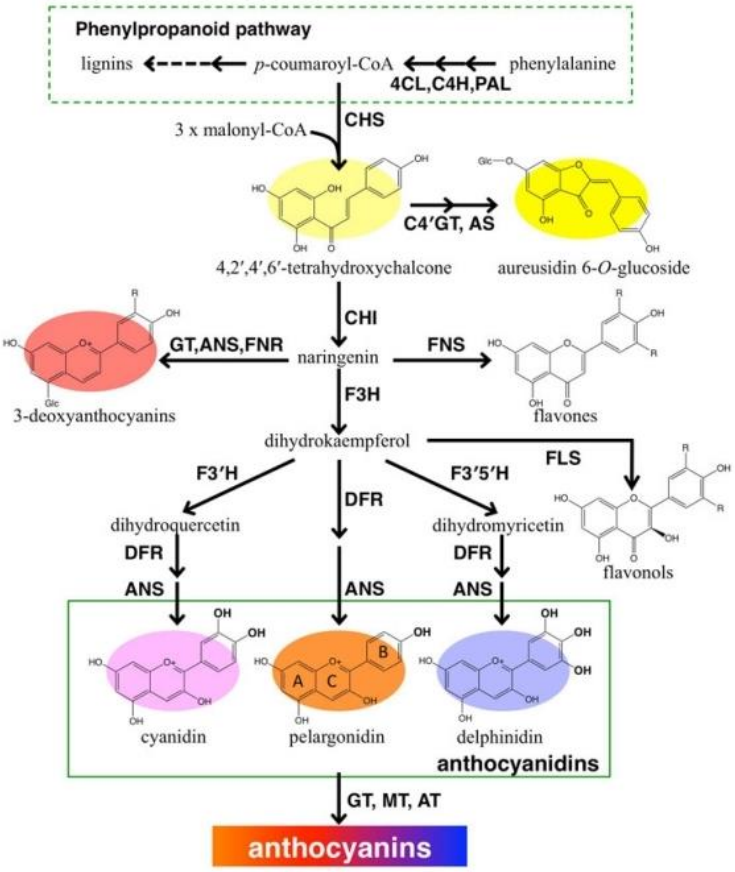


Fig. 2. *The anthocyanin biosynthesis pathway. After Nishihara and Nakatsuka (2011).*

In addition to a shift towards accumulation of delphinidin-based anthocyanins, an elevation of vacuolar pH to at least 4.0 (Tanaka et al. 2009), preferably >5.5 (Davies 2009), and appropriate co-pigment and metal ion interactions, is important for formation of bluer flowers. Genes such as *PH5* in *Petunia* spp. (Verweij et al. 2008), which reduce vacuolar acidification in petals, *Vit1* in *Tulipa gesneriana* (Momonoi et al. 2009), which regulate vacuolar iron concentrations, and many more, directly impact blue flower colour, making engineering of true blue flowers a complex task (Davies 2009; Tanaka et al. 2009; Zhao & Tao 2015).

1.3 Genetic engineering and genome editing of plants

Genetic engineering and genome editing offers the opportunity to insert genes and other genetic elements into the genome of a host organism, as well as regulating, deleting, or disrupting endogenous genes and other genetic elements. These technologies make it possible to achieve breeding goals not achievable by traditional breeding methods, to speed up the breeding process, and to make the breeding process more precise (Doudna & Charpentier 2014; Ma et al. 2016; Slater, A. et al. 2008).

Various methods for genetic engineering of plants have been developed, such as *Agrobacterium*-mediated transformation, direct DNA transfer (using a variety of techniques such as biolistics, electroporation, electrophoresis and microinjection), and, more recently, programmable sequence-specific nucleases such as zinc finger nucleases (ZFNs), transcriptor activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) (Jinek et al. 2012; Ma et al. 2016; Slater, A. et al. 2008). *Agrobacterium*-mediated transformation (Bevan 1984) is the most commonly used method for non-specific stable insertion and expression of transgenes (Slater, A. et al. 2008), while CRISPR/Cas9 (Jinek et al. 2012) is currently considered to be the easiest, most efficient, and most versatile method for site-specific gene editing (Ma et al. 2016).

1.3.1 *Agrobacterium*-mediated transformation

The most common and well-studied system for stable transformation of plants is *Agrobacterium*-mediated transformation. It is thought to generally result in less rearrangement and a lower copy number of the transgene than direct DNA delivery methods, which can lead to unintentional transgene silencing (Slater, A. et al. 2008). It was also the method of choice in all reported transformation of begonia (Einset & Kopperud 1995; Kishimoto et al. 2002; Kiyokawa et al. 1996; Ohki et al. 2009). As such, this method was chosen for insertion of *F3’5’H* in *B. tuberhybrida*.

Agrobacterium tumefaciens has the natural ability to transfer a segment of its DNA called the T-DNA into the nuclear genome of susceptible dicotyledonous host plants and cause tumorous growth. A tumour inducing (Ti) plasmid harbours the T-DNA bordered by 25 bp repeats, known as the right (RB) and left border (LB), and a *vir* region responsible for transfer of the T-DNA (Bevan 1984). As removal of the genes within the T-DNA does not interfere with *A. tumefaciens*’ ability to transfect plants, but prevent the formation of tumours, non-oncogenic Ti plasmids have been developed, enabling researchers to replace the bacterial T-DNA with DNA of choice for transfection

(Zambryski et al. 1983). Furthermore, it has been discovered that the T-DNA and the *vir* region can be situated on separate plasmids, forming the basis of modern binary Ti vectors (Hellens et al. 2000b; Hoekema et al. 1983). In binary vector systems, the T-DNA and the *vir* region are separated onto plasmids commonly termed the binary and the helper plasmid, respectively. The binary plasmid normally contain origins of replication and genes encoding antibiotic resistance in addition to the T-DNA flanked by RB and LB, enabling replication and selection in both *Escherichia coli* and *A. tumefaciens* (Hellens et al. 2000b). By using a binary system, it is possible to drastically reduce the size of the plasmid carrying the T-DNA and hence increase transformation efficiency, ease of use, and the bacterial copy number, which increase the extractable plasmid yield (Hellens et al. 2000a; Hoekema et al. 1983; Sambrook et al. 1989).

The T-DNA integrates fairly randomly and in variable copy numbers into the plant genome, although selection pressure may shift the T-DNA insertions into gene-rich or transcriptionally active regions of chromatin (Gelvin 2003; Kim et al. 2007). Furthermore, unwanted “non-T-DNA” might also be transferred (Gelvin 2003). The random nature of the insertion and the variable copy number of inserted genes may cause mutation of genes at the integration site and silencing of transgenes, in addition to variable expression levels (Gelvin 2003; Jia et al. 2012; Slater, A. et al. 2008). This lack of precision has been a concern for researchers, regulators, and activists (Gelvin 2003; Robinson et al. 2015; Slater, A. et al. 2008), and can explain some of the massive interest in site-specific gene editing technologies such as CRISPR/Cas9 (Jinek et al. 2012)

1.3.2 The CRISPR/Cas9 gene editing system

Several options are available to render a gene non-functional; RNA interference (RNAi) or antisense suppression have been the most common methods used to silence genes involved in development of flower coloration (Nishihara & Nakatsuka 2011; Tanaka & Brugliera 2013), but modern direct genome modification makes it easier to achieve irreversible gene disruption and stably altered phenotypes (Doudna & Charpentier 2014; Ma et al. 2016).

The most accessible, precise, and easy-to-use method for gene editing today is the CRISPR/Cas9 system (Fig. 3), in which CRISPR/Cas9 from the type II CRISPR/Cas adaptive immunity system in *Streptococcus pyogenes* have been repurposed to make edits to the genome of virtually any organism (Doudna & Charpentier 2014; Jinek et al. 2012). As such, the CRISPR/Cas9 system was chosen to attempt to disrupt *F3'H* in *B. tuberhybrida*.

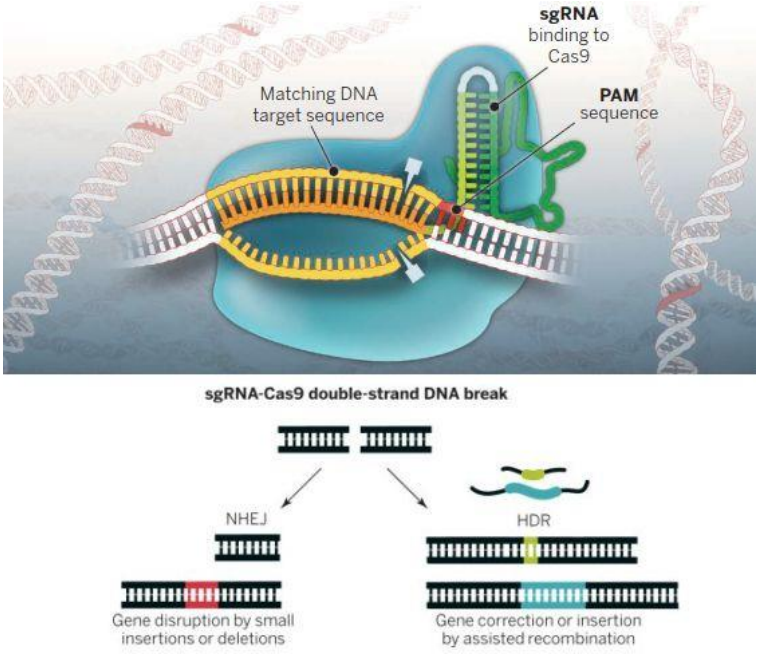


Fig. 3. *Top: The Cas9 enzyme (blue) generates a DSB in a DNA target site (gold) three bp upstream of PAM (red). Bottom: The DSB is repaired by the endogenous cellular repair machinery by either NHEJ or HDR, depending on whether a homologous DNA sequence is available or not. Both figures after Doudna and Charpentier (2014)*

Cas9 is an endonuclease able to introduce double-stranded breaks (DSBs) at sites complementary to a guide sequence within a RNA duplex consisting of CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA). The tracrRNA:crRNA duplex was engineered as a single RNA chimera, commonly termed single guide RNA (sgRNA), that retains the ability to bind to the target sequence at the 5' side and Cas9 at the 3' side. The guide sequence at the 5' side of the sgRNA can be changed to target any site directly upstream of a protospacer adjacent motif (PAM), which in the case of Cas9 is NGG, and direct Cas9 to induce a blunt DSB three base pairs directly upstream of the PAM (Jinek et al. 2012). The endogenous cellular DNA machinery will then, unless homologous DNA is present, attempt to repair the DSB by non-homologous end joining (NHEJ), usually resulting in single-base insertions or 1-50 bp deletions (indels) which most of the time cause frame-shift and gene disruption (Doudna & Charpentier 2014; Ma et al. 2016).

Other site-specific gene editing technologies such as ZFNs and TALENs require considerable protein engineering to target new DNA sequences, while only the sgRNA needs to be changed in the CRISPR/Cas9 system, enabling faster and cheaper development (Doudna & Charpentier 2014). However, it is necessary to know the DNA sequence of the target gene to design appropriate sgRNAs (Jinek et al. 2012), and preferentially also the full genome DNA sequence of the host organism to check for possible off-target effects, although off-target mutations are less problematic

in plants where such events can be eliminated by backcrossing (Barakate & Stephens 2016; Ma et al. 2016).

In contrast to CRISPR/Cas9 genome editing of animals and bacteria, CRISPR/Cas9 genome editing of plants usually depend on stable transformation with constructs expressing *Cas9* and sgRNA(s), although it is possible to obtain heritable genome modifications by transiently expressing *Cas9* and sgRNA(s) in the target cells by using DNA delivery methods such as biolistics, agroinfiltration, or viral vectors (Bortesi & Fischer 2015; Ma et al. 2015). CRISPR/Cas9 has already been applied to disrupt targeted genes in several monocotyledonous and dicotyledonous plants, most commonly using the *Agrobacterium*-mediated transformation method to integrate both the *Cas9* and sgRNA expression cassettes into the plant genome (Ma et al. 2016). However, there are still many unknown factors affecting the systems' efficiency, and it is hence recommended to select more than one sgRNA to target multiple sites in the gene of interest to ensure efficient editing (Liang et al. 2016),

By transiently expressing Cas9 and sgRNA(s), or by removing stably inserted CRISPR/Cas9 constructs via segregation, it is possible to obtain transgene-free mutated plants using the CRISPR/Cas9 system (Bortesi & Fischer 2015). The debate whether such genome edited transgene-free plants should be regulated in the same strict manner as transgenic plants is currently an unresolved issue in most European countries, including Norway, which complicates plant breeding efforts because it makes regulation unpredictable (Bortesi & Fischer 2015; Eriksson et al. 2017 (In Press.)). Because the economics of breeding a transgenic plant is enormously different from non-regulated cultivars, if Europe, or parts of Europe, chooses to regulate genome edited transgene-free plants as transgenic plants, this will have vast implications for the competitiveness of the agricultural industries in these countries (Eriksson et al. 2017 (In Press.)).

1.4 Study questions

It is speculated that the absence of *F3'5'H* is the main cause for the lack of delphinidin-based anthocyanins, and hence no blue or purple flowers, throughout the *Begonia* genus (T Hvoslef-Eide pers. comm. 2016).

Based on previous research on engineering of blue flowers in other species, it is further speculated that introduction of *F3'5'H* alone will not produce true blue flowers, although a shift towards bluish might occur, and that disrupting *F3'H*, which encodes an enzyme that competes with F3'5'H for substrate, will further increase the accumulation of delphinidin-based anthocyanins and hence

produce a bluer phenotype (Brugliera et al. 2013; Katsumoto et al. 2007; Noda et al. 2013; Tanaka & Brugliera 2013; Zhao & Tao 2015).

This thesis work will use *Agrobacterium*-mediated transformation to insert *F3'5'H* into *B. tuberhybrida* and CRISPR/Cas9 to disrupt *F3'H* in *B. tuberhybrida* and *Arabidopsis thaliana*. Because no sequence data for any *Begonia* species is available in the National Center for Biotechnology Information (NCBI) Gene database (Brown et al. 2015), and gene sequencing is unfeasible given the constraints on this thesis work, *F3'H* sequences from species related to *Begonia* will be used to find possible sgRNA targets likely conserved in *B. tuberhybrida*. As use of such a strategy has not been previously reported, *A. thaliana* will be used as a proof of concept for *F3'H* disruption.

The study questions are;

1. Is it possible to shift the flower colouration of *B. tuberhybrida* towards blue by introduction of *F3'5'H* from *Petunia x hybrida* using *Agrobacterium*-mediated transformation?
2. Can *F3'H* in *B. tuberhybrida* be disrupted by using CRISPR/Cas9 to target a sequence that is found by aligning *F3'H* in related species?
3. Is it possible to disrupt *F3'H* in *A. thaliana* by using CRISPR/Cas9?

2 Materials and methods

2.1 *Agrobacterium*-mediated transformation of *B. tuberhybrida* ‘Urban Bicolor Pink’ with plasmid pIA291

2.1.1 Plasmid pIA291

Escherichia coli strain DH5α harbouring the plasmid pIA291, kindly provided by Cathie Martin’s group at the John Innes Centre, Norwich, England, had been previously preserved as glycerol stocks stored at -80°C by Anders Wulff-Vester, NMBU. The plasmid pIA291 contains the *flavonoid 3',5'-hydroxylase* (*F3'5'H)* gene, encoding the enzyme F3'5'H, which is essential for biosynthesis of the delphinidin-based anthocyanins. The gene originates from *Petunia x hybrida* (*PhF3'5'H*) and has a double 35S promoter from *Cauliflower mosaic virus* (CaMV 35S) in a pB7WG2 backbone (Karimi et al. 2002) (Fig. 4).

Furthermore, the plasmid contains the *bar* gene from *Streptomyces hygroscopius*, which encodes resistance to the herbicide glufosinate (Deblock et al. 1987; Thompson et al. 1987) enabling selection for transformed plants by applying glufosinate to the medium or by spraying the plants directly. The plasmid pIA291 also contains the *Sm/Spr* gene for bacterial selection, which encodes resistance to the antibiotics streptomycin and spectinomycin (Karimi et al. 2002). Antibiotic resistance is not only necessary for selection of transformed bacteria, but also to make sure that selection pressure for the plasmid is maintained. Because a metabolic cost is associated with harbouring the plasmid, stable maintenance of the plasmid in the bacteria can only be achieved if the plasmid confers a net fitness advantage by for example conveying resistance to an antibiotic the bacteria is exposed to (Slater, F. et al. 2008).

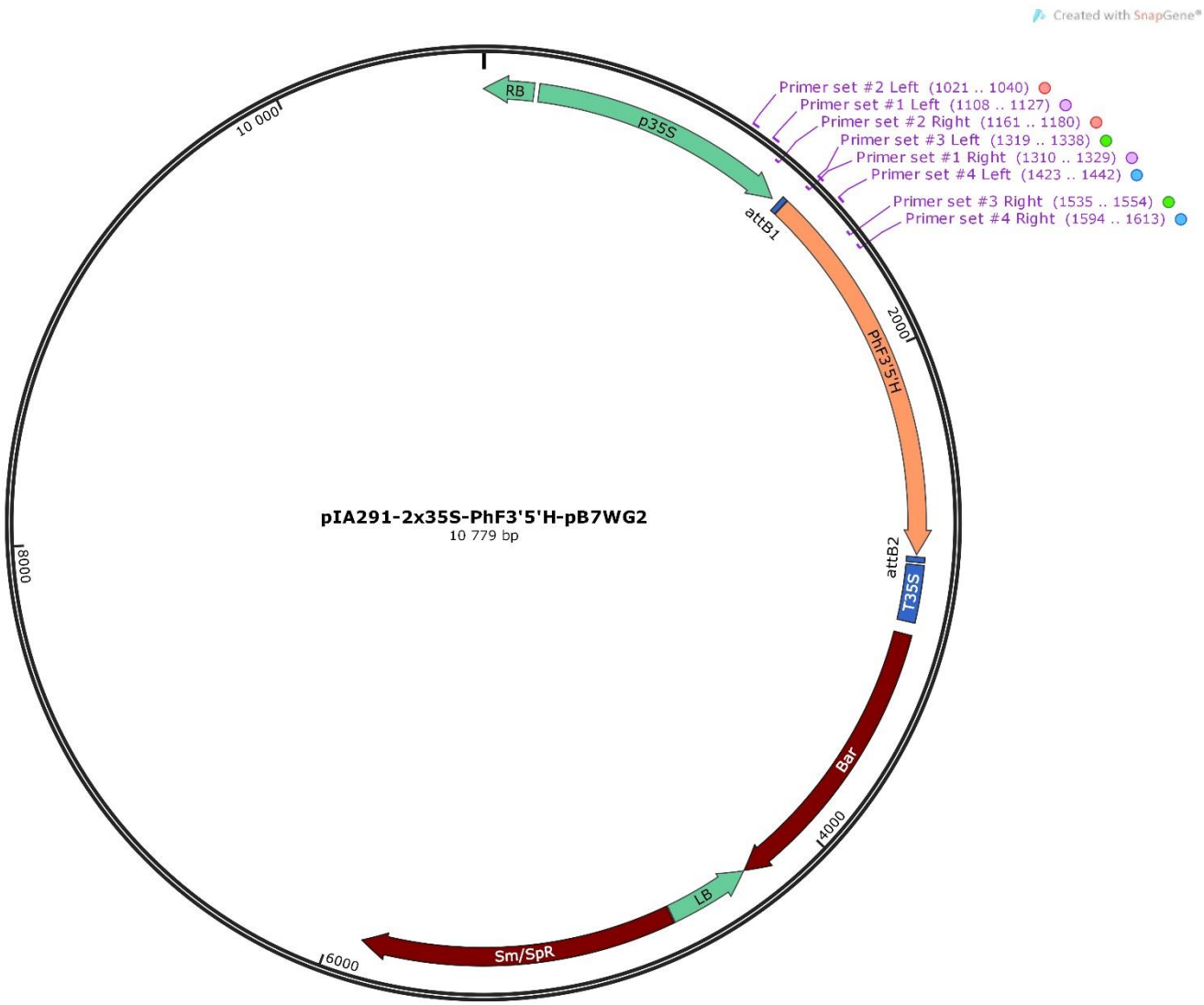


Fig. 4. *Map of the plasmid pIA291 containing the gene encoding F3'5'H as well as a double 35S promoter, the bar gene (resistance to glufosinate) and the Sm/SpR gene (resistance to streptomycin and spectinomycin). The plasmid was used as a vector for transformation of B. tuberhybrida 'Urban Bicolor Pink'. Primer set #1-4 (Table 1).*

The plasmid map was created using SnapGene v3.3.4 (from GSL Biotech; available at snapgene.com).

Regenerating E. coli harbouring plasmid pIA291 from glycerol stock

Glycerol stock of *E. coli* harbouring pIA291 was used to inoculate 5 ml liquid Lysogeny Broth (LB) medium (Bertani 1951) (Appendix II) in 13 ml Snap Caps and incubated at 37°C and 275 rpm overnight. These cultures were used the following day to inoculate solid LB medium in 9 cm Petri dishes and incubated upside down at 37°C overnight to produce single colonies. A single colony was then used to inoculate 5 ml liquid LB in a 13 ml Snap Cap and grown at 37°C and 275 rpm

overnight. All medium contained 50 mg/l spectinomycin to maintain selection pressure for the plasmid pIA291.

Isolation of plasmid DNA from E. coli

Plasmid DNA was isolated from the final 5 ml *E. coli* culture using Genomed JETquick Plasmid Miniprep Spin Kit according to the kit protocol (Appendix I). 75 µl TE-buffer was used for final elution. The plasmid yield was determined to 1.83 µg (24.4 ng/µl) high-purity DNA (A_{260}/A_{280} ≥1.8, A_{260}/A_{230} 2.0-2.2) (Thermo Fisher Scientific) by using Nanodrop ND-1000 spectrophotometer. A second run was later performed, yielding 4.24 µg (56.5 ng/µl) high-purity DNA.

2.1.2 Transforming *A. tumefaciens* with plasmid pIA291

Regenerating A. tumefaciens from stab cultures

Agrobacterium tumefaciens strain GV3101 (pMP90), which is resistant to rifampicin and gentamicin, (Koncz & Schell 1986) was kindly provided by Cathie Martin's group at the John Innes Centre, Norwich, England as a stab culture. The stab culture was used to inoculate solid LB medium containing 50 mg/l rifampicin and 25 mg/l gentamicin in 9 cm Petri dishes and incubated at 28°C for three days to produce single colonies.

Transformation protocol for A. tumefaciens

A slightly modified version of the protocol from Wise et al. (2006) for "Transformation of Agrobacterium Using the Freeze/Thaw Method" was used to transform *A. tumefaciens* with the plasmid pIA291. The following changes were made to the protocol;

- The optical density at 600 nm (OD_{600}) of the *A. tumefaciens* suspension used was measured to 1.15 using a Unicam Heλios α spectrophotometer. That is slightly higher than the suggested 0.5-1.0, as the overnight culture grew faster than anticipated.

- 0.97 µg (40 µl 24.4 ng/µl) high-purity pIA291 DNA was used because that was all the pIA291 DNA available at that moment. The authors suggested the use of 1 µg (100-1000 ng/µl) purified plasmid DNA.
- A 2 ml Eppendorf tube containing 100 µl competent *A. tumefaciens* in solution, but no plasmid DNA, and a 2 ml Eppendorf tube containing 100 µl non-competent *A. tumefaciens* in solution and no plasmid DNA, was used as negative controls.
- Centrifugation was performed in a Eppendorf 5810R centrifuge at 4°C for 13 minutes at 1 811*g* instead of the suggested 10 000*g* for 8-10 minutes, as centrifugation at such high speeds can cause damage to the bacteria (Peterson et al. 2012).
- 50 mg/l rifampicin, 25 mg/l gentamicin and 100 mg/l spectinomycin were used as selective agents.

Verification of transformed A. tumefaciens by colony PCR

Primers used to detect pIA291 DNA

Primers were designed using Primer3Plus (Untergasser et al. 2007). Four different sets of primers were designed (Table 1) and tested on high-purity pIA291 plasmid DNA.

Table 1. *Primers used for PCR to verify stable insertion of pIA291 DNA. Primer set #1 covers the transition from promoter to protein-coding gene. Primer set #2 covers only the promoter, while primer set #3 and #4 only covers the protein-coding gene.*

Primer set #	Target region	Forward primer	Reverse primer	Amplicon size (BP)
#1	CaMV 35S + *PhF3'5'H*	CGCACAATCCC ACTATCCTT	TGCTGCACCAA GCTCAGTAA	222
#2	CaMV 35S	GAGGAGCATCG TGGAAAAAG	CGGAGTCCTCT CCAAATGAA	160
#3	*PhF3'5'H*	TTGGTGCAGCA ACTTCAATC	AGCATCAGGGG TAGAAGCAA	236
#4	*PhF3'5'H*	GTGATCGGAGC ACTTCCACT	CCTGCATTAGG TGGACGATT	191

A temperature gradient experiment was performed to establish the most appropriate annealing temperature. The temperature gradient ranged from 45 to 60°C (12x reactions per set of primers, ~1.25°C steps). 24.4 ng high-purity plasmid DNA (1 µl 24.4 ng/µl) was added to each PCR reaction (Appendix Ig) for a total individual reaction volume of 25 µl in thin-walled PCR tubes and transferred to a DNA Engine Tetrad 2 thermocycler for thermocycling. The thermocycling conditions used are described in Table 2, except that in this particular case the annealing temperatures ranged from 45 to 60°C. The thermocycling conditions used were as suggested by co-supervisor Tone Melby. The PCR products were analysed by gel electrophoresis on a 200 ml 1% (w/v) agarose gel stained with 4 µl GelRed (Appendix II) and the DNA visualised by ImageLab v5.0 and BioRad ChemiDoc MP.

Primer sets #1 and #4, and an annealing temperature 54°C were considered most suited, as these reactions exhibited the most intense bands.

Colony PCR

A single colony from a Petri dish containing putatively transformed *A. tumefaciens* was used to inoculate 5 ml liquid LB containing 50 mg/l rifampicin, 25 mg/l gentamicin, and 100 mg/l spectinomycin, and incubated at 28°C and 225 rpm in a 13 ml Snap Cap overnight.

20 µl of the solution was transferred to a 1.5 ml Eppendorf tube containing 100 µl Milli-Q water and the solution boiled for two minutes to release the DNA (I Appelhagen pers. comm. 5 September 2016). 1 µl of the solution was added to each PCR reaction (Appendix Ig) for a total individual reaction volume of 25 µl in thin-walled PCR tubes and transferred to a DNA Engine Tetrad 2 thermocycler for thermocycling. The thermocycling conditions used are described in Table 2. Primer set #1 and #3 were used to detect pIA291 DNA (Table 1). 24.4 ng high-purity plasmid DNA (1 µl 24.4 ng/µl) was used as a positive control and 1 µl of Milli-Q H_2O was used as a negative control. The PCR products were analysed by gel electrophoresis on 2x 50 ml 1% (w/v) agarose gels stained with 1 µl GelRed and the DNA visualised by ImageLab v5.0 and BioRad ChemiDoc MP.

Table 2. *Thermocycling conditions used for PCR with OneTaq DNA polymerase (lid control mode: Constant at 100°C).*

Step	Temperature	Time (min:sec)
#1 Initial denaturation	94°C	03:00
#2 Denaturation	94°C	01:00
#3 Primer annealing	54°C	00:30
#4 Extension	72°C	01:00
#5 Cycle to step #2 34x times		
#6 Final extension	72°C	10:00
#7 Cooling	4°C	Forever

Making glycerol stock of transformed A. tumefaciens

Glycerol stocks were made to preserve the *A. tumefaciens* harbouring plasmid pIA291. 850 µl autoclaved glycerol and 150 µl *A. tumefaciens* growing in liquid LB supplemented with 25 mg/l gentamicin and 100 mg/l spectinomycin were transferred to 2 ml screw-cap vials, the contents mixed and the vials subsequently submerged in liquid N_2 for approximately 2 minutes. The vials were then stored at -80°C.

2.1.3 *Agrobacterium*-mediated transformation of *B. tuberhybrida* 'Urban Bicolor Pink' with plasmid pIA291

Plant material and culture conditions

The plant material used was from i*n vitro* cultures of *B. tuberhybrida* 'Urban Bicolor Pink', a hanging begonia. 'Urban Bicolor Pink' is a new Norwegian cultivar, which was introduced to the international ornamental market in 2015, bred through a joint NMBU-industry project, and owned by Tiboplant AS (Tiboplant 2015).

The plantlets were growing in semi-solid (0.35% (w/v) Gelrite) half-strength (½x) Murashige and Skoog (MS) medium (Murashige & Skoog 1962) (Appendix II) from Duchefa, supplemented with 2% sucrose (w/v) (MS2), 0.5 mg/l IBA, and 0.1 mg/l BAP, at 20°C under an 18-hour photoperiod

(cool white fluorescent light (Philips Master TL-D 58W/840), approximately 14 µmol m^{-2} s^{-1}). The plant material was subcultured onto fresh medium every six weeks. The culture conditions were as suggested by technicians Astrid Sivertsen and Gry Skjeseth (A Sivertsen & G Skjeseth pers. comm. 2016).

Establishing dose-response to glufosinate in B. tuberhybrida 'Urban Bicolor Pink'

Concentrations of 0.00, 0.25, 0.50, 1.00, 2.50, and 5.00 mg/l were tested to establish the most appropriate concentration of glufosinate for screening of transformants on selective media *in vitro*, and, because there might be a difference in glufosinate sensitivity between different tissues, separate experiments were performed for explants of petioles and leaves. Glufosinate stock solutions were filter sterilised and added to semi-solid MS3 medium supplemented with 0.1 mg/l NAA and 0.5 mg/l BAP after autoclaving.

Explants were obtained from *in vitro* cultures of *B. tuberhybrida* 'Urban Bicolor Pink' grown as described under Plant material and culture conditions. The edges of the leaves were excised using a scalpel to increase the cut surface area, and the remaining leaf material were cut into approximately 0.5-1 cm^2 pieces and transferred to the medium with the adaxial side touching the medium. Petioles were cut into approximately 0.5-1 cm pieces and placed vertically into the medium. 25 explants of each tissue type were used per concentration of glufosinate tested, totalling 150 explants (including 25 explants on medium without glufosinate as a positive control). The explants were subcultured every 14 days onto fresh medium. After eight weeks, the number of explants exhibiting formation of adventitious shoots was recorded.

Transformation protocol for B. tuberhybrida

A modified version of the protocol for transformation of *B. tuberhybrida* developed by Kiyokawa et al. (1996) was used. Because the protocol developed by Kiyokawa et al. (1996) was lacking some details, the whole protocol performed is written up here:

A single colony of *A. tumefaciens* strain GV3101 (pMP90) harbouring plasmid pIA291 was used to inoculate 5 ml liquid LB in Snap Cap and incubated at 28°C and 225 rpm for approximately 5 hours. 1 ml of this starter culture was then used to inoculate 100 ml liquid LB in a 500 ml

Erlenmeyer flask and incubated at 28°C and 225 rpm until an OD_{600} of 0.5-1.0 was preferentially achieved (usually approximately 20 hours). 50 mg/l rifampicin, 25 mg/l gentamicin, and 100 mg/l spectinomycin, or just 100 mg/l spectinomycin were used as selective agents in the LB medium. Table 3 contains the specific OD_{600} and antibiotics used for the various transformation experiments performed.

The *A. tumefaciens* suspension was transferred to 2x 50 ml Falcon tubes and centrifuged at 1 635*g* for 10 minutes to pellet the cells. The supernatant was discarded and the collected bacteria washed by resuspending in 20 ml liquid MS3 medium and centrifuged again at 1 635*g* for 5 minutes. The supernatant was discarded and the bacterial pellets finally resuspended in 8 ml liquid MS3, totalling 16 ml bacterial suspension which were transferred to 3x 5 cm Petri dishes.

0.5-1.0 cm^2 segments of leaves (with the edges excised to increase cut surface area) and 0.5-1.0 cm petioles cut from *B. tuberhybrida* 'Urban Bicolor Pink', both growing as described under Plant material and culture conditions on page 15, were submerged in the bacterial suspension for 5 minutes while shaking on a shaker, transferred to sterile filter paper to remove excess bacterial suspension, and then placed on semi-solid MS3 medium supplemented with 0.1 mg/l NAA and 0.5 mg/l BAP. The cultures were kept in the dark at 23°C for three days of co-cultivation, after which the explants were transferred to semi-solid MS3 medium supplemented with 0.1 mg/l NAA, 0.5 mg/l BAP, 500 mg/l cefotaxime, and 1 mg/l glufosinate. Some explants were always transferred to medium without glufosinate as positive controls. Cefotaxime was used to inhibit overgrowth of *A. tumefaciens*.

The cultures were kept at 20°C under 18-hour photoperiod (cool white fluorescent light (Philips Master TL-D 58W/840), approximately 14 µmol m^{-2} s^{-1}) and subcultured onto fresh medium every 14 days. The leaf explants were placed adaxial side facing the medium and the petiole explants placed vertically in the medium. Swollen explants were cut into smaller pieces during subculturing to increase contact with the medium and to avoid shoots growing into the lids of the Petri dishes.

Table 3. *Information related to transformation of B. tuberhybrida 'Urban Bicolor Pink' with the plasmid pIA291. The experiment was repeated nine times and a total of 1 692 explants were treated. The table shows the various amounts of explants treated per experiment, the antibiotics used during growth of A. tumefaciens and the* OD_{600} *of the bacterial solution (before washing and resuspending).*

R = Rifampicin, G = Gentamicin, S = Spectinomycin (mg/l).

Date	**Experiment #**	**Leaf explants**	**Petiole explants**	**Antibiotics used during growth of *A. tumefaciens***	**OD_{600} of *A. tumefaciens***
23.09.16	#1	129	47	50R/25G/100S	1.711
28.09.16	#2	130	1	50R/25G/100S	1.124
04.10.16	#3	84	0	50R/25G/100S	0.616
07.10.16	#4	157	0	50R/25G/100S	0.873
11.10.16	#5	210	1	50R/25G/100S	1.160
21.10.16	#6	289	48	100S	0.674
21.10.16	#7	200	2	100S	0.545
01.11.16	#8	170	14	100S	0.786
01.11.16	#9	210	0	100S	0.669
SUM		**1 579**	**113**		

Treatment of contaminations in in vitro cultures of B. tuberhybrida 'Urban Bicolor Pink'

The *B. tuberhybrida* 'Urban Bicolor Pink' explants were visually inspected at least once a week (except during the Christmas holidays between 22.12.16 and 04.01.17). Various contaminations arose, mainly caused by fungi or overgrowth of *A. tumefaciens* (Fig. 5).

When the first bacterial overgrowth of what was presumed to be *A. tumefaciens* was discovered the bacterial overgrowth was tested by colony PCR as described under 2.1.2 Colony PCR, and confirmed to be *A. tumefaciens* transformed with pIA291. Subsequent contaminations of similar characteristic cloudy brownish thick liquid appearance were also considered to be caused by *A. tumefaciens*, even though they were not all verified by PCR.

Fig. 5. *Examples of A. tumefaciens overgrowth (top) and fungal infection (bottom).*

The images were obtained using a Leica M205 C stereo microscope and Leica application suite v4.3.0.

When an infection was discovered the seemingly unaffected explants in the same Petri dish, if any, were immediately transferred to fresh medium. Any explants infected by fungi were discarded. When *A. tumefaciens* overgrowth was discovered the amount of damage was assessed on a case by case basis.

According to Maheshwari and Kovalchuk (2016) it is possible to treat overgrowth of *A. tumefaciens* by washing the explant in sterile water containing antibiotics. The explant was discarded if the overgrowth detected was considered as total. The explant was treated in the following manner if the overgrowth was considered as minimal:

Rinsed in Milli-Q H_2O, then submerged and shaken in Milli-Q H_2O + 500 mg/l cefotaxime for approximately 1 minute, again rinsed in Milli-Q H_2O, again submerged and shaken in Milli-Q H_2O + 500 mg/l cefotaxime for approximately 1 minute, and again rinsed in Milli-Q H_2O. Finally, excess liquid was removed by placing the explant on sterile filter paper, and the explant transferred to fresh medium.

2.1.4 Screening and analyses of *B. tuberhybrida* 'Urban Bicolor Pink' transformants

Extraction of DNA from leaves of B. tuberhybrida 'Urban Bicolor Pink' by CTAB method

The protocol for extraction of organic acids from leaves of *B x cheimantha* by Kopperud and Einset (1995) was combined with the protocol used for DNA extraction from tobacco at the International Centre for Genetic Engineering and Biotechnology (ICGEB) (R Pathak pers. comm. 6 December 2016).

100 mg (fresh weight) leaf tissue of *B. tuberhybrida* 'Urban Bicolor Pink' was collected and ground to a fine powder in the presence of liquid N_2 using a mortar and pestle or in a Retsch MM301 TissueLyser at 20 hz for 30 seconds. The powder was immediately transferred to pre-chilled 2 ml Eppendorf tubes.

1 ml of cold T10E10 buffer (10 mM Tris-HCl, 10 mM EDTA, pH 8) was added, the mixture vortexed for 10 seconds and then centrifuged in an Eppendorf 5810R centrifuge at 4°C for 10 minutes at 10 600*g*. The supernatant was discarded and the pellet resuspended in 800 µl extraction buffer (2% (v/v) CTAB, 1.4 M NaCl, 100 mM Tris-HCl, 20 mM EDTA, 1% (v/v) 2-Mercaptoethanol) and kept at 65°C for 45 minutes with intermittent mixing.

700 µl of Chloroform:Isoamyl alcohol (24:1) was added, the tube inverted to mix thoroughly, and then centrifuged at 10 600*g* for 10 minutes. The supernatant was transferred to a new 2 ml Eppendorf tube and 700 µl of Chloroform:Isoamyl alcohol (24:1) was added, the tube inverted to mix, and the suspension again centrifuged at 10 600*g* for 10 minutes. The supernatant was transferred to a new 2 ml Eppendorf tube, 3/4th volume of Isopropanol added (usually approximately 530 µl) and the suspension again centrifuged at 10 600*g* for 10 minutes.

The supernatant was immediately discarded and the pellet washed twice by adding 1 ml 70% EtOH, centrifuging at 10 600*g* for 30 seconds, and removing EtOH by pipette. The pellet was finally air dried and dissolved in 50 µl Milli-Q H_2O, to be diluted further when used.

The DNA yield was determined by using Nanodrop ND-1000 spectrophotometer.

Locating a positive control for Extract-N-Amp PCR of putatively transformed B. tuberhybrida 'Urban Bicolor Pink'

Because begonia is particularly difficult material for extraction of DNA (Kopperud & Einset 1995), and it was desired to simplify the verification of *B. tuberhybrida* 'Urban Bicolor Pink' transformants by using the Extract-N-Amp kit from Sigma-Aldrich for DNA extraction and verification by PCR, it was decided to find a set of primers that could be used as a positive control for DNA extraction when performing the Extract-N-Amp protocol to avoid false negatives.

Because no sequence data for any *Begonia* species is available in the National Center for Biotechnology Information (NCBI) Gene database (Brown et al. 2015), an alternative approach had to be used. Dajana Blagojevic, Sylvia Sagen Johnsen, and Tone Melby, all from NMBU, kindly provided me with primer sets targeting regions likely conserved in many plant species (Table 4). 188 ng (1 µl 188 ng/µl) high-purity *B. tuberhybrida* 'Urban Bicolor Pink' DNA, and 162 ng (1 µl 162 ng/µl) high-purity *A. thaliana* DNA for control, in addition to 1 µl Milli-Q H_2O used as negative controls, were added to each PCR reaction (Appendix Ig (primer set #9-12), Appendix Ih (primer set #13)) for a total individual reaction volume of 25 µl. OneTaq DNA polymerase were used for primer set #9-12. For primer set #13 Taq DNA polymerase was used, as this was the only polymerase available at the time. An annealing temperature of 57°C was used, as suggested by the researchers who provided me with the primer sets, otherwise the thermocycling conditions were as described in Table 2.

Table 4. *Primers used for PCR to find a putative positive control for subsequent use when verifying B. tuberhybrida 'Urban Bicolor Pink' transformants. As no information on any Begonia sequence in the NCBI Gene database was available, several primers targeting regions likely conserved between many plant species were tested.*

Primer set #	Species previously tested on	Target region	Forward primer	Reverse primer	Amplicon size (BP)
#9	*A. thaliana*	*EF-1α*	CCCAGGCTGATTGTGCTGT	GGGTAGTGGCATCCATCTTGTT	159
#10	*A. thaliana*	*Actin 2*	TCAGATGCCCAGAAGTCTTGTTCC	CCGTACAGATCCTTCCTGATATCC	121
#11	*I. batatas*	*β-tubulin*	CAACTACCAGCCACCAACTGT	CAGATCCTCACGAGCTTCAC	Unknown
#12	*I. batatas*	*18S*	GTGACGGGTGACGGAGAATTA	ACACTAAAGCGCCCGGTATTG	Unknown
#13	*L. usitatissimum*	*ITS*	GGAAGGAGAAGTCGTAACAAGG	GCAATTCACACCAAGTATCGC	382

It was discovered that only primer set #13 yielded bands in both *B. tuberhybrida* 'Urban Bicolor Pink' and *A. thaliana*. Primer set #13 was then used to test the Extract-N-Amp kit on 'Urban Bicolor Pink' and *A. thaliana*. The procedure was performed as described in the Extract-N-Amp kit protocol (Appendix Ic) by using 0.38 cm^2 leaf discs from 'Urban Bicolor Pink' and *A. thaliana*. Standard PCR was performed in parallel as a positive control by using the same primer set (#13); 188 ng (1 µl 188 ng/µl) high-purity 'Urban Bicolor Pink' DNA and 162 ng (1 µl 166 ng/µl) high-purity *A. thaliana* DNA added to each PCR reaction (Appendix Ih) for a total individual reaction volume of 25 µl in thin-walled PCR tubes and transferred to a DNA Engine Tetrad 2 thermocycler for thermocycling. 1 µl Milli-Q H_2O was used as negative controls with both the Extract-N-Amp protocol and the standard PCR protocol. The same thermocycler program (Table 2, but with an annealing temperature of 57°C) were used for both the standard PCR and the Extract-N-Amp reactions. The PCR products were analysed by gel electrophoresis on a 50 ml 1.5% (w/v) agarose gel stained with 1 µl GelRed and the DNA visualised by ImageLab v5.0 and BioRad ChemiDoc MP.

Transplanting mature putatively transformed B. tuberhybrida 'Urban Bicolor Pink' shoots for root induction

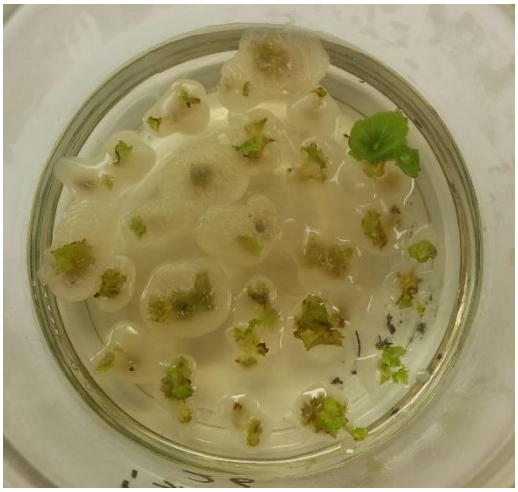


Fig. 6. *Overgrowth by A. tumefaciens on excised shoots six days after transfer to semi-solid MS3 without any additives for rooting.*

The image was obtained using a Leica M205 C stereo microscope and Leica application suite v4.3.0.

Adventitious shoots were excised and transferred to jars containing semi-solid MS3 without any additives for rooting when they had elongated to approximately 2-5 mm, as described by Kiyokawa et al. (1996). After six days *A. tumefaciens* overgrowth was discovered on 72 out of 230 shoots, as shown in Fig. 6. Consequently, it was decided to transfer the remaining unharmed shoots immediately to potting mix (Appendix II) in a greenhouse under a 16-hour photoperiod (supplemental lighting (Philips Master Son-T Pia Plus 400W E E40, approximately 100 µmol m^{-2} s^{-1}) during time periods with low levels of solar radiation (<100 µmol m^{-2} s^{-1}), 70% air humidity, 22°C during the day, 20°C during the night). The plantlets were covered with a plastic sheet to maintain plant turgor until rooted and vigorous growth was observed (usually 2-3 weeks). Subsequent elongated *in vitro* adventitious shoots were also transferred directly to potting mix for root induction.

Verification of B. tuberhybrida 'Urban Bicolor Pink' transformants by PCR

The 28 largest plants were chosen for PCR screening (2 from experiment #1, 20 from experiment #2, 6 from experiment #3 (Table 3)). The procedure was performed as described in the Extract-N-Amp protocol (Appendix I) by using 0.25-0.50 cm^2 leaf explants. Primer set #13 (Table 4) was used as a positive control to test whether the Extract-N-Amp DNA extraction worked as intended. Primer set #1 (Table 1) was used to verify transformants. Unfortunately, no positive control for verification of transformants was employed as DNA from *B. tuberhybrida* 'Urban Bicolor Pink' was mistakenly used instead of pIA291 plasmid DNA. 1 µl Milli-Q H_2O was used as a negative control. The

reactions were transferred to a DNA Engine Tetrad 2 thermocycler for thermocycling as described in Table 2, but with an annealing temperature of 57°C for primer set #13. The PCR products were analysed by gel electrophoresis on a 200 ml 1.5% (w/v) agarose gel stained with 4 µl GelRed and the DNA visualized by ImageLab v5.0 and BioRad ChemiDoc MP.

Eight of the plants which the initial PCR showed to be transformed with the plasmid pIA291 (1 from experiment #1, 5 from experiment #2, 2 from experiment #3 (Table 3)) were tested again by Extract-N-Amp PCR. The procedure was performed as described above, but this time both primer set #1 and #4 (Table 1) were used, and 57 ng (1 µl 56.5 ng/µl) high-purity pIA291 DNA was used as a positive control. 1 µl Milli-Q H_2O was used as a negative control. The PCR products were analysed by gel electrophoresis on 2x 50 ml 1.5% (w/v) agarose gels stained with 1 µl GelRed and the DNA visualized by ImageLab v5.0 and BioRad ChemiDoc MP.

pH and colour measurements

To measure the pH and colour of sap from *B. tuberhybrida* 'Urban Bicolor Pink' sepals and petals, equal parts of sepals and petals from staminate and pistillate flowers were collected and ground to a powder using a mortar and pestle in the presence of liquid N_2, transferred to 50 ml Falcon tubes and thawed at room temperature. The ground tissues were then centrifuged at 2 000*g* for 4 minutes and the supernatant transferred to glass test tubes for measurement of pH using a Thermo Electron Corporation Orion 420A+ pH-meter.

To determine if the pH of the sap was stable at room temperature the pH of sap from wild type sepals and petals was measured immediately after extraction of sap, and at 6, 24, 48, and 72 hours after extraction.

A Color Striker colorimeter (software v1.0.14) from It's Mathai employing CIE Standard Illuminant D65 (International Commission on Illumination 1999) was used to measure colour. Colour differences (Δ values) were calculated using the DIN99o formula (Cui et al. 2002) (DIN99o was denoted DIN99b by Cui et al. (2002), but has later been referred to as DIN99o (Dibakar Raj et al. 2013)). The colour of sepals and petals from both the wild type and the eight transformed plants chosen for second screening by PCR were measured.

Furthermore, sap was extracted from the wild type and from two different transformed plants, which tested positive for insertion of the plasmid pIA291 in all PCR screenings performed. The

colour of the sap was measured at pH 1.5 (unadjusted), 3.2, 5.1, 7.3, and 9.9, using 1 M NaOH and 0.5 M HCl to adjust the pH of the sap.

2.2 Disruption of F3'H using CRISPR/Cas9

2.2.1 Design of sgRNA and plasmids

Two custom CRISPR/Cas9 plasmids designed for *Agrobacterium*-mediated transformation and containing *Cas9* codon optimised for dicotyledonous plants were ordered from Sigma-Aldrich for disruption of *F3'H* in *B. tuberhybrida* 'Urban Bicolor Pink' and *A. thaliana* (Fig. 7). The only difference between the two CRISPR/Cas9 plasmids is the incorporated sgRNA sequence, which guides the Cas9 endonuclease to the target sequence (Jinek et al. 2012). An sgRNA length of 19 nt was chosen for both plasmids, as truncated sgRNAs have been found to decrease off-target effects without reducing editing efficiencies (Fu et al. 2014; Osakabe et al. 2016), and was the length recommended by the technicians at Sigma-Aldrich (G Jackson pers. comm. 16 September 2016). The *A. thaliana* AtU6-1 promoter was chosen as sgRNA expression promoter, as suggested by Sigma-Aldrich (Sigma-Aldrich 2016). The *bar* gene conveying resistance to glufosinate was chosen as a selectable marker for selection of transformed plants, as glufosinate already had been acquired for selection of *B. tuberhybrida* transformed with pIA291. CaMV 35S promoters were chosen for expression of *Cas9* and *bar*. The plasmid also includes the *KanR* gene conveying resistance to kanamycin for bacterial selection.

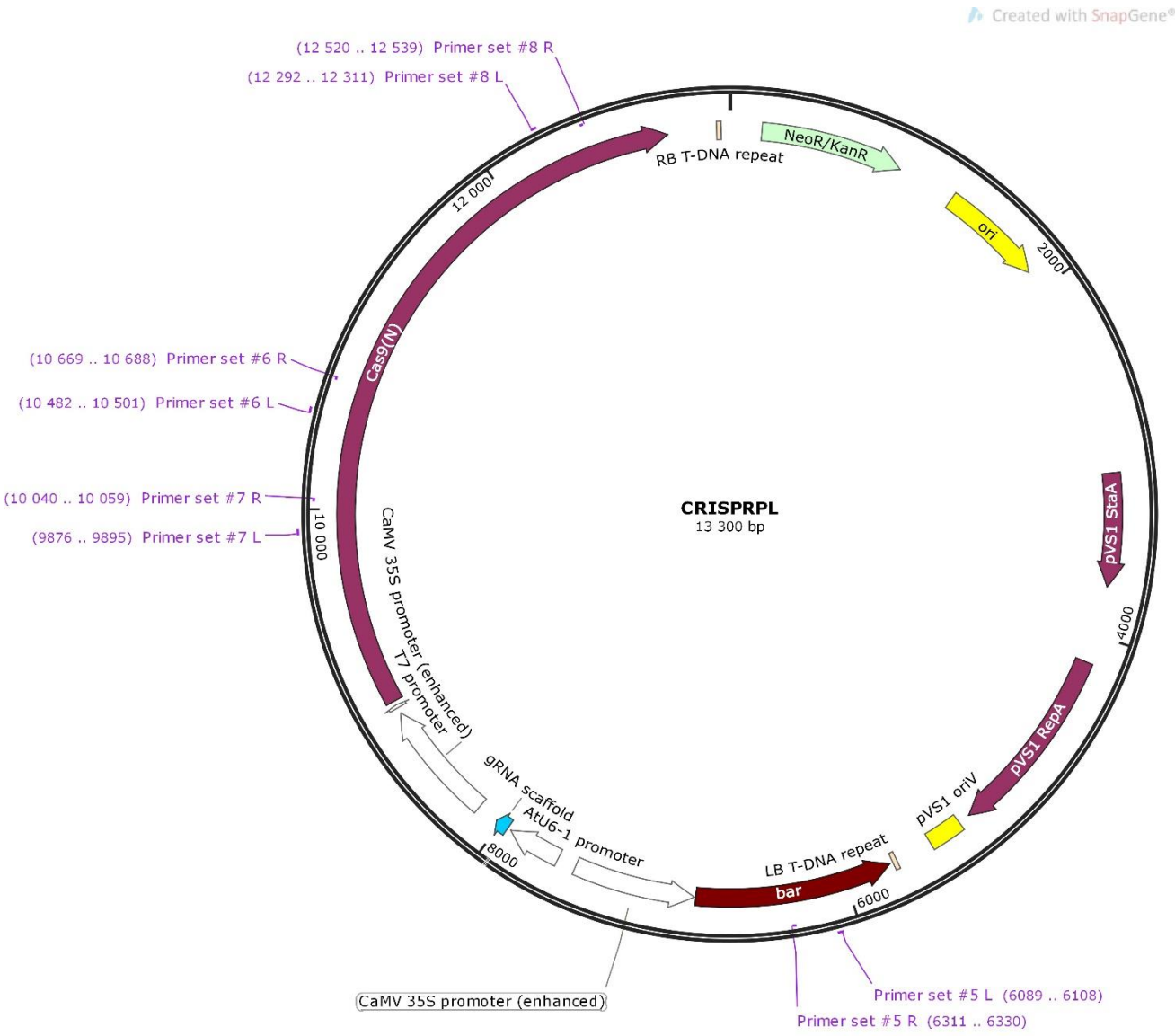


Fig. 7. *Map of the custom CRISPRPL plasmid from Sigma-Aldrich containing sgRNA, the gene encoding Cas9, as well as two 35S promoters, the bar gene (resistance to glufosinate) and the KanR gene (resistance to kanamycin). Two plasmids with different sgRNAs targeting different F3'H sequences were made. The plasmids were used to disrupt F3'H in B. tuberhybrida and A. thaliana. Primer set #5-8 (Table 5).*

The plasmid map was created using SnapGene v3.3.4 (from GSL Biotech; available at snapgene.com).

Design of sgRNA for use in A. thaliana (CRISPR/Cas9 plasmid #1)

The NCBI Gene database (Brown et al. 2015) was used to access the genetic code for the gene *flavonoid 3'-hydroxylase* (*F3'H*) in *A. thaliana* (Col) (National Center for Biotechnology Information 2000). CRISPR Design (Zhang Lab 2015), CRISPRdirect (Naito et al. 2015), and

CRISPOR (Haeussler et al. 2016) were used to scan the genetic code for possible target sequences and estimate their specificity.

CCTCCTAGCCACTGTCCTCTTC was chosen as the target sequence as zero likely off-target effects were discovered (three off-targets with four mismatches were indeed found, but none of those were in the 12 bp seed sequence adjacent to PAM and none of them in exons). This target is located in the very first exon of the gene, minimizing the chance of getting a truncated functional protein, as the desired indel-mutation is more to likely cause a full knock-out of the gene if the transcription is halted as early as possible (G Jackson pers. comm. 16 September 2016). Furthermore, the corresponding sgRNA sequence GAAGAGGACAGTGGCTAGG, which is complementary to the target sequence and does not include PAM (AGG), begins with a guanine nucleotide which is thought to be ideal for U6 promoters to initiate transcription (Jinek et al. 2013; Kim et al. 2016), even though this has not been proven for plant applications (Liang et al. 2016). The 58% G/C content of the sgRNA is also within the recommended 50-70% range (Liang et al. 2016; Ma et al. 2015; Tsai et al. 2015). This specific sgRNA sequence was furthermore considered as excellent by the bioinformaticians working at Sigma-Aldrich (M Sankala pers. comm. 27 September 2016).

Design of sgRNA for use in A. thaliana and B. tuberhybrida 'Urban Bicolor Pink' (CRISPR/Cas9 plasmid #2)

The aim was to design one sgRNA that could work in *B. tuberhybrida* 'Urban Bicolor Pink' as well as in *A. thaliana*. Unfortunately, it was not feasible to perform sequencing of *F3'H* in *B. tuberhybrida* as a part of this thesis work, due to time and financial constraints.

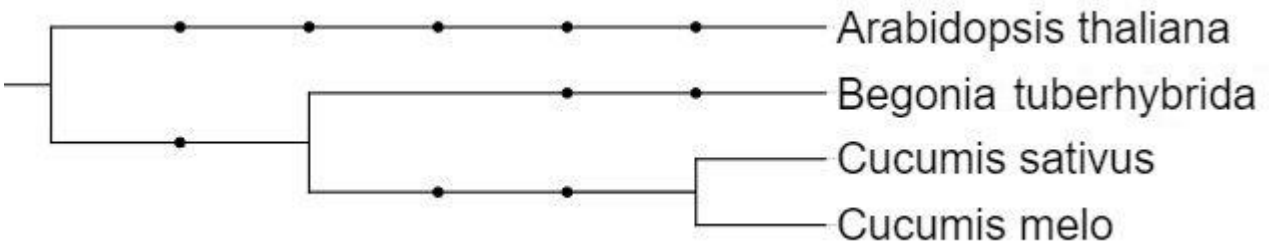


Fig. 8. *Phylogenetic tree showing the evolutionary relationship between A. thaliana, B. tuberhybrida, C. sativus, and C. melo. The phylogenetic tree was created using the Interactive tree of life (Letunic & Bork 2016).*

Because the NCBI Gene database (Brown et al. 2015) does not contain any information about any *Begonia* species, searches were performed for *F3'H* sequences conserved between other species in the Cucurbitales order, of which *Begonia* is a member, and *A. thaliana*, all of which are members of the clade Rosids.

The genomes of *Cucumis melo* and *Cucumis sativus*, both members of the Cucurbitales order, are both sequenced and available in the NCBI Gene database. When aligning the *F3'H* sequence from these two species (National Center for Biotechnology Information 2009; National Center for Biotechnology Information 2012) and *A. thaliana* (National Center for Biotechnology Information 2000) using BLAST (Boratyn et al. 2013), only one possible sgRNA target sequence 100% conserved between all three species was found; TACAGAACATGTTCACAGCTGG (TGG is the PAM sequence, which is not included in the sgRNA). As when designing the sgRNA for CRISPR/Cas9 plasmid #1, CRISPR Design (Zhang Lab 2015), CRISPRdirect (Naito et al. 2015), and CRISPOR (Haeussler et al. 2016) were used to estimate the sgRNA specificity, although only estimates for specificity in *A. thaliana* could be obtained, as a reference genome for *B. tuberhybrida* is not available. The target sequence is covering an intron/exon border, but the predicted cut site is located in the beginning of the exon and zero off-target effects were discovered (five off-targets with four mismatches were indeed found, but none of those were in the 12 bp seed sequence adjacent to PAM and none of them in exons).

Unfortunately, this target is located in the third out of a total of four exons, reducing the chance of successfully producing a full knock-out (G Jackson pers. comm. 16 September 2016). No extra G was added to the 5’ of the sgRNA, as Liang et al. (2016) could not find it to be beneficial, and the technicians at Sigma-Aldrich suggested it was not necessary (G Jackson pers. comm. 5 October 2016). The G/C content of 42% is within the 30-80% range suggested by Liang et al. (2016), but lower than the >50% suggested by Ma et al. (2015) for optimal efficiency.

2.2.2 Transformation of *E. coli* by heat shock

NovaBlue *E. coli* from Merck Millipore, a K-12 strain derivative, provided in single-use 50 µl aliquots were transformed with the two CRISPR/Cas9 plasmids (plasmid #1 and plasmid #2) separately in parallel. The provided “detailed transformation protocol” was followed (Appendix Ib) and the provided test plasmid conveying ampicillin resistance was used as a positive control. 20 ng (1 µl 20 ng/µl) of CRISPR/Cas9 plasmid DNA #1 and #2, as well as 0.2 ng (1 µl 0.2 ng/µl) test plasmid DNA were used. 50 mg/l kanamycin was used in the medium to select for *E. coli*

transformed with CRISPR/Cas9 plasmid #1 and #2, and 50 mg/l carbenicillin was used in the medium to select for *E. coli* transformed with the test plasmid. Putatively transformed *E. coli* were also plated on medium containing the "wrong" (opposite) type of antibiotic as negative controls.

Verification of transformed E. coli by colony PCR

Primers used to detect CRISPR/Cas9 DNA

Primers were designed using Primer3Plus (Untergasser et al. 2007). Four different sets of primers were designed (Table 5) and tested directly on putatively transformed *E. coli*. Because running a temperature gradient is time- and reagent-consuming, especially when testing several sets of primers, New England Biolabs' Tm Calculator v1.9.6 (New England BioLabs 2016c) was used to calculate suggested annealing temperatures (T_a). The software suggested the use of T_a = 54°C for primer set #5 and #6 and T_a = 52°C for primer set #7 and #8.

Table 5. *Primers used for PCR to verify stable insertion of CRISPR/Cas9 DNA.*

Primer set #	Target region	Forward primer	Reverse primer	Amplicon size (BP)
#5	*bar*	GAAGTCCAGCTGCCAGAAAC	AGTCCACCGTGTACGTCTCC	242
#6	*Cas9*	AGTACGTGACCGAGGGAATG	GATCGTGGTAGGTTCCGAGA	207
#7	*Cas9*	ATGAGCACCACCAGGATCTC	TCGGTTCCATCCATCTTCTC	184
#8	*Cas9*	TGGTTTCGATTCTCCTACCG	CGAGCATCCTCTTTCTACCG	248

Colony PCR

The primer sets described in Table 5 were tested directly on single colonies from the Petri dishes containing *E. coli* putatively transformed with CRISPR/Cas9 plasmid #1 and #2 as described under 2.1.2 Colony PCR on page 14. 20 ng plasmid DNA (1 µl 20 ng/µl) was used as a positive control and 1 µl of Milli-Q H_2O was used as a negative control. The PCR products were analysed by gel

electrophoresis on a 200 ml 1% (w/v) agarose gel stained with 4 µl GelRed and the DNA visualized by ImageLab v5.0 and BioRad ChemiDoc MP.

Making glycerol stock of transformed E. coli

Glycerol stocks were made to preserve the *E. coli* harbouring CRISPR/Cas9 plasmid #1 and #2. 850 µl autoclaved glycerol and 150 µl *E. coli* growing in liquid LB supplemented with 50 mg/l kanamycin were transferred to 2 ml screw-cap vials, the contents mixed and the vials subsequently submerged in liquid N_2 for approximately 2 minutes. The vials were then stored at -80°C.

2.2.3 Isolation of plasmid DNA from *E. coli*

The single *E. coli* colonies that were verified as transformed by PCR were used to inoculate 2x 5 ml liquid LB containing 50 mg/l kanamycin in 13 ml Snap Caps (one Snap Cap with *E. coli* transformed with CRISPR/Cas9 plasmid #1 and one Snap Cap with *E. coli* transformed with CRISPR/Cas9 plasmid #2) and incubated at 37°C and 300 rpm overnight. Plasmid DNA was isolated from the 5 ml *E. coli* cultures (aliquoted into 2x 3 suspensions) using Genomed JETquick Plasmid Miniprep Spin Kit according to the kit protocol (Appendix I). 75µl TE-buffer was used for final elution. The yield of high-purity plasmid DNA was determined to 48.29 µg (182.7, 174.1 and 287.2 ng/µl) of CRISPR/Cas9 plasmid #1 and 51.08 µg (167.3, 315.9, 197.9 ng/µl) of CRISPR/Cas9 plasmid #2 by using Nanodrop ND-1000 spectrophotometer.

2.2.4 Transformation of *A. tumefaciens* with the CRISPR/Cas9 plasmids

The procedure was performed as previously described under 2.1.2 Transformation protocol for A. tumefaciens on page 12. The only differences were;

- 25 mg/l gentamicin and 50 mg/l kanamycin were used as selective agents.
- The OD_{600} was measured to 1.37 when preparing freeze/thaw-competent cells.
- Amount of plasmid DNA used (higher amounts of DNA results in better transformation efficiencies (I Appelhagen pers. comm. 5 September 2016)):

- 2.87 µg (10 µl 287.15 ng/µl) of CRISPR/Cas9 plasmid #1.
- 3.16µg (10 µl 315.90 ng/µl) of CRISPR/Cas9 plasmid #2.

Verification of transformed A. tumefaciens by colony PCR

Single colonies of *A. tumefaciens* putatively transformed with CRISPR/Cas9 plasmid #1 and #2 were each used to inoculate 5 ml liquid LB containing 25 mg/l gentamicin and 50 mg/l kanamycin separately, and incubated at 28°C and 225 rpm in 13 ml Snap Caps overnight.

Colony PCR was performed as described under 2.1.2 Colony PCR on page 14. Primer set #6 (Table 5) was the only primer set used. 20 ng plasmid DNA (1 µl 20 ng/µl) was used as a positive control, 1 µl of Milli-Q H_2O and 1 µl solution of wild type *A. tumefaciens* strain GV3101 (pMP90) growing in liquid LB containing 25 mg/l gentamicin were used as negative controls. The PCR products were analysed by gel electrophoresis on a 50 ml 1.5% (w/v) agarose gels stained with 1 µl GelRed and the DNA visualized by ImageLab v5.0 and BioRad ChemiDoc MP.

Making glycerol stock of A. tumefaciens transformed with *CRISPR/Cas9* plasmid #1 and #2

Glycerol stocks were made to preserve the *A. tumefaciens* harbouring CRISPR/Cas9 plasmid #1 and #2. 850 µl autoclaved glycerol and 150 µl *A. tumefaciens* growing in liquid LB supplemented with 25 mg/l gentamicin and 50 mg/l kanamycin were transferred to each of their own 2 ml screw-cap vials, the contents mixed and the vials subsequently submerged in liquid N_2 for approximately 2 minutes. The vials were then stored at -80°C.

2.2.5 *Agrobacterium*-mediated transformation of *B. tuberhybrida* ‘Urban Bicolor Pink’ with CRISPR/Cas9 plasmid #2

Agrobacterium-mediated transformation of *B. tuberhybrida* ‘Urban Bicolor Pink’ with the CRISPR/Cas9 plasmid #2 was performed as described under 2.1.3 Transformation protocol for B. tuberhybrida on page 16. The only differences were that *A. tumefaciens* harbouring CRISPR/Cas9

plasmid #2 was used, the OD_{600} of the bacterial culture was measured to 0.861 (before washing and resuspending), and 50 mg/l kanamycin was the only antibiotic used in the LB medium. Because of time constraints only one batch of 'Urban Bicolor Pink' explants were treated. Contaminations were handled in the same manner as described under 2.1.3 Treatment of contaminations on page 19.

Table 6. *Information related to the transformation of B. tuberhybrida 'Urban Bicolor Pink' with CRISPR/Cas9 plasmid #2. A total of 112 explants were treated. The table shows the amounts of explants treated, the antibiotics used during growth of A. tumefaciens and the OD_{600} of the bacterial solution (before washing and resuspending).*

Date	**Experiment #**	**Leaf explants**	**Petiole explants**	**Antibiotics used during growth of *A. tumefaciens***	**OD_{600} of *A. tumefaciens***
10.01.17	#1	85	27	50 mg/l kanamycin	0.861

2.2.6 Transformation of *A. thaliana* with CRISPR/Cas9 plasmid #1 and #2

Plant material and growth conditions

Seeds of *A. thaliana* (Col) were stratified at 4°C for approximately 48 hours to break seed dormancy and hence increase germination rates. The seeds were then sown in potting mix at approximately 10-day intervals, to ensure that at least a few plants had developed to the appropriate stage for transformation, and kept under 18-hour photoperiod in a greenhouse (supplemental lighting (Philips Master Son-T Pia Plus 400W E E40, approximately 100 µmol m^{-2} s^{-1}) during time periods with low levels of solar radiation (<100 µmol m^{-2} s^{-1}), 70% air humidity, 22°C during the day, 20°C during the night). Two-three weeks after germination the plants were transferred to separate pots, three plants in each pot. The first bolts were clipped five days prior to transformation to stimulate proliferation of secondary bolts, which increase the number of floral buds per plant and the transformation efficiency (Clough & Bent 1998; Narusaka et al. 2010).

Transformation of A. thaliana by "floral drip"

A modified version of the *A. thaliana* transformation protocols developed by Clough and Bent (1998), Hvoslef-Eide et al. (2016), Narusaka et al. (2010), and Zhang et al. (2006) was used.

Single colonies of *A. tumefaciens* harbouring CRISPR/Cas9 plasmid #1 and #2, respectively, were used separately to inoculate each of their own 5 ml liquid LB in 13 ml Snap Caps and grown at 28°C and 225 rpm for approximately six hours. 500 µl of these starter cultures were then used to inoculate each of their own 50 ml liquid LB in 250 ml Erlenmeyer flasks and incubated at 28°C and 225 rpm for approximately 24 hours. 25 mg/l gentamicin and 50 mg/l kanamycin were used as selective agents.

The OD_{600} of the cultures were measured to 1.57 (plasmid #1) and 1.73 (plasmid #2) using a Unicam Heλios α spectrophotometer. The cells were pelleted by centrifugation at 3 000*g* for five minutes, the supernatant discarded and the pellets washed in 30 ml infiltration medium (½x MS5, 0.05% Silwet L-77, pH=5.8). The suspensions were again centrifuged at 3 000*g* for five minutes, the supernatant discarded and the pellets resuspended in 10 ml infiltration medium.

Any siliques and open flowers were removed to improve the transformation efficiency ratio. A plastic Pasteur pipette was used to drip one drop of bacterial solution on each flower bud. The plants were covered in plastic film and kept in the dark at 22°C for approximately 24 hours to retain humidity without causing overheating and to increase the transformation efficiency. Afterwards the plants were kept under 18-hour photoperiod at 22°C (cool white fluorescent light (Osram L 36W/840 Lumilux), approximately 70 µmol m^{-2} s^{-1}) in a Termaks environmental chamber (Type KBP 2324 V).

The protocol was repeated once more five days later. For the second procedure, the OD_{600} of the cultures were measured to 1.68 (plasmid #1) and 1.58 (plasmid #2) before washing and resuspending in infiltration medium.

Harvesting and surface-sterilization of A. thaliana seeds

After approximately two months, the plants had turned almost completely brown and the siliques were dry. The plants were not watered the last week before harvesting. The seeds were harvested from individual plants by rubbing the siliques over a sheet of paper, sieving the collected material over another sheet of paper and the seeds finally transferred to 15 ml Falcon tubes, one tube per plant.

The seeds were surface sterilized according to the protocol taught in the BIO244 course at NMBU (Hvoslef-Eide et al. 2016).

5 ml of wash solution (70% EtOH + 0.05% (v/v) TritonX-100) were added to the 15 ml Falcon tubes containing the seeds. The tubes were inverted every 1-2 minutes. After six minutes, the seeds were allowed to sink to the bottom of the tube and the wash solution removed by pipette. 5 ml wash solution were again added to the tubes, the tubes inverted every 1-2 minutes, and the wash solution finally removed by pipette after six minutes. 5 ml of 100% EtOH were then added, the tubes inverted every 1-2 minutes, and the EtOH finally removed by pipette after five minutes. The contents of the tubes were transferred onto sterile filter paper and the EtOH was allowed to evaporate. When the seeds were dry, they were transferred to new 15 ml Falcon tubes and kept at room temperature in the dark.

2.2.7 Screening and verification of CRISPR/Cas9 gene edited plants

Establishing dose-response to glufosinate in A. thaliana

A relatively wide range of glufosinate concentrations (5-25 mg/l) have been reported as the most appropriate concentrations for screening of transformants on selective media *in vitro* (Abdeen & Miki 2009; Glazebrook & Weigel 2002). Consequently, it was decided to test the following concentrations; 0.0, 2.5, 5.0, 10.0, 15.0, 20.0, and 30.0 mg/l glufosinate.

25 wild type seeds of *A. thaliana* (Col) were used per concentration of glufosinate tested, totalling 175 seeds (including 25 seeds on medium without glufosinate as a positive control). All the seeds were from the same plant and vial as had been used for transformation purposes.

The seeds were sown in 9 cm Petri dishes containing semi-solid MS0.7 medium supplemented with the various concentrations of glufosinate, in aliquots of five seeds per Petri dish. The Petri dishes were kept at 4°C for approximately 48 hours to stratify the seeds. The Petri dishes were then transferred to a growth chamber and kept at 20°C under 18-hour photoperiod (cool white fluorescent light (Philips Master TL-D 58W/840), approximately 14 µmol m^{-2} s^{-1}). The amount of seeds that had germinated and were actively growing were counted after 14 days.

Screening of A. thaliana transformants by seed colour/phenotype

Seeds lacking F3'H activity should be possible to distinguish from wild type seeds by observing the seed colour (Appelhagen et al. 2014); mutants lacking F3'H activity, termed *tt7*-mutants by Appelhagen et al. (2014), have a lighter seed colour than the wild type seeds. As such, a large number of *A. thaliana* seeds were observed under a Leica M205 C stereo microscope to find putative transformants.

Screening of A. thaliana transformants on medium containing glufosinate

The screening procedure was initially performed as described by Glazebrook and Weigel (2002). The concentration of glufosinate used was increased to 10 mg/l during the screening process because 5 mg/l glufosinate proved insufficient in suppressing growth of non-transformed seedlings. Furthermore, 500 mg/l cefotaxime was eventually added to the medium used to avoid overgrowth of *A. tumefaciens*.

A. thaliana-seeds, harvested and surface-sterilized as described under 2.2.6 Harvesting and surface-sterilization of A. thaliana seeds on page 33, were sowed in 9 cm Petri dishes containing semi-solid MS0.7 medium supplemented with either 5 mg/l glufosinate, 10 mg/l glufosinate, or 10 mg/l and 500 mg/l cefotaxime, and kept at 4°C for approximately 48 hours to stratify the seeds. Approximately 50 mg dry seeds were sown evenly spread out per Petri dish. Seeds were also sown on medium without any glufosinate as a positive control, and wild type seeds were sown on medium containing glufosinate as a negative control. The Petri dishes were then transferred to a growth chamber and kept at 20°C under 18-hour photoperiod (cool white fluorescent light (Philips Master TL-D 58W/840), approximately 14 µmol m^{-2} s^{-1}). Healthy green plants were transferred to potting mix when they could be clearly distinguished from moribund seedlings, and then kept under 18-hour photoperiod at 22°C (cool white fluorescent light (Osram L 36W/840 Lumilux), approximately 70 µmol m^{-2} s^{-1}) in a Termaks environmental chamber (Type KBP 2324 V).

Screening of A. thaliana transformations in potting mix

The screening procedure was performed as described by Glazebrook and Weigel (2002).

A. thaliana seeds harvested as described under 2.2.6 Harvesting and surface-sterilization of *A. thaliana* seeds on page 33 were sowed in potting mix, stratified at 4°C for approximately 48 hours, and then kept under 18-hour photoperiod at 22°C (cool white fluorescent light (Osram L 36W/840 Lumilux), approximately 70 µmol m^{-2} s^{-1}) in a Termaks environmental chamber (Type KBP 2324 V). After six days, the seedlings were sprayed with Milli-Q H_2O + 40 mg/l glufosinate using a bottle that produces a fine mist, and then sprayed three more times at three-day intervals. Healthy green plants could then be clearly distinguished from moribund seedlings and were transferred to individual pots.

Extraction of DNA from leaves of A. thaliana by CTAB method

Extraction of DNA from *A. thaliana* was performed as described under 2.1.4 Extraction of DNA from leaves of *B. tuberhybrida* 'Urban Bicolor Pink' by CTAB method on page 20, except omitting the use of T10E10 buffer; extraction buffer was added directly to the ground leaf tissue.

Verification of A. thaliana transformants by PCR

Three *A. thaliana* plants putatively transformed with CRISPR/Cas9 plasmid #1, labelled plant #1, #3, and #5, and two putatively transformed with CRISPR/Cas9 plasmid #2, labelled plant #2 and #4, were randomly selected to be screened for insertion of the CRISPR/Cas9 system by PCR. Plant #4 and #5 had been growing *in vitro* on medium containing 10 mg/l glufosinate and 500 mg/l cefotaxime, while plant #1, #2, and #3 had been growing in potting mix and sprayed with 40 mg/l glufosinate. Primer set #6 (Table 5) was used to detect CRISPR/Cas9 DNA.

1 µl high-purity DNA (1141.62, 1338.18, 2302.64, 1032.70, and 1149.67 ng/µl for plant #1-6, respectively) were added to each PCR reaction (Appendix Ih) separately for a total individual reaction volume of 25 µl in thin-walled PCR tubes and transferred to a DNA Engine Tetrad 2 thermocycler for thermocycling. 1 µl high-purity plasmid DNA (174 and 167 ng/µl, CRISPR/Cas9 plasmid #1 and #2 respectively) were used as positive controls and 1 µl of Milli-Q H_2O was used as a negative control. Thermocycling conditions used are described in Table 7. The PCR products were analysed by gel electrophoresis on 2x 50 ml 1.5% (w/v) agarose gel stained with 1 µl GelRed and the DNA visualized by ImageLab v5.0 and BioRad ChemiDoc MP.

Table 7. *Thermocycling conditions used for PCR with Taq DNA polymerase and primer set #6 (lid control mode: Constant at 100°C).*

Step	Temperature	Time (min:sec)
#1 Initial denaturation	95°C	00:30
#2 Denaturation	95°C	00:30
#3 Primer annealing	54°C	00:30
#4 Extension	68°C	01:00
#5 Cycle to step #2 30x times		
#6 Final extension	68°C	05:00
#7 Cooling	4°C	Forever

Verification of mutations in F3'H in transformed A. thaliana by enzyme mismatch cleavage assays

Primers used to amplify *F3'H* regions flanking the predicted mutation sites

Primers were designed using Primer3Plus (Untergasser et al. 2007). Five different sets of primers were designed (Table 8) to amplify the regions flanking the predicted Cas9-induced mutations, with the mutations hypothetically occurring at least 100 bp away from the amplicon ends. New England Biolabs' Tm Calculator v1.9.6 (New England BioLabs 2016c) was used to calculate suggested annealing temperatures (T_a). The software suggested the use of T_a = 66°C for primer set #14, #16, #17, #18 and T_a = 65°C for primer set #15, when using New England BioLabs Q5 High-Fidelity DNA Polymerase.

All primers were tested by adding 37 ng high-purity DNA (1 µl 37.0 ng/µl) to each PCR reaction (Appendix Ii) for a total individual reaction volume of 25 µl in thin-walled PCR tubes and transferred to a DNA Engine Tetrad 2 thermocycler for thermocycling. The thermocycler conditions are listed in Table 9, except that T_a = 65°C was used for primer set #15. 1 µl Milli-Q H_2O was used as a negative control. The PCR products were analysed by gel electrophoresis on a 50 ml 1.5%

(w/v) agarose gel stained with 1 µl GelRed and the DNA visualized by ImageLab v5.0 and BioRad ChemiDoc MP.

Table 8. *Primers used for PCR to amplify regions flanking the predicted Cas9-induced mutations in F3'H.*

Primer set #	Target region	Forward primer	Reverse primer	Amplicon size (BP)
#14	*F3'H (sgRNA #1)*	AAACCCAACACTATGGCAACTC	GTTTAGCTCCTGAGTTTGGTGGTC	334
#15	*F3'H (sgRNA #1)*	CTCCCATATTCTCCACGGTTTA	AGGATAGGGCCGTAAGTAGTAACC	567
#16	*F3'H (sgRNA #1)*	GAGAATCTAAGGCGCTTAAGGAAG	AGGATAGGGCCGTAAGTAGTAACC	664
#17	*F3'H (sgRNA #2)*	GAGGAAGCTTAACGGATACTGAGA	ATGTGTGGTAACGAGAGTGGTGT	429
#18	*F3'H (sgRNA #2)*	GAGGAAGCTTAACGGATACTGAGA	GTTAACGGTACCTGAAGGTAAGGAAG	301

Table 9. *Thermocycling conditions used for PCR with Q5 High-Fidelity DNA polymerase (lid control mode: Constant at 100°C).*

Step	Temperature	Time (min:sec)
#1 Initial denaturation	98°C	00:30
#2 Denaturation	98°C	00:10
#3 Primer annealing	66°C	00:30
#4 Extension	72°C	00:30
#5 Cycle to step #2 30x times		
#6 Final extension	72°C	02:00
#7 Cooling	4°C	Forever

Surveyor Mismatch Cleavage Assay

IDT Surveyor Mutation Detection Kit was used in an attempt to detect Cas9-induced mutations in *F3'H* in *A. thaliana* plant #1, #2, #3, #4, and #5. The assay was performed as described in the provided protocol (Appendix Ie), except that 1x TAE buffer was used instead of 1x TBE buffer, and 1 µl GelRed per 50 ml agarose gel was used instead of 0.2 µg/ml ethidium bromide to stain the gels.

Primer set #16 (Table 8) was used when testing the plants transformed with CRISPR/Cas9 plasmid #1 (plant #1, #3, and #5). Primer set #17 (Table 8) was used when testing the plants transformed with CRISPR/Cas9 plasmid #2 (plant #2 and #4). New England BioLabs Q5 High-Fidelity DNA Polymerase and the corresponding kit protocol (Appendix Ii) was used to amplify the regions of interest. 1 µl high-purity DNA (57.8, 60.6, 65.2, 47.2, and 63.9 ng/µl for plant #1, #2, #3, #4, and #5, respectively, and 36.3 ng/µl wild type DNA) was added separately to each PCR reaction (Appendix Ii) for a total individual reaction volume of 50 µl in thin-walled PCR tubes and transferred to a DNA Engine Tetrad 2 thermocycler for thermocycling as described in Table 9. 1 µl Milli-Q H_2O was used as a negative control. The control provided in the IDT Surveyor Mutation Detection Kit was used as a positive control when performing the cleavage assay. The PCR products and digestion of heteroduplexes was analysed by gel electrophoresis on 50 ml 1.5% (w/v) agarose gels stained with 1 µl GelRed and the DNA visualized by ImageLab v5.0 and BioRad ChemiDoc MP.

T7E1 Mismatch Cleavage Assay

IDT Alt-R Genome Editing Detection Kit, containing T7E1, was used in an attempt to detect Cas9-induced mutations in *F3'H* in *A. thaliana* plant #1, #2, #3, #4, and #5. The assay was performed as described in the provided protocol (Appendix If), except that New England BioLabs Q5 High-Fidelity DNA Polymerase and the corresponding PCR protocol (Appendix Ii), was used instead of KAPA HiFi HotStart PCR Kit and its corresponding PCR protocol.

Primer set #16 (Table 8) was used when testing the plants transformed with CRISPR/Cas9 plasmid #1 (plant #1, #3, and #5). Primer set #17 (Table 8) was used when testing the plants transformed with CRISPR/Cas9 plasmid #2 (plant #2 and #4). 1 µl high-purity DNA (57.8, 60.6, 65.2, 47.2, and 63.9 ng/µl for plant #1, #2, #3, #4, and #5, respectively, and 36.3 ng/µl wild type DNA) was added separately to each PCR reaction (Appendix If) for a total individual reaction volume of 50 µl in thin-walled PCR tubes and transferred to a DNA Engine Tetrad 2 thermocycler for thermocycling as described in Table 9. 1 µl Milli-Q H_2O was used as a negative control. The control provided in the IDT Alt-R Genome Editing Detection Kit was used as a positive control when performing the cleavage assay. The PCR products and digestion of heteroduplexes was analysed by gel electrophoresis on 50 ml 1.5% (w/v) agarose gels stained with 1 µl GelRed and the DNA visualized by ImageLab v5.0 and BioRad ChemiDoc MP.

Verification of mutations in F3'H in transformed A. thaliana by Sanger sequencing

Sanger sequencing was used in an attempt to detect Cas9-induced mutations in *F3'H*. Regions flanking the predicted mutations induced by both CRISPR/Cas9 plasmid #1 and #2 was sequenced in all transformed *A. thaliana* plants (plant #1, #2, #3, #4, and #5), even though they had only been transformed with one of the plasmids, in case mislabelling of plants or plasmid had occurred at any point of time.

The regions flanking the predicted Cas9-induced mutations in *F3'H* were amplified using both primer set #16 and #17 (Table 8) for all transformed plants. 1 µl high-purity DNA (57.8, 60.6, 65.2, 47.2, and 63.9 ng/µl for plant #1, #2, #3, #4, and #5, respectively) was added separately to each PCR reaction (Appendix Ii) for a total individual reaction volume of 50 µl in thin-walled PCR tubes

and transferred to a DNA Engine Tetrad 2 thermocycler for thermocycling as described in Table 9. 36.3 ng/µl wild type DNA was used as a positive control and 1 µl Milli-Q H_2O was used as a negative control. The PCR products were purified using Qiagen MinElute Reaction Cleanup Kit as described in the kit protocol (Appendix Id). The final purified DNA concentration ranged from 43 to 90 ng/µl of high-purity DNA. 5 µl high-purity DNA, from each plant for both amplified regions, was mixed with 5 µl (5 µM) forward primer separately and shipped to GATC Biotech for Sanger sequencing.

The sequence data and quality was analysed using SnapGene v3.34 (from GSL Biotech; available at snapgene.com) and GATC Viewer v1.00 (from GATC Biotech).

2.3 Statistical analyses

All statistical analyses were performed using R v3.4.1 (R Core Team 2017). To establish if glufosinate, and in the case of *B. tuberhybrida* 'Urban Bicolor Pink', type of explant tissue and interaction between glufosinate and type of explant tissue, had a significant effect on regeneration in selective media, analysis of variances (ANOVA) were carried out using generalised linear models.

To compare the effect of the various concentrations of glufosinate, a Tukey's test was performed post hoc.

The R package mixlm: Mixed Model ANOVA and Statistics for Education (Liland 2017) was used to perform the ANOVAs.

The full outputs from the analyses can be found under Appendix III on page 107.

3 Results

3.1 *Agrobacterium*-mediated transformation of *B. tuberhybrida* ‘Urban Bicolor Pink’ with plasmid pIA291

3.1.1 Verification of transformed *A. tumefaciens* by colony PCR

As shown in Fig. 9, the *A. tumefaciens* was verified as transformed with the plasmid pIA291 by colony PCR. The size of the DNA amplicons from both the positive control (plasmid DNA) and the transformed *A. tumefaciens* corresponded to the expected sizes; 222 bp for primer set #1 and 191 bp for primer set #4 (Table 1).

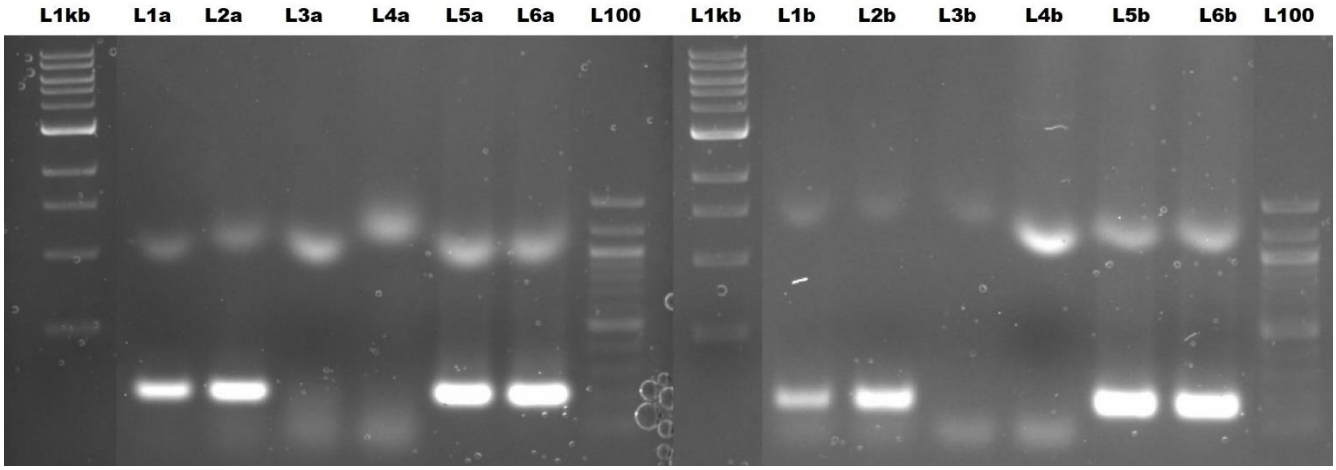


Fig. 9. *Gel documentation of PCR products following transformation of A. tumefaciens with plasmid pIA291.*

Left: Primer set #1; L1a+L2a transformed A. tumefaciens, L3a+L4a neg. control, L5a+L6a pos. control.

Right: Primer set #4; L1b+L2b transformed A. tumefaciens, L3b+L4b neg. control, L5b+L6b pos. control.

L1kb = 1000bp ladder, L100 = 100bp ladder.

3.1.2 Establishing dose-response to glufosinate in *B. tuberhybrida* ‘Urban Bicolor Pink’

The *B. tuberhybrida* ‘Urban Bicolor Pink’ explants were visually inspected every week, and after eight weeks the number of explants exhibiting formation of adventitious shoots were recorded.

Considering the tight time frame for this thesis work, rapid regeneration was of the essence, and as such only explants that developed adventitious shoots during the first eight weeks were considered as “regenerating explants” for the purpose of this experiment. See Fig. 11 for examples of explants considered as regenerating and Fig. 10 for explants considered as not regenerating**.**

Fig. 11. *Development of adventitious shoots in B. tuberhybrida ‘Urban Bicolor Pink’ from eight-week-old leaf explants growing in various concentrations of glufosinate, as examples of regenerating explants.*

Top left: Explant in 0.50 mg/l. Top right: Explant in 0.50 mg/l. Bottom left: Explant in 0.25 mg/l. Bottom right: Explant in 0.00 mg/l (control).

The images were obtained using a Leica M205 C stereo microscope and Leica application suite v4.3.0.

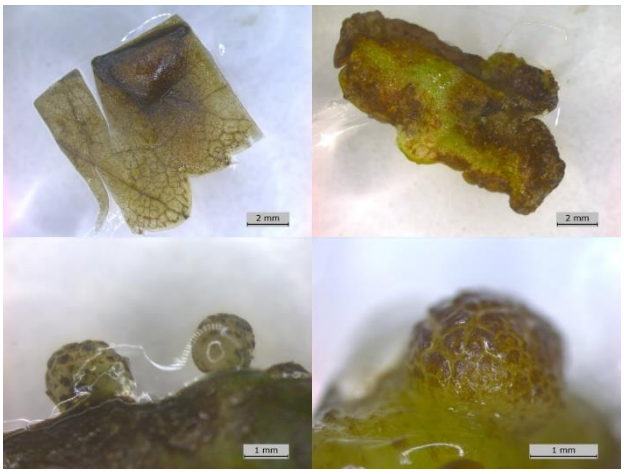


Fig. 10. *Eight-week-old B. tuberhybrida ‘Urban Bicolor’ leaf explants in various concentrations of glufosinate, as examples of non-regenerating explants.*

Top left: Explant in 1.00 mg/l. Top right: Explant in 0.50 mg/l. Bottom left: Explant in 0.50 mg/l. Bottom right: Explant growing in 0.25 mg/l.

The images were obtained using a Leica M205 C stereo microscope and Leica application suite v4.3.0.

Table 10 shows that no leaf explants developed adventitious shoots at concentrations of 1.00 mg/l glufosinate or higher. Hence this concentration ensures few, if any, escapes. In concentrations of 2.50 and 5.00 mg/l, all the explants died during the first two weeks. When grown on 1.00 mg/l, 14 out of 25 explants died during the experiment. The other 11 explants still had some pigmentation, but did not appear to be growing. At 0.50 mg/l, six explants developed adventitious shoots. However, they exhibited retarded growth compared to growth at lower concentrations of glufosinate. Six other explants exhibited callus-like growth, but did not regenerate any shoots. At 0.25 and 0.00 mg/l both treatments had a relatively large number of shoots developing at comparable rates. Ten additional explants exhibited callus-like growth at 0.00 mg/l while only four additional explants exhibited callus-like growth at 0.25 mg/l. The shoots that emerged at 0.25 and 0.00 mg/l appeared to be growing at comparable rates.

Table 10. *Glufosinate dose-response of non-transformed leaf explants in B. tuberhybrida 'Urban Bicolor Pink'.*

Selection pressure	Total number of explants	# of regenerating explants	% regeneration
0.00 mg/l (control)	25	14	56
0.25 mg/l	25	13	52
0.50 mg/l	25	6	24
1.00 mg/l	25	0	0
2.50 mg/l	25	0	0
5.00 mg/l	25	0	0

Table 11 shows that three petiole explants developed adventitious shoots on 1.00 mg/l glufosinate, indicating a chance of escapes at this concentration. The results of the dose-response experiment on petiole explants were statistically significantly different from the dose-response experiment on leaf explants ($p=0.0017$), but exhibited a similar trend. At 2.50 and 5.00 mg/l all the explants died during the first two weeks. Even though formation of shoots on three petiole explants on 1.00 mg/l was observed, the growth was very stunted compared to growth at lower concentrations. At 0.50 mg/l, 12 explants had developed adventitious shoots. However, the growth was retarded compared to the growth at lower concentrations, as also observed with the leaf explants at this concentration. Callus-like growth was observed in four additional explants at 0.50 mg/l. At 0.25 mg/l, 17 explants developed adventitious shoots that were larger than those at 0.50 and 1.00 mg/l, but noticeably smaller than those at 0.00 mg/l. Six additional explants at 0.25 mg/l exhibited callus-like growth. At 0.00 mg/l 20 explants developed shoots and one additional explant exhibited callus-like growth.

Table 11. *Glufosinate dose-response of non-transformed petiole explants in B. tuberhybrida 'Urban Bicolor Pink'.*

Selection pressure	Total number of explants	# of regenerating explants	% regeneration
0.00 mg/l (control)	25	20	80
0.25 mg/l	25	17	68
0.50 mg/l	25	12	48
1.00 mg/l	25	3	12
2.50 mg/l	25	0	0
5.00 mg/l	25	0	0

When performing ANOVA tests (Appendix III), the interaction effect between type of explant tissue and concentration of glufosinate was found not to be significant ($p=0.7946$). Hence, a model without the interaction effect was used to estimate if type of explant tissue and glufosinate had significant effects on regeneration. Both type of tissue ($p=0.0017$) and the concentration of glufosinate ($p<2.2e-16$) was found to be statistically significant variables. Furthermore, as shown in Fig. 12, no significant differences were found between any concentrations of glufosinate ≥1 mg/l when performing a Tukey's test.

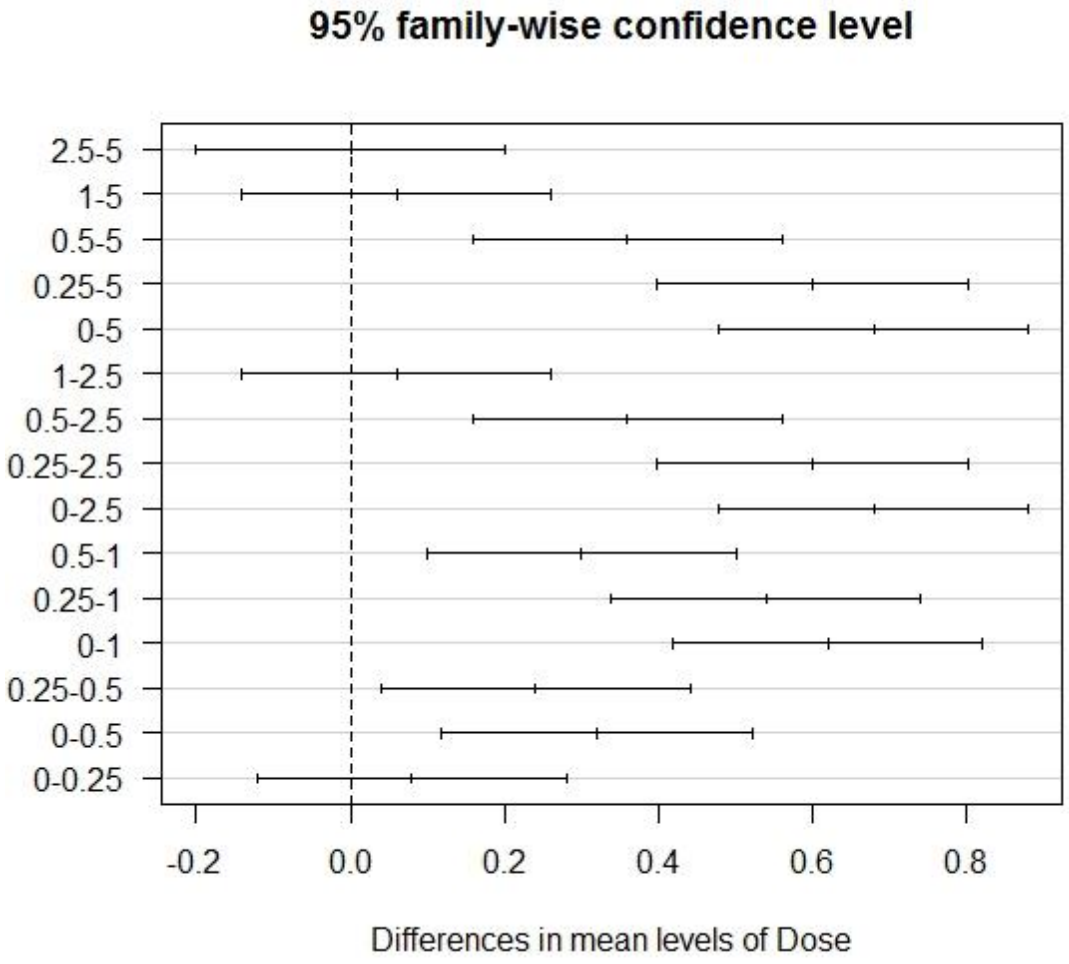


Fig. 12. *Differences in effect of glufosinate concentrations (Dose) on regeneration of B. tuberhybrida 'Urban Bicolor Pink' leaf and petiole explants calculated by performing Tukey's test. Compared doses of glufosinate (mg/l) is displayed on the y-axis, while the 95% confidence interval is displayed on the x-axis.*

R v.3.4.1 (R Core Team 2017) was used to create the figure and perform the calculations.

3.1.3 Treatment of contaminations in *in vitro* cultures of *B. tuberhybrida* 'Urban Bicolor Pink'

The first bacterial overgrowth was tested by colony PCR with primers targeting sequences found in pIA291. As shown in Fig. 13, the size of the DNA amplicons from the bacterial overgrowth tested corresponded to the sizes expected for pIA291; 222 bp for primer set #1 and 191 bp for primer set #4 (Table 1). Consequently, the bacterial overgrowth was considered to be transformed *A. tumefaciens*. Subsequent bacterial contaminations of similar characteristic cloudy brownish thick liquid appearance (Fig. 5 top) were also considered to be transformed *A. tumefaciens*.

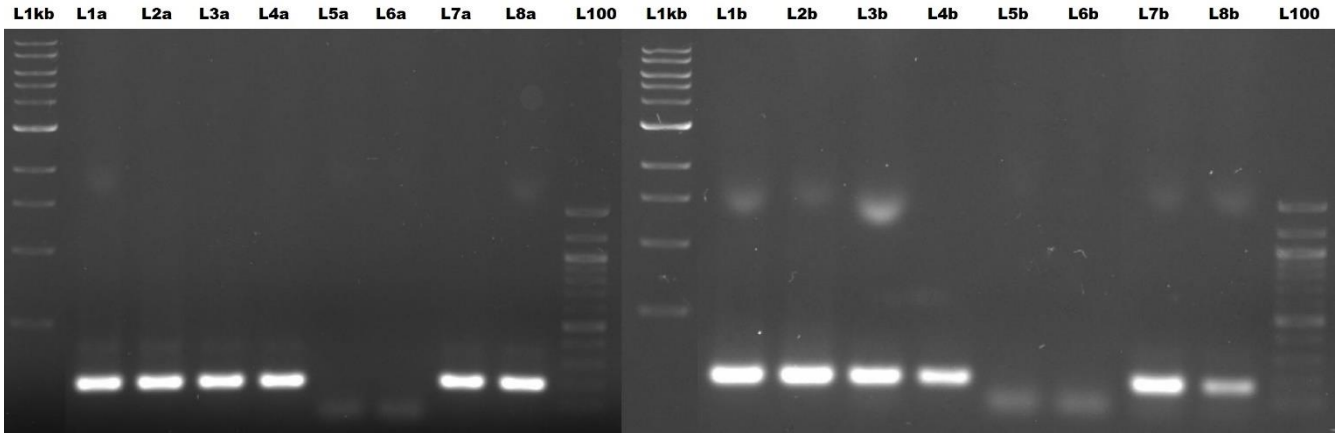


Fig. 13. *Gel documentation of PCR products from bacterial overgrowth of B. tuberhybrida 'Urban Bicolor Pink' explants.*

Left: Primer set #1; L1a+L2a pos. control (plasmid DNA), L3a+L4a bacterial infection, L5a+L6a neg. control, L7a+L8a pos. control (transformed A. tumefaciens).

Right: Primer set #4; L1b+L2b pos. control (plasmid DNA), L3b+L4b bacterial infection, L5b+L6b neg. control, L7b+L8b pos. control (transformed A. tumefaciens).

L1kb = 1000bp ladder, L100 = 100bp ladder.

Approximately 50 *B. tuberhybrida* 'Urban Bicolor Pink' explants were treated as described under 2.1.3 Treatment of contaminations in in vitro cultures of B. tuberhybrida 'Urban Bicolor Pink' on page 19. Unfortunately, it was too cumbersome and not a priority to keep track of individual explants, so it is not possible to accurately consider the efficiency of the treatment. A substantial amount of the treated explants got reinfected within a few weeks, but it is not possible to state whether the fresh overgrowth of *A. tumefaciens* originated from the treated explant or from an adjacent explant.

3.1.4 Extraction of DNA from leaves of *B. tuberhybrida* 'Urban Bicolor Pink'

When combining the protocol for organic acid extraction developed by Kopperud and Einset (1995) and the protocol used for DNA extraction from tobacco at the International Centre for Genetic Engineering and Biotechnology (ICGEB) (R Pathak pers. comm. 6 December 2016), it was possible to extract 6-30 µg of high-purity DNA per 100 mg *B. tuberhybrida* 'Urban Bicolor Pink' leaf tissue (fresh weight). If DNA extraction was performed without initial extraction of organic acids the DNA yield was usually higher, but the quality always poorer. Fig. 14 shows the comparison of DNA yield and quality between leaf tissue treated with extraction of organic acids and untreated leaf tissue measured with a Nanodrop ND-1000 spectrophotometer. The leaf tissue not treated with the extraction of organic acids exhibited absorption peaks in the 220-230 nm range, indicating poor nucleic acid purity (Thermo Fisher Scientific).

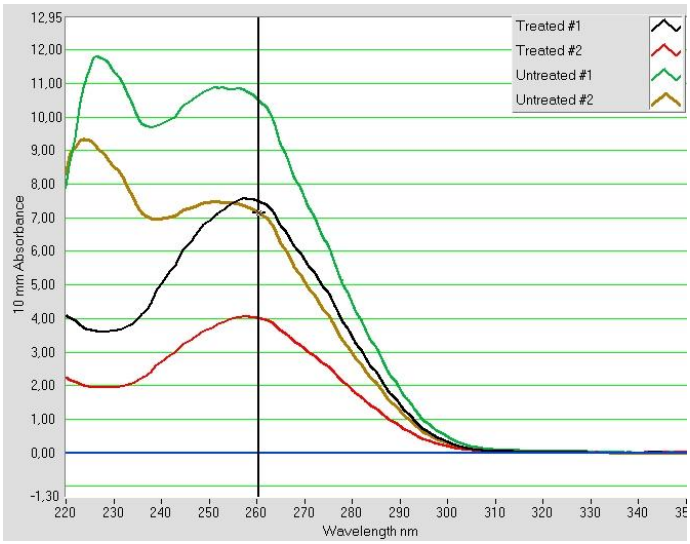


Fig. 14. *Comparison of DNA quality between B. tuberhybrida 'Urban Bicolor Pink' leaf tissue treated with organic acid extraction (Treated #1 and #2) and DNA extracted directly from leaf tissue not treated with organic acid extraction (Untreated #1 and #2). The peaks at 220-230 nm indicates contamination. The peaks at 260 nm indicates DNA yield.*

Nanodrop ND-1000 spectrophotometer was used to analyse the DNA and NanoDrop Operating Software was used to create the graph.

3.1.5 Locating a positive control for Extract-N-AMP PCR of putatively transformed *B. tuberhybrida* 'Urban Bicolor Pink'

As shown in Fig. 15, primer set #13 (Table 4) yielded bands of similar intensity in both *B. tuberhybrida* 'Urban Bicolor Pink' and *A. thaliana* when performing standard PCR with previously extracted DNA and Extract-N-AMP PCR directly from leaf tissue. Consequently, it was decided to use Extract-N-Amp PCR for subsequent verification of transformed 'Urban Bicolor Pink' by PCR.

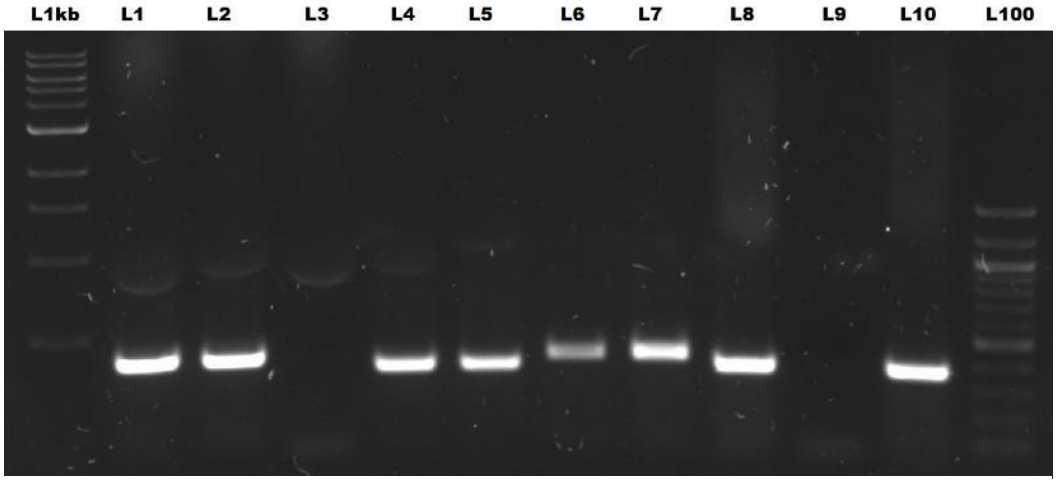


Fig. 15. *Gel documentation of PCR products from B. tuberhybrida 'Urban Bicolor Pink' and A. thaliana using primer set #13.*

L1+L2 std. A. thaliana, L3 std. neg. control, L4+L5 std. B. tuberhybrida, L6+L7 ENA A. thaliana, L8 ENA B. tuberhybrida, L9 ENA neg. control, L10 ENA B. tuberhybrida.

Std. = Standard PCR.

ENA = Extract-N-Amp PCR.

L1kb = 1000bp ladder, L100 = 100bp ladder.

3.1.6 Transplanting mature *B. tuberhybrida* 'Urban Bicolor Pink' shoots for root induction

Out of 616 *B. tuberhybrida* 'Urban Bicolor Pink' plantlets transplanted to potting mix 163 survived. Several additional adventitious shoots growing *in vitro* were too small to be excised and transplanted to potting mix at the time of conclusion.

Table 12. *The number of surviving B. tuberhybrida 'Urban Bicolor Pink' plants at the time of conclusion and the total number of plantlets transplanted to potting mix. Experiment # refers to the various transformation experiments performed (Table 6).*

Experiment #	Surviving	Transplanted to potting mix
#1	20	60
#2	47	93
#3	34	59
#4	0	26
#5	5	67
#6	29	114
#7	9	111
#8	19	86
SUM	**163**	**616**

3.1.7 Verification of *B. tuberhybrida* 'Urban Bicolor Pink' transformed with pIA291 by PCR screening

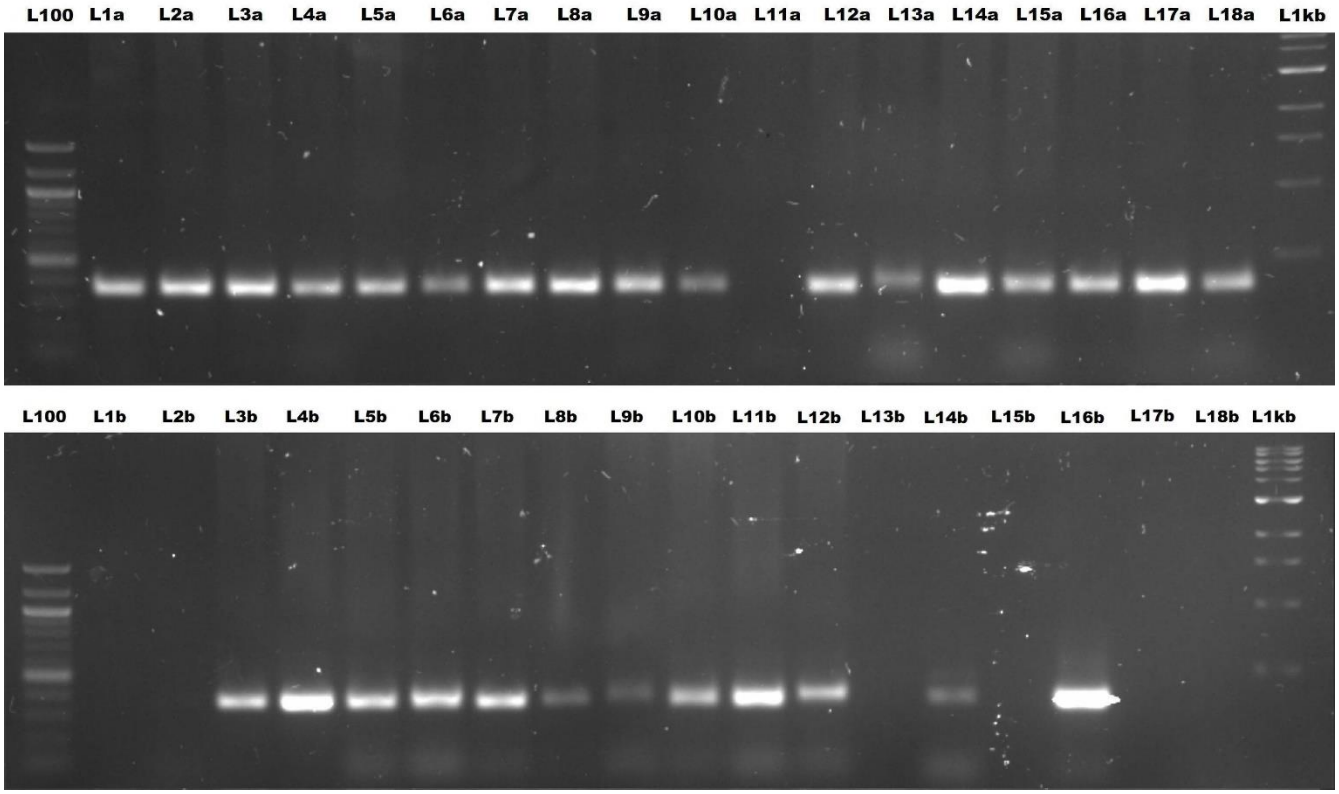


Fig. 16. *Gel documentation of PCR products from initial screening for B. tuberhybrida 'Urban Bicolor Pink' transformed with plasmid pIA291. These are the positive controls (primer set #13) amplifying ITS, used to verify that DNA extraction using the Extract-N-Amp worked.*

Top: L1a plant #1-2, L2a plant #1-4, L3a plant #1-5, L4a plant #1-7, L5a plant #2-1, L6a plant #2-4, L7a plant #2-5, L8a plant #2-7, L9a plant #2-8, L10a plant #3-5, L11a plant #3-6, L12a plant #4-4, L13a plant #4-6, L14a plant #5-5, L15a plant #5-6, L16a plant #5-7, L17a plant #6-1, L18a plant #6-2.

Bottom: L2b plant #6-6, L3b plant #6-7, L4b plant #7-4, L5b plant #8-1, L6b plant #9-2, L7b plant #9-3, L8b plant #9-4, L9b plant #9-6, L10b plant #10-3, L11b plant #10-5, L12b plant #10-6, L14b pos. control, L15b neg. control, L16b pos. control.

L1kb = 1000bp ladder, L100 = 100bp ladder.

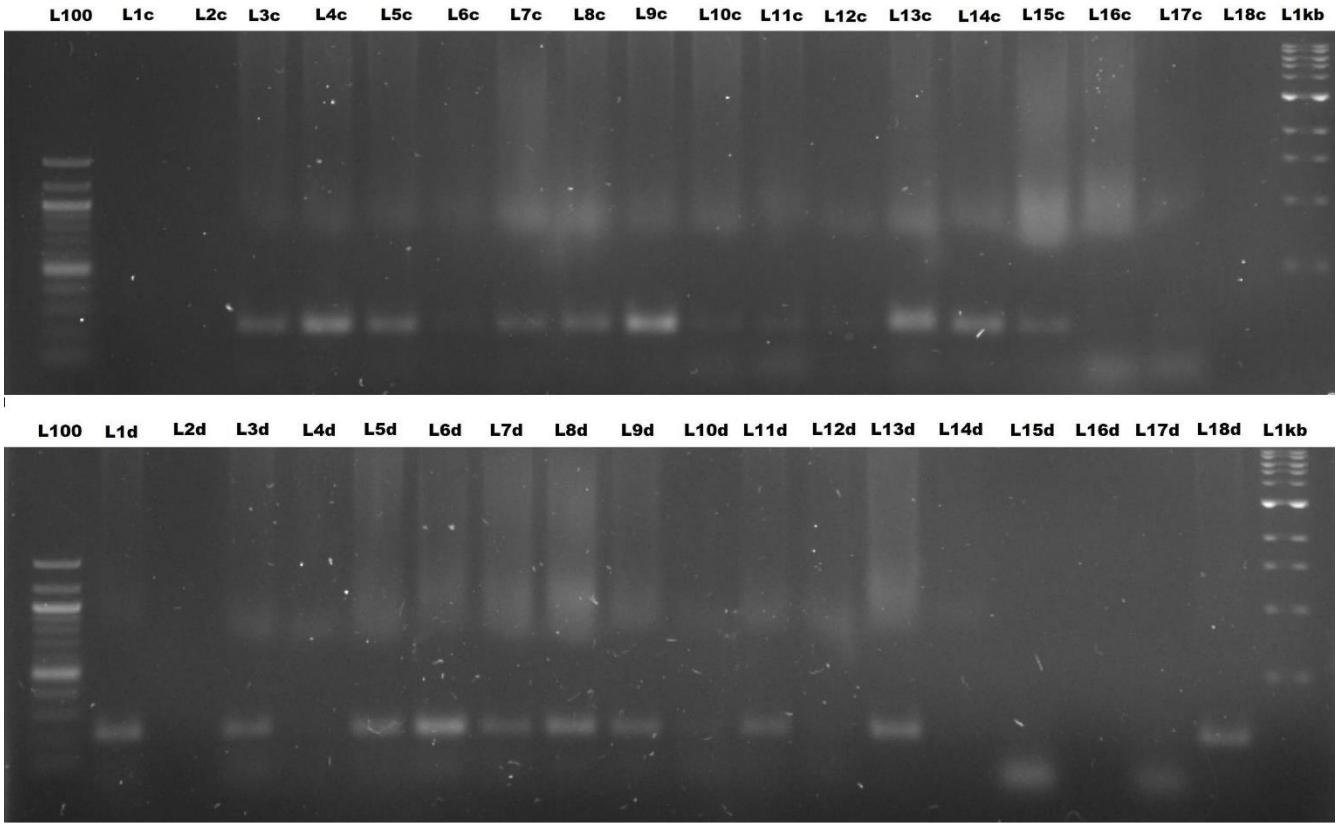


Fig. 17. *Gel documentation of PCR products from initial screening for B. tuberhybrida 'Urban Bicolor Pink' transformed with plasmid pIA291. Primer set #1 was used to detect the presence of the F3'5'H transgene.*

Top: L3c plant #1-2, L4c plant #1-4, L5c plant #1-5, L6c plant #1-7, L7c plant #2-1, L8c plant #2-4, L9c plant #2-5, L10c plant #2-7, L11c plant #2-8, L12c plant #3-5, L13c plant #3-6+4-4, L14c plant #4-6, L15c plant #5-5, L16c plant #5-6, L17c plant #5-7,

Bottom: L1d plant #6-1, L3d plant #6-2, L4d plant #6-6, L5d plant #6-7, L6d plant #7-4, L7d plant #8-1, L8d plant #9-2, L9d plant #9-3, L10d plant #9-4, L11d plant #9-6, L12d plant #10-3, L13d plant #10-5, L14d plant #3-6, L15d neg. control (wt DNA), L17d neg. control (H2O), L18d plant #4-4.

L1kb = 1000bp ladder, L100 = 100bp ladder.

As shown in Fig. 17, 18 of the 28 plants (two from experiment #1, 20 from experiment #2, six from experiment #3 (Table 6)) that were chosen for initial PCR screening produced 222 bp bands with primer set #1 (Table 1), consistent with what was expected from plants transformed with pIA291. Although most of the bands with primer set #1 were quite faint, they were clearly present and distinct from the primer-dimers found in the lanes with negative controls. Unfortunately, a positive control was mistakenly omitted in this PCR screen.

Primer set #13 (Table 4) was used as a positive control for the extraction of DNA by the Extract-N-Amp kit (Fig. 16). As shown in Fig. 16 and Fig. 17, two samples, plant #3-6 and #6-6, did not produce any bands with primer set #13 or #1, indicating that the DNA extraction failed. Plant #9-4

produced a very faint band with primer set #13 and no band with primer set #1, indicating weak DNA extraction and a possible false negative.

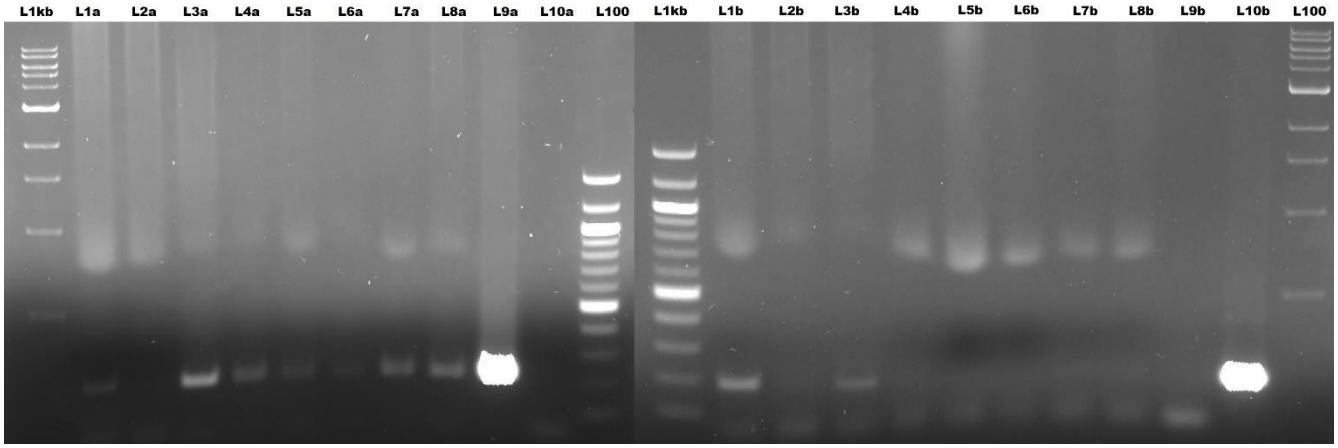


Fig. 18. *Gel documentation of PCR products from second screening for B. tuberhybrida 'Urban Bicolor Pink' transformed with plasmid pIA291.*

Left: Primer set #1; L1a plant #10-5, L2a plant #9-2, L3a plant #7-4, L4a plant #6-1, L5a plant #5-5, L6a plant #4-6, L7a plant #2-5, L8a plant #1-4, L9a pos. control, L10a neg. control.

Right: Primer set #4; L1b plant #10-5, L2b plant #9-2, L3b plant #7-4, L4b plant #6-1, L5b plant #5-5, L6b plant #4-6, L7b plant #2-5, L8b plant #1-4, L9b neg. control, L10b pos. control.

L1kb = 1000bp ladder, L100 = 100bp ladder.

The results from the second screening of eight of the transformed plants (one from experiment #1, five from experiment #2, two from experiment #3) seven (one from experiment #1, five from experiment #2, one from experiment #3) from the first screening are shown in Fig. 18. Seven (one from experiment #1, five from experiment #2, one from experiment #3) produced the expected 222 bp bands with primer set #1 (although one sample #4-6 produced a very faint band). Two (one from experiment #1 and one from experiment #3) produced the expected 191 bp bands with primer set #4 (Table 1). The positive controls (plasmid DNA) produced bands of seemingly identical size compared to the positive plant samples.

3.1.8 Observed phenotypical differences in *B. tuberhybrida* 'Urban Bicolor Pink' transformants

As shown in Fig. 19, some variation in *B. tuberhybrida* 'Urban Bicolor Pink' flower colouration was observed, both among flowers from the same plant, and between flowers from different plants. However, the observed variation in sepal and petal colour was mainly between very light pink to

dark pink and this range in colour was observed in all plants. No shift towards bluer sepals or sepals or petals could be observed in any of the 163 plants which developed flowers.

Fig. 19. *Left: Sepals and petals from wild type B. tuberhybrida 'Urban Bicolor Pink' exhibiting natural variation in colour from very light pink to dark pink. All sepals and petals were harvested from the same wild type plant at the same time.*

Right: Transformed flowers (Transformant #1 bottom left, Transformant #2 bottom right) could not be distinguished from the wild type flowers (top)

The colour of sepals and petals from both the wild type and the eight transformed plants chosen for second screening by PCR were measured, but, as shown in Fig. 19, the natural variation of sepal and petal colour, even in the same sepal or petal, was too large to provide accurate information for comparisons between plants unless they were homogenised.

When the sepals and petals were homogenised and the colour of the extracted sap was measured it was possible to detect differences in colour, as shown in Table 13, but it was not possible to distinguish the colour of the wild type sap from the colour of the sap from transformants by visual observation alone. Because pH greatly affects the colour of anthocyanins (Davies 2009; Pina 2014; Tanaka et al. 2009), and the pH of the extracted sap was extremely low (pH=1.5), the sap was adjusted to higher pH and the colour measured at pH 1.5, 3.2, 5.2, 7.3, and 9.8 (Fig. 20 and Table 13). The pH of the extracted sap did not vary by more than ±0.3 when measured 6, 24, 48, and 72 hours after extraction.

As shown in Table 13, at the natural pH level (1.5) and pH 3.2, the sap from both transformant #1 and #2 were found to be bluer than the wild type (negative Δb-values). Sap from transformant #2 was also measured to be bluer than the wild type at pH 7.3. However, the sap from the wild type

was found to be bluer than sap from both transformants at pH 5.2 and 9.8. The amount of variation caused by sampling error and natural variation was not estimated by repeated measurements.

As the extracted sap from the transformants did not consistently exhibit bluer colouration than the sap extracted from the wild type at elevated pH levels, it was assumed that the transformants tested did not accumulate significant amounts of delphinidin-based anthocyanins.

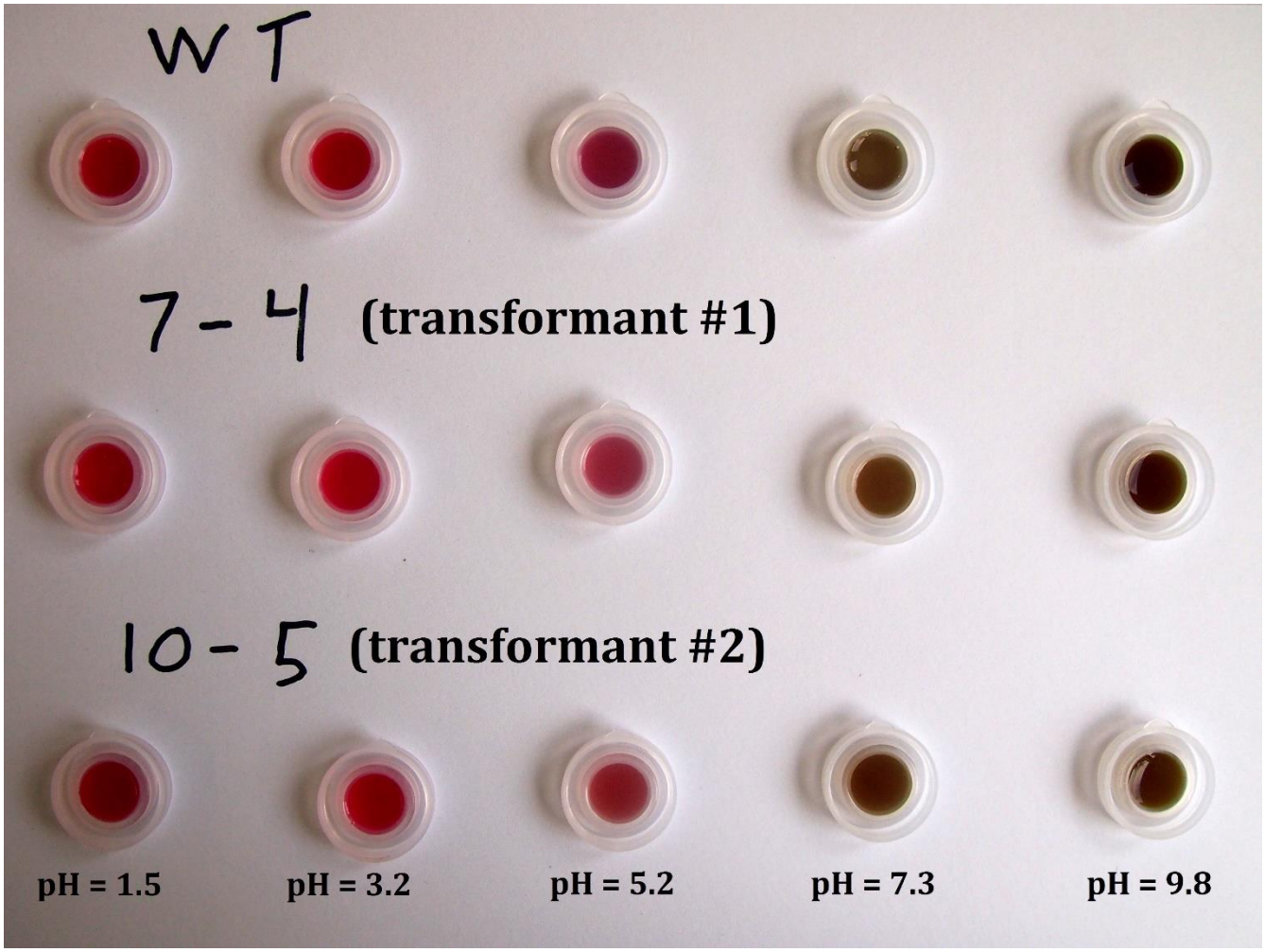


Fig. 20. *Colour of extracted sap from sepals and petals of wild type and transformed B. tuberhybrida 'Urban Bicolor Pink' at different pH levels. pH 1.5 is the natural pH of the extracted sap.*

Table 13. *Measured colour differences of extracted B. tuberhybrida 'Urban Bicolor Pink' sap from sepals and petals from transformant #1 and #2 compared to extracted sap of approximately the same pH from wild type sepals and petals.*

ΔL=difference in lightness and darkness (+=lighter, -=darker),

Δa=difference in red and green (+=redder, -=greener),

Δb=difference in yellow and blue (+=yellower -=bluer),

ΔE=total colour difference (Cui et al. 2002).

Sample	pH	ΔL	Δa	Δb	ΔE
Transformant #1	1.5	-5.07	-1.44	-4.33	5.97
Transformant #2	1.5	0.12	-3.97	-2.04	1.83
Transformant #1	3.2	3.08	0.96	-2.26	3.63
Transformant #2	3.2	4.41	0.10	-0.52	4.41
Transformant #1	5.2	5.89	-2.84	0.41	6.40
Transformant #2	5.2	4.29	-4.46	5.13	7.09
Transformant #1	7.3	1.08	2.16	1.09	2.57
Transformant #2	7.3	-1.75	1.72	-1.91	2.78
Transformant #1	9.8	3.61	-0.89	3.08	4.46
Transformant #2	9.8	7.01	-2.89	7.86	9.33

3.2 Disruption of *F3'H* using CRISPR/Cas9

3.2.1 Verification of transformed *E. coli* by colony PCR

As shown in Fig. 21, separate *E. coli* colonies were verified as transformed with CRISPR/Cas9 plasmid #1 and #2. The size of the DNA amplicons corresponded to the expected sizes; 242 bp for primer set #5, 207 bp for primer set #6, 184 bp for primer set #7, and 248 bp for primer set #8 (Table 5). For further experiments only primer set #6 was used, as this set yielded good bands and was the only set tested with both positive and negative controls.

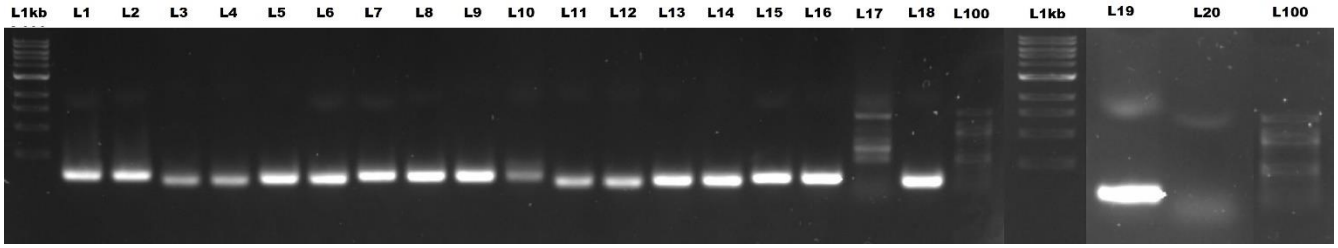


Fig. 21. *Gel documentation of PCR products following transformation of E. coli with CRISPR/Cas9 plasmid #1 and #2.*

L1 g2 P8, L2 g1 P8, L3 g2 P7, L4 g1 P7, L5 g2 P6, L6 g1 P6, L7 g2 P5, L8 g1 P5, L9 g2 P8, L10 g1 P8, L11 g2 P7, L12 g1 P7, L13 g2 P6, L14 g1 P6, L15 g2 P5, L16 g1 P5, L17 neg. control P6, L18 pos. control g1 P6, L19 pos. control g2 P6, L20 neg. control P7.

g1 = E. coli transformed with CRISPR/Cas9 plasmid #1.

g2 = E. coli transformed with CRISPR/Cas9 plasmid #2.

PX = Primer set X.

L1kb = 1000bp ladder, L100 = 100bp ladder.

3.2.2 Verification of transformed *A. tumefaciens* by colony PCR

As shown in Fig. 22, separate *A. tumefaciens* colonies were verified as transformed with CRISPR/Cas9 plasmid #1 and #2. The size of the DNA amplicons from both the positive control (plasmid DNA) and the transformed *A. tumefaciens* corresponded to the expected size of 207 bp with primer set #6 (Table 5). A less intense band of the same size was observed in the lane with non-transformed *A. tumefaciens*, and a very faint band of the same size was observed in the lane with H_2O, both negative controls. These bands are probably the result of spill over from inaccurate loading in the gel, or cross-contamination during preparation of the reactions.

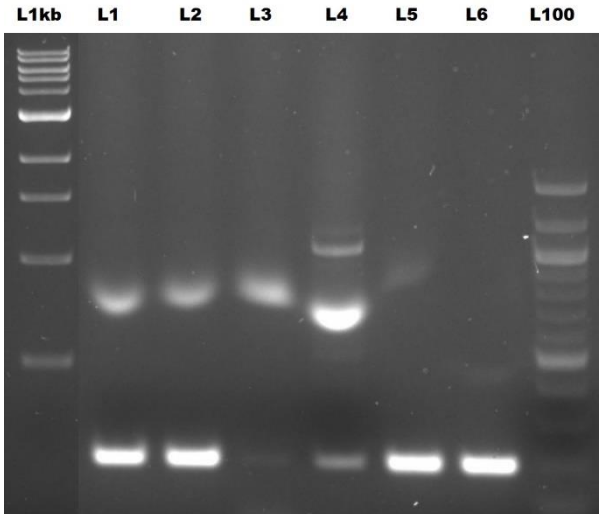


Fig. 22. *Gel documentation of PCR products following transformation of A. tumefaciens with CRISPR/Cas9 plasmid #1 and #2.*

L1 pos. control g2, L2 pos. control g1, L3, neg. control (H_2O), L4 neg. control (non-transformed A. tumefaciens), L5 A. tumefaciens transformed with g2, L6 A. tumefaciens transformed with g1.

g1 = CRISPR/Cas9 plasmid #1.

g2 = CRISPR/Cas9 plasmid #2.

L1kb = 1000bp ladder, L100 = 100bp ladder.

3.2.3 *Agrobacterium*-mediated transformation of *B. tuberhybrida* 'Urban Bicolor Pink' with CRISPR/Cas9 plasmid #2

At the time of conclusion, the putatively transformed *B. tuberhybrida* 'Urban Bicolor Pink' explants were still growing *in vitro* in medium containing 1 mg/l glufosinate as a selective agent. Unfortunately, no adventitious shoots were large enough to be transplanted to potting mix or screened by PCR. Fig. 23 shows the development of putatively transformed adventitious shoots in four different 'Urban Bicolor Pink' explants 18 weeks after transformation.

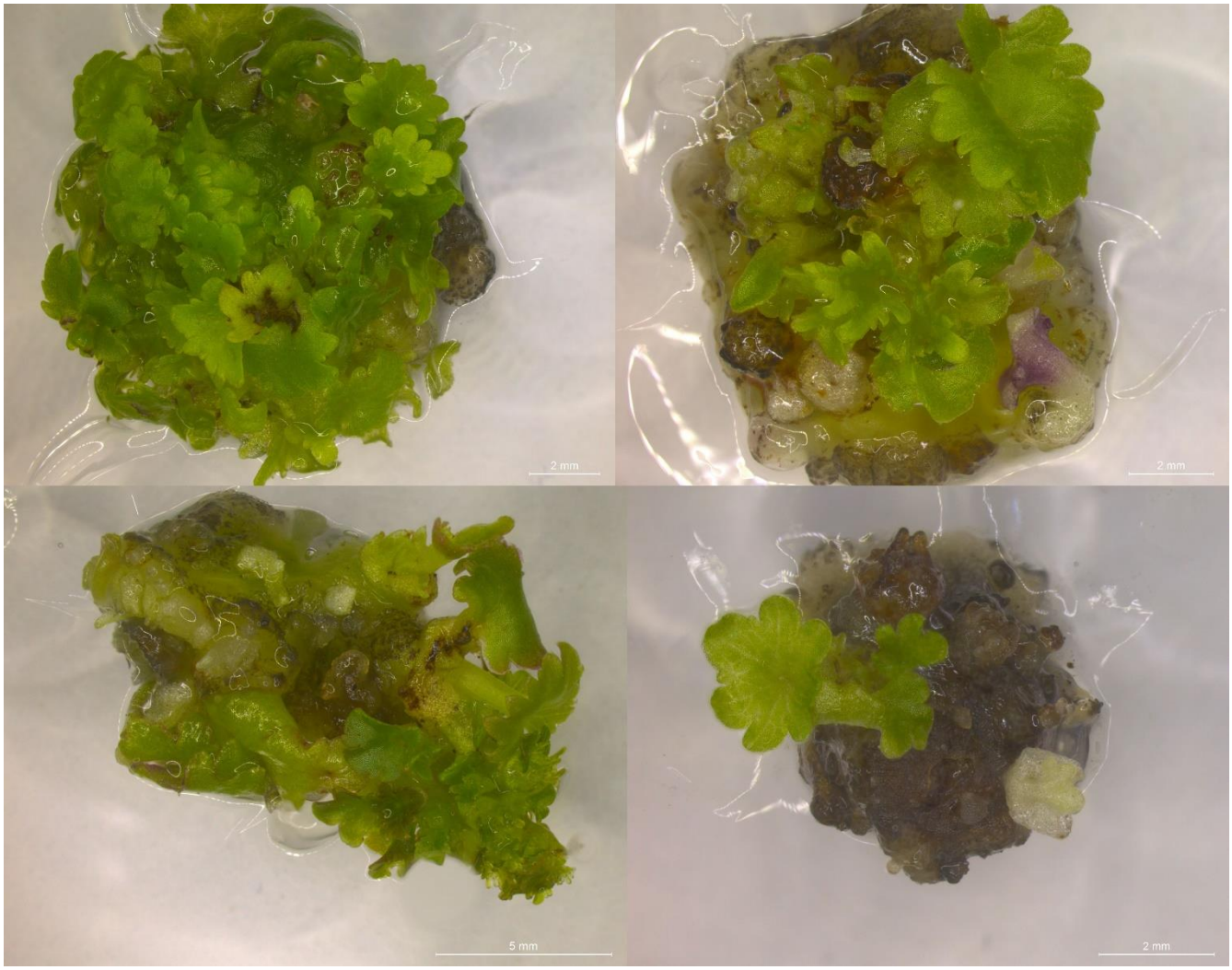


Fig. 23. *Development of putatively transformed adventitious shoots in B. tuberhybrida 'Urban Bicolor Pink' explants growing on 1 mg/l glufosinate, 18 weeks after transformation.*

The images were obtained using a Leica M205 C stereo microscope and Leica application suite v4.3.0.

3.2.4 Transformation of *A. thaliana* with CRISPR/Cas9 plasmid #1 and #2

Establishing dose-response to glufosinate in A. thaliana

As shown in Table 14 and Fig. 24, 173 of 175 seeds germinated, but the seedlings quickly turned pale and died on medium containing ≥5.0 mg/l glufosinate. Hence this concentration should ensure few, if any, escapes. As shown in Fig. 25, the growth on medium containing 2.5 mg/l glufosinate was very stunted compared to the growth observed on medium without glufosinate (control). It was possible to distinguish the growth on medium without any glufosinate (control) from growth on medium containing ≥2.5 mg/l glufosinate already six days after sowing.

Glufosinate was confirmed to be a statistically significant variable ($p<2.2e-16$) when performing an ANOVA test (Appendix III). Furthermore, as shown in Table 14 and Fig. 26, no significant differences were found between any concentrations of ≥5.0 mg/l glufosinate when performing a Tukey's test. Consequently, it was decided to use 5.0 mg/l glufosinate for subsequent screening of *A. thaliana* transformed with CRISPR/Cas9 plasmid #1 and #2.

Table 14. *Glufosinate dose-response on germination and growth of non-transformed A. thaliana seeds.*

Selection pressure	Number of seeds	Germinating seeds	Actively growing plants after 14 days	% plants viable
0.0 mg/l (control)	25	25	25	100%
2.5 mg/l	25	24	20	80%
5.0 mg/l	25	25	0	0%
10.0 mg/l	25	25	0	0%
15.0 mg/l	25	25	0	0%
20.0 mg/l	25	25	0	0%
30.0 mg/l	25	24	0	0%

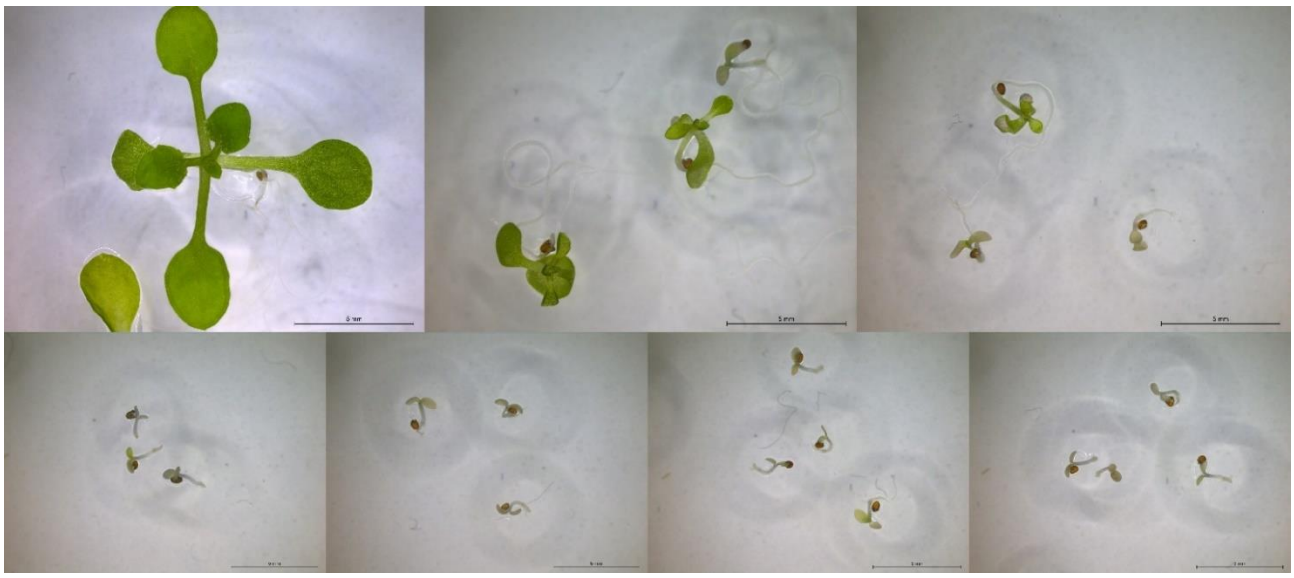


Fig. 24. *Non-transformed A. thaliana plant development 14 days after sowing.*

Top left to right: Seedlings growing on medium containing 0.0, 2.5, 5.0 mg/l glufosinate.

Bottom left to right: Seedlings growing on medium containing 10.0, 15.0, 20.0, 30.0 mg/l glufosinate.

The images were obtained using a Leica M205 C stereo microscope and Leica application suite v4.3.0.

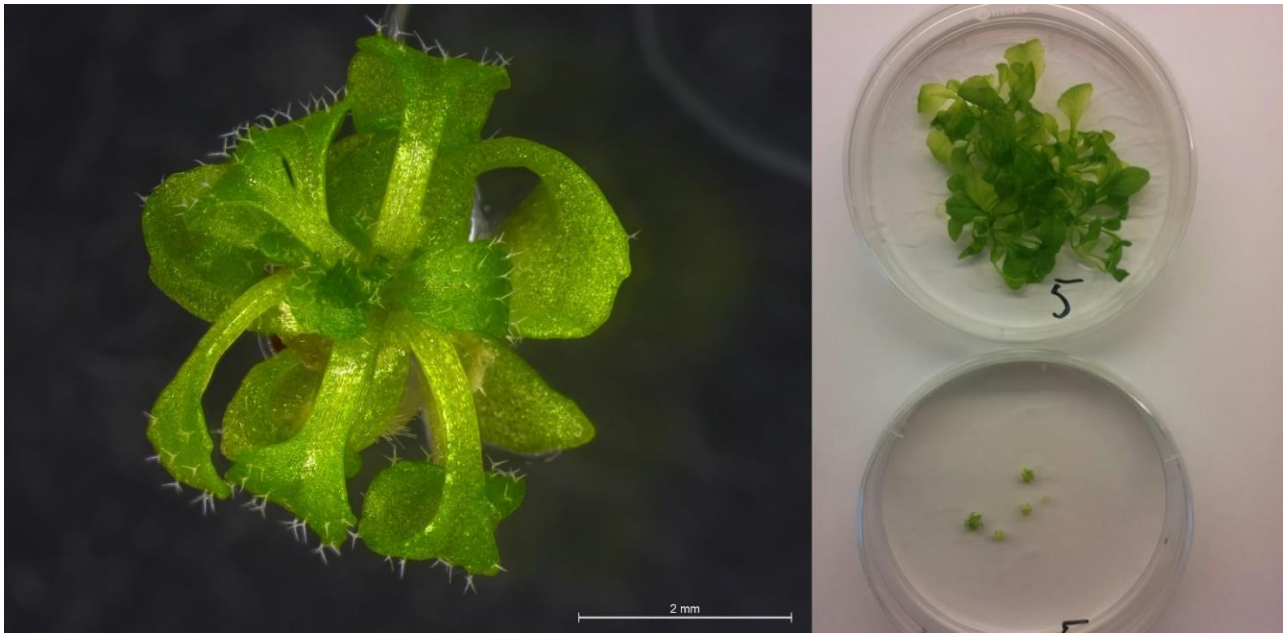


Fig. 25. *Comparison of non-transformed A. thaliana seedling development on medium containing 0.0 and 2.5 mg/l glufosinate, 39 days after sowing.*

Left: Plant growing in 2.5 mg/l glufosinate.

Top right: Plants growing in 0.0 mg/l glufosinate (control).

Bottom right: Plants growing in 2.5 mg/l glufosinate.

The images were obtained using a Leica M205 C stereo microscope and Leica application suite v4.3.0.

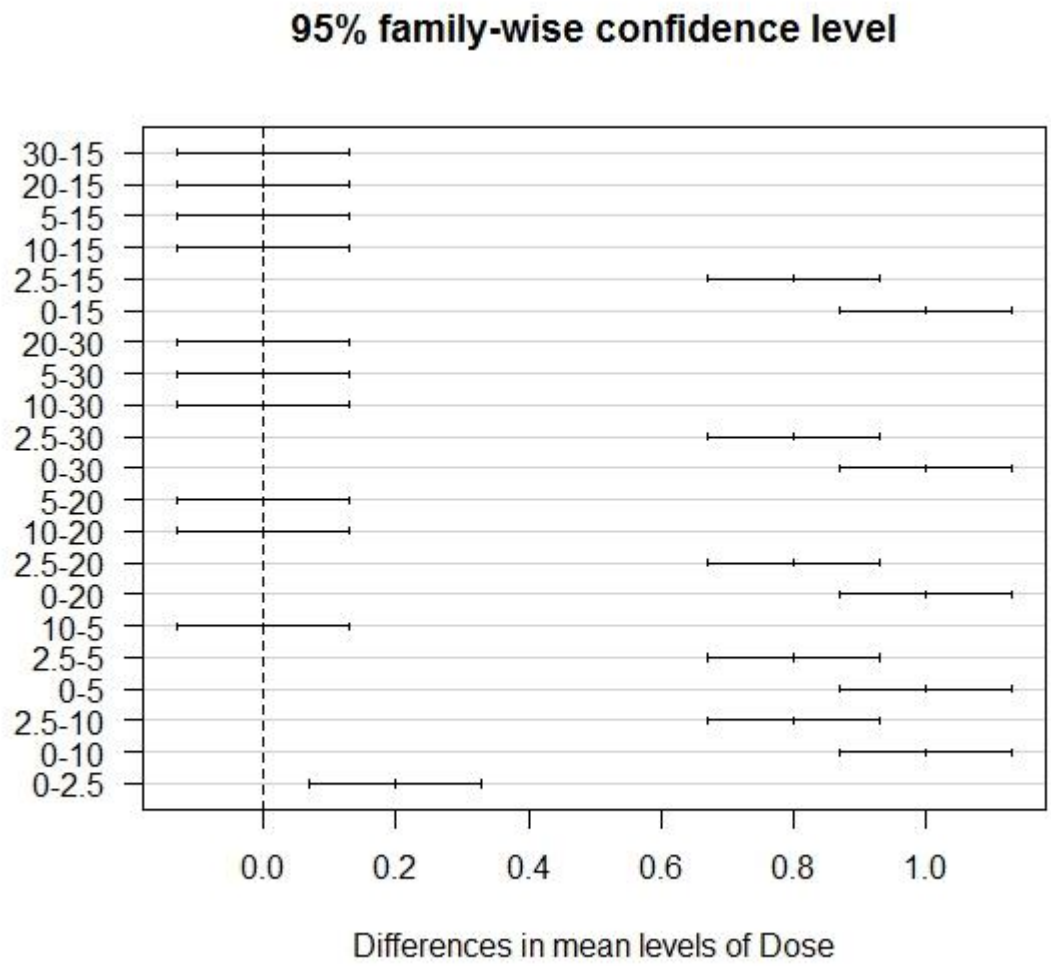


Fig. 26. *Differences in effect of glufosinate concentrations on germination and viability of A. thaliana seeds calculated by performing Tukey's test. Compared doses of glufosinate (mg/l) is displayed on the y-axis, while the 95% confidence interval is displayed on the x-axis.*

R v.3.4.1 (R Core Team 2017) was used to create the figure and perform the calculations.

Screening of A. thaliana transformants by seed colour

A large number of seeds were observed under a Leica M205 C stereo microscope to find putatively transformed seeds with lighter seed colour, but no putatively transformed seeds were found using this screening method. Considering an expected transformation efficiency of ≤3% (Clough & Bent 1998; Zhang et al. 2006), the small size of the seeds, and some natural variation in seed morphology, it was simply too difficult to find putative transformants by visual observation of seed colour.

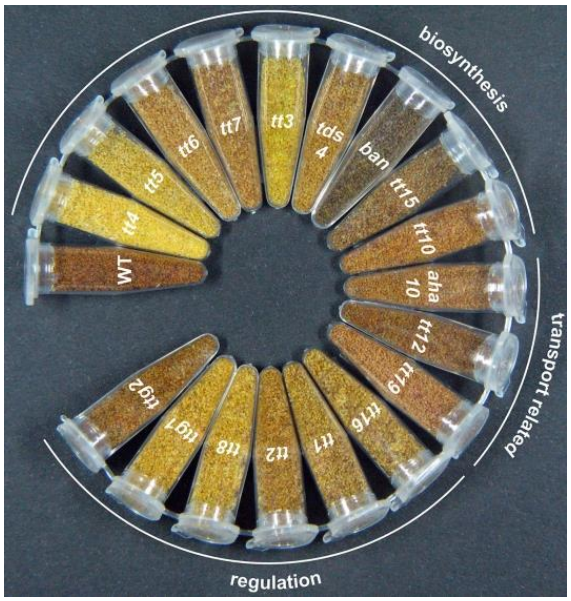


Fig. 27. *A. thaliana mutant seeds. tt7 mutants are lacking F3'H activity and can be distinguished from wild type seeds by their lighter seed colour. After Appelhagen et al. (2014).*

Screening of A. thaliana transformants in vitro on medium containing glufosinate

Almost all seeds germinated on medium containing 5 mg/l glufosinate, and putative transformants were impossible to distinguish from the wild type ten days after sowing, as all seedlings were growing reasonably well. Between 10-14 days after germination, the experiment was ruined by various contaminations that could be observed in all the 24 Petri dishes containing germinated seeds, except for the wild type seeds sown as controls in medium without and with 5 mg/l glufosinate. Approximately 4/5 of the observed contaminations seemed to be caused by overgrowth of *A. tumefaciens*, as the visual appearance of the contamination was similar to previously observed and verified overgrowth of *A. tumefaciens*, but no molecular tests were performed to verify the contaminations.

When it was discovered that all seeds were germinating and growing reasonably well in medium containing 5 mg/l glufosinate, it was decided to sow a new batch of seeds in medium containing 10 mg/l glufosinate. In medium containing 10 mg/l glufosinate it was also impossible to distinguish putative transformants from wild type ten days after sowing, as almost all seeds germinated and were growing reasonably well. Fifteen days after sowing it was possible to distinguish putative transformants from moribund seedlings, but at the same time it was also discovered that all the seedlings, growing in 23 separate Petri dishes, had been contaminated, except for the wild type seeds sown in medium without any glufosinate as controls, as observed in medium containing 5 mg/l glufosinate.

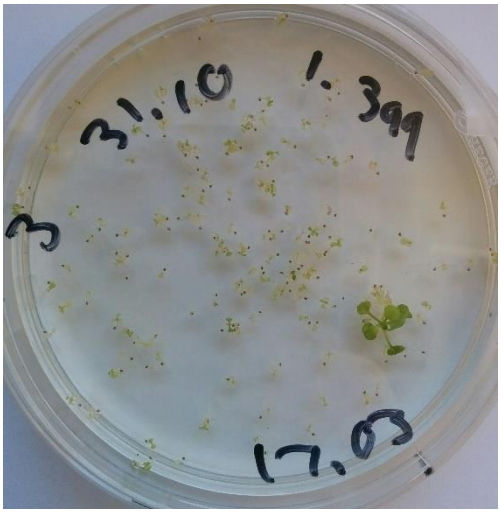


Fig. 28. *Putatively transformed A. thaliana seedling growing in vitro on medium containing 10 mg/l glufosinate and 500 mg/l cefotaxime, easily distinguishable from moribund seedlings.*

When it was discovered that all germinated seeds were lost to contaminations it was decided to sow a new batch of seeds on medium containing 10 mg/l glufosinate and 500 mg/l cefotaxime. Fourteen days after sowing, contaminations were discovered in 9 out of 20 Petri dishes containing germinated seeds. Two putative transformants could clearly be distinguished from moribund seedlings three weeks after sowing, as shown in Fig. 28, and were transferred to soil in separate pots for continued growth and subsequent screening by PCR.

Screening of A. thaliana transformants in potting mix

When it was discovered that a large proportion of the seedlings were lost due to contaminations when performing screening *in vitro* on medium containing glufosinate, even in medium containing 500 mg/l cefotaxime, it was decided to sow the remaining seeds directly in potting mix and select for transformed plants by spraying with 40 mg/l glufosinate.

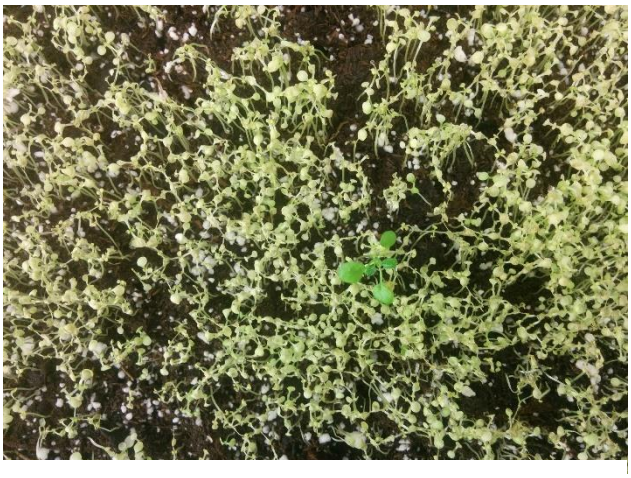


Fig. 29. *Putatively transformed A. thaliana seedling growing in potting mix and sprayed with 40 mg/l glufosinate, easily distinguishable from moribund seedlings.*

As shown in Fig. 29, putative transformants could easily be distinguished from moribund seedlings after four treatments of spraying with Milli-Q H_2O + 40 mg/l glufosinate at three-day intervals. Fourteen putative transformants were transferred to individual pots for continued growth and subsequent analysis.

Verification of A. thaliana transformed with CRISPR/Cas9 plasmid #1 and #2 by PCR

As shown in Fig. 30, all five plants chosen for screening for insertion of CRISPR/Cas9 DNA by PCR were verified as transformed. Plant #1, #3, and #5 had been transformed with CRISPR/Cas9 plasmid #1, while plant #2 and #4 had been transformed with CRISPR/Cas9 plasmid #2. The size of the DNA amplicons from both the positive controls (plasmid DNA) and the transformed *B. tuberhybrida* corresponded to the expected size of 207 bp with primer set #6 (Table 5), although the CRISPR/Cas9 plasmid #1 positive control was very faint.

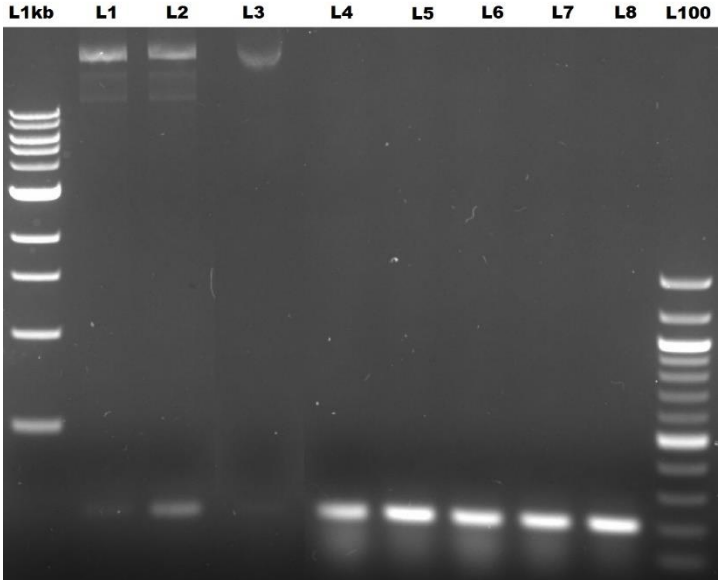


Fig. 30. *Gel documentation of PCR products following transformation of A. thaliana with CRISPR/Cas9 plasmid #1 and #2.*

L1 pos. control (CRISPR/Cas9 plasmid #1), L2 pos. control (CRISPR/Cas9 plasmid #2), L3 neg. control, L4 plant #1, L5 plant #2, L6 plant #3, L7 plant #4, L8 plant #5.

L1kb = 1000bp ladder, L100 = 100bp ladder.

Verification of mutations in F3'H in transformed A. thaliana by enzyme mismatch cleavage assays

Surveyor Mismatch Cleavage Assay

No results were obtained when performing the Surveyor Mismatch Cleavage Assay, even when using the controls supplied in the IDT Surveyor Mutation Detection Kit. After contacting technical support at IDT, it was suggested that it was difficult to troubleshoot the assay and it was suggested that a T7E1 mismatch cleavage assay should be performed instead (A Ling pers. comm. 1 March 2017).

T7E1 Mismatch Cleavage Assay

As shown in Fig. 31 right, no digested heteroduplexes could be observed when analysing the products from *A. thaliana* plant #1, #2, #3, #4, or #5 by gel electrophoresis (only results from plant #2 and #4 are shown). As shown by the three distinct bands in L1a in Fig. 31 left, the controls supplied in the IDT Alt-R Genome Editing Detection Kit worked as intended. DNA from control B contain a 6 bp deletion, but is otherwise identical to DNA from control A.

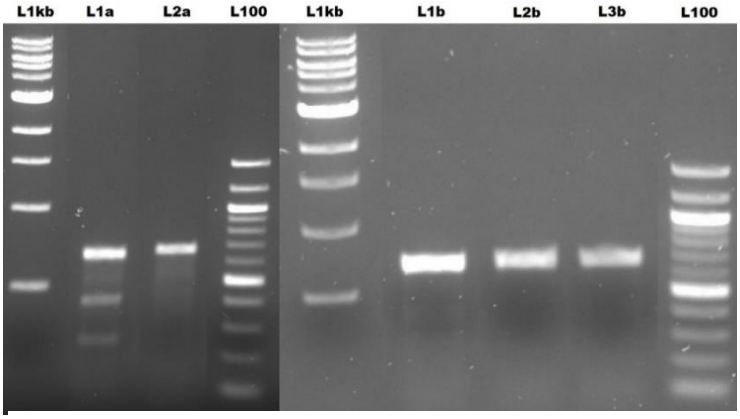


Fig. 31. *Gel documentation of the results from treatment of PCR products with T7E1.*

Left: Controls supplied in the IDT Alt-R Genome Editing Detection Kit; L1a mixture of DNA from control A and control B, L2a DNA from control A (control).

Right: Primer set #16; L1b DNA from plant #2 (control), L2b mixture of DNA from plant #4 and wild type DNA, L3b mixture of DNA from plant #2 and wild type DNA.

L1kb = 1000bp ladder, L100 = 100bp ladder.

Verification of mutations in F3'H in transformed A. thaliana by Sanger sequencing

As shown in Fig. 32, no mutations were detected in any of the *F3'H* fragments from transformed *A. thaliana* plants that were sequenced. *F3'H* regions covering the predicted cut sites for both CRISPR/Cas9 construct #1 and #2 were sequenced in plant #1, #2, #3, #4, and #5.

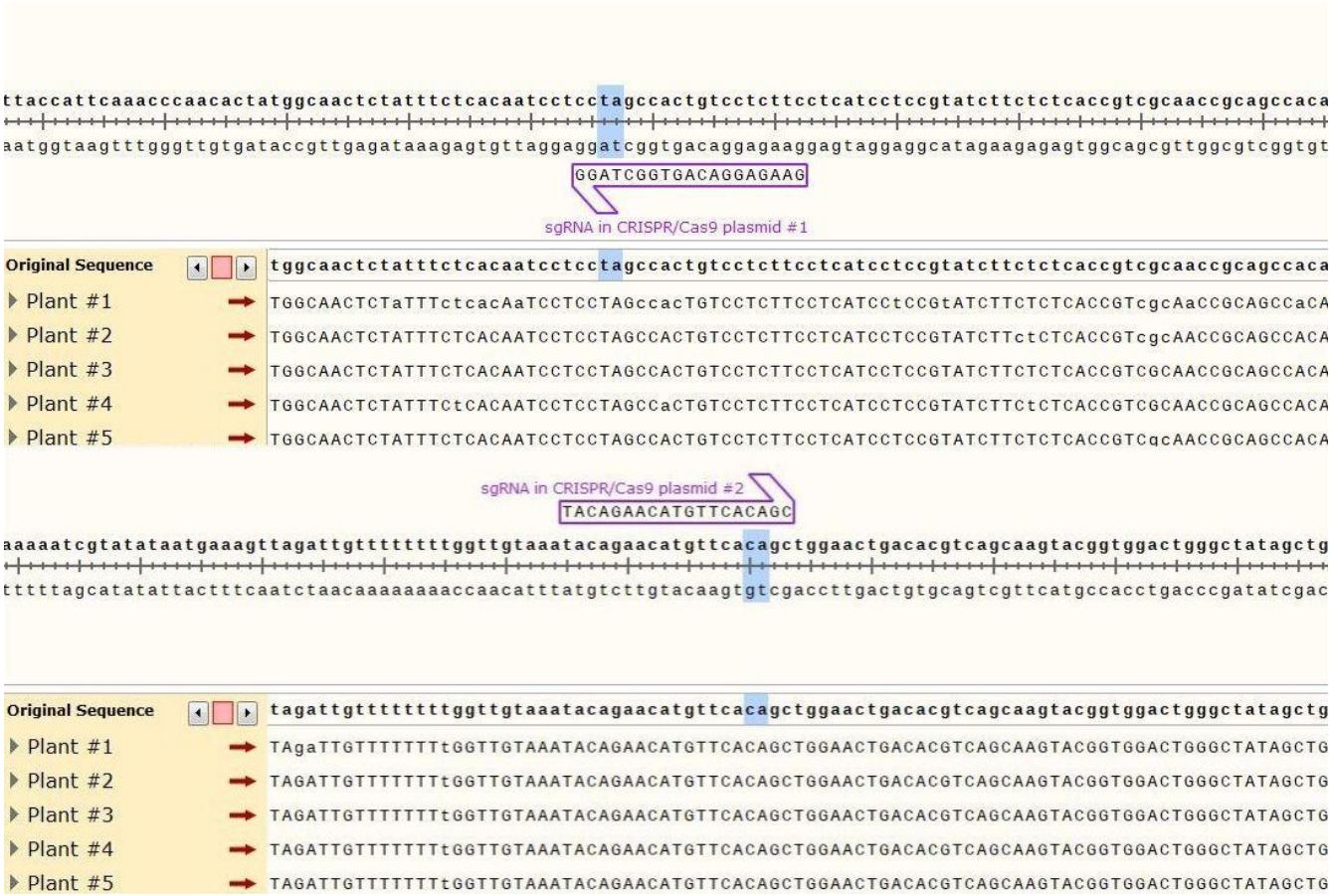


Fig. 32. *Sequence data covering the predicted cut sites (highlighted in blue) for CRISPR/Cas9 construct #1 (top) and #2 (bottom) in F3'H from A. thaliana plant #1, #2, #3, #4, and #5.*

Upper case letters indicate Phred20 quality (99% accuracy), lower case letters indicate Phred10-19 (90-98% accuracy) (Ewing & Green 1998; Ewing et al. 1998). The original sequence was taken from the NCBI Gene database (National Center for Biotechnology Information 2000).

The figure was created using SnapGene v3.3.4 (from GSL Biotech; available at snapgene.com).

4 Discussion

At least 18 *B. tuberhybrida* ‘Urban Bicolor Pink’ plants transformed with *F3’5’H* were regenerated. A slight change towards bluer flower colour was measured with a colorimeter in transformed plants, but no significant shift in flower colour towards blue could be observed. The lack of significant change in phenotype can be due to several reasons; (1) it is probable that the vacuolar pH in the sepals and petals is too low, (2) the *F3’5’H* transgene is possibly not sufficiently expressed, (3) the endogenous F3’H and/or DFR might out-compete F3’5’H for substrate, and/or (4) the endogenous DFR might not efficiently catalyse conversion of DHM. These possible explanations will be discussed in the following sections.

If this had been a study with a longer time frame and an allocated budget, several initial steps could have been taken to secure a positive result: For a start, it would have been better to have a sequenced genome, or at least sequence data on some of the genes involved in the anthocyanin biosynthesis pathway, of *B. tuberhybrida* or a related *Begonia* sp. Secondly, we could have started by analysing the pigment composition and substrate specificity of DFR in a selection of *B. tuberhybrida* cultivars, to search for a suitable candidate to transform. Furthermore, several different constructs containing *F3’5’H* from different sources under the control of different promoters could have been used. All of these tasks could have been a master project in itself.

The results from this thesis work showed that, on average, 0.36 putatively transformed *B. tuberhybrida* ‘Urban Bicolor Pink’ plantlets were obtained per explant 15-23 weeks after performing *Agrobacterium*-mediated transformation. Even when considering that only 18 out of 28 putative transformants tested could be verified as transformed, this result is at least as good as what was reported in the only other publication on *Agrobacterium*-mediated transformation of *B. tuberhybrida*, which reported obtaining 0.24 transformed plantlets per explant (19 transformed plantlets from 80 explants) (Kiyokawa et al. 1996), especially when taking into consideration the significant amount of explants lost due to contaminations and the high probability of several false negatives resulting from weak DNA extraction with the Extract-N-Amp kit. As such, the refined transformation protocol used in this thesis work must be considered as excellent, although improvements can be made with regards to the culture conditions to speed up the regeneration process and lower the contamination rates.

One of the aims of this thesis work was to explore the use of CRISPR/Cas9 to edit the anthocyanin biosynthesis pathway in *B. tuberhybrida*, as this relatively novel technology holds great potential for altering complex biosynthesis pathways and it is also a very valuable skillset to acquire for all researchers involved in genetics. As it was, no *B. tuberhybrida* ‘Urban Bicolor Pink’ plants were

verified as transformed with the CRISPR/Cas9 construct targeting *F3'H* during the time frame of this thesis work, but several putative transformants were growing *in vitro* on selective medium at the time of conclusion. However, the large number of putatively transformed 'Urban Bicolor Pink' plantlets obtained during this thesis work indicates that *B. tuberhybrida* is easily transformed with *Agrobacterium*-mediated transformation, which is also the most commonly used delivery system for the CRISPR/Cas9 system (Ma et al. 2016), and could possibly be an amenable species to work with for future CRISPR/Cas9 gene editing, if sequence data becomes available and both *Cas9* and sgRNA is shown to be readily expressed at sufficient levels in *B. tuberhybrida* cells.

A. thaliana was also transformed with CRISPR/Cas9 constructs targeting *F3'H* as a proof of concept, as *F3'H* sequence data was not available for *B. tuberhybrida*. At least five *A. thaliana* plants transformed with the CRISPR/Cas9 constructs were regenerated (three were verified as transformed with CRISPR/Cas9 plasmid #1, two were verified as transformed with CRISPR/Cas9 plasmid #2), but no mutations could be detected near the predicted cut sites after performing enzyme mismatch cleavage assays and Sanger sequencing. These results add to the growing body of reports showing that *A. thaliana* plants transformed with the CRISPR/Cas9 system exhibit much lower mutation efficiencies than other plants (Ma et al. 2016). The absence of the predicted mutations could be explained by; low expression levels of *Cas9* or sgRNA, the nucleotide composition of the sgRNA, or any unknown factor(s) affecting the efficiency of the CRISPR/Cas9 system. These possible explanations will be discussed in the following sections.

4.1 Regarding selection of plant material and genetic elements for engineering of the anthocyanin biosynthesis pathway

Selection of suitable host cultivar(s) and transgenic elements is very important to successfully engineer blue flowers, especially considering the vast differences in *B. tuberhybrida* flower morphology (Haegeman 1979; Hvoslef-Eide & Munster 2006). To increase the probability of engineering a desirable blue-hued phenotype, a large number of cultivars are commonly screened and several vectors containing different combinations of transgenic elements have been tested for other species; 75 cultivars and seven constructs in the case of transgenic *C. x morifolum* engineered by Brugliera et al. (2013), two cultivars and 25 constructs in the case of transgenic *C. x morifolum* engineered by Noda et al. (2013), 169 cultivars and four constructs in the case of transgenic *R. hybrida* engineered by Katsumoto et al. (2007).

Disruption of endogenous genes using CRISPR/Cas9, as in the case of the attempt to disrupt *F3'H* in *B. tuberhybrida* 'Urban Bicolor Pink' and *A. thaliana* in this thesis work, should be possible no matter what cultivar is chosen, as Cas9 is able to introduce DSB in any DNA sequence of interest (Doudna & Charpentier 2014). However, it is necessary to know the target sequence to be able to design appropriate sgRNAs, and both *Cas9* and the sgRNA must be sufficiently expressed in the cell of interest. Furthermore, the ease of delivery and mutation efficiency varies vastly between plant species (Bortesi & Fischer 2015; Liang et al. 2016; Ma et al. 2016), but there is less chance of significant differences between various cultivars from the same species, since there is more sequence homology in these cases. However, it is well known that there are genotype differences with regards to transformation efficiency when performing *Agrobacterium*-mediated transformations (Komari et al. 2004), but it is unclear whether genotype differences directly affect the efficiency of the CRISPR/Cas9 system, or if it is just the ease of delivery of the system that is affected.

4.1.1 Selection of host cultivar

The vacuolar pH of sepals and petals, total flavonoid composition, F3'H activity, and DFR substrate specificity are some of the most important factors which should be considered when selecting genotypes suitable for engineering of blue flowers by introduction of *F3'5'H*.

The most appropriate course of action for selection of appropriate *B. tuberhybrida* cultivar(s) would have been to perform precursor feeding experiments to determine the substrate specificity of DFR, analyse the flavonoid composition by HPLC, and measure the pH of sap from sepals and petals in as many relevant cultivars as feasible to find the most promising cultivars, before performing the transformation procedures. Sepals and petals from promising cultivars should; (1) be able to accumulate delphinidin-based anthocyanins when incubated on medium containing DHM, indicating that the endogenous DFR is able to catalyse the conversion of DHM to delphinidin (Brugliera et al. 2013), (2) contain no/very little cyanidin, indicating low F3'H activity (Katsumoto et al. 2007), (3) contain high amounts of flavones and flavonols (co-pigments) which can enhance blue colour development (Brugliera et al. 2013; Fukui et al. 2003; Katsumoto et al. 2007; Yabuya et al. 1997), and (4) have a higher vacuolar pH (Davies 2009; Tanaka et al. 2009).

Unfortunately, it was unfeasible to perform a thorough analysis of *B. tuberhybrida* cultivars within the constraints of this thesis work. However, the pink-flowered 'Urban Bicolor Pink' was chosen as host material based on the results from Brugliera et al. (2013) and Katsumoto et al. (2007), which

found that pink cultivars in general tended to contain the most appropriate flavonoid composition, endogenous enzyme activity, and vacuolar environments. Given the constraints on available time and resources, this was considered to be the best educated guess and the most appropriate approach to selection of *B. tuberhybrida* cultivar for engineering of blue flowers.

4.1.2 Selection of gene sources and promoters

It is probable that *F3'5'H* from *P. x hybrida* under the control of the CaMV 35S promoter is not the most effective combination for maximum accumulation of delphinidin-based anthocyanins in *B. tuberhybrida* 'Urban Bicolor Pink' sepals and petals. To assess the efficiency of the construct it would be necessary to analyse the flavonoid composition of the transformed 'Urban Bicolor Pink' sepals and petals, and to transform other 'Urban Bicolor Pink' plants with different constructs for comparison. Furthermore, as reported by several research groups, CaMV 35S is not the most appropriate promoter for expression of *Cas9* in *A. thaliana* (Ma et al. 2016; Tsutsui & Higashiyama 2017).

Because the colour of sepal and petal sap extracted from the two transformed 'Urban Bicolor Pink' plants tested did not consistently exhibit bluer colouration than the sap extracted from the wild type at elevated pH levels, it was apparent that delphinidin-based anthocyanins were not being accumulated in significant amounts in sepal or petal tissues, at least in these two transformants tested. One probable explanation is low expression levels of the *F3'5'H* transgene, as a result of not using the most appropriate promoter and/or gene source.

It was evident that CaMV 35S was effective at expressing the *bar* selectable marker gene in vegetative tissues in *A. thaliana* and *B. tuberhybrida* 'Urban Bicolor Pink', as *A. thaliana* seedlings transformed with the CRISPR/Cas9 plasmids and 'Urban Bicolor Pink' plantlets transformed with pIA291 and CRISPR/Cas9 plasmid #2 were able to grow on medium containing glufosinate or survive spraying with glufosinate. However, even though CaMV 35S can effectively control the selectable marker gene in developing plantlets and seedlings throughout the screening process, it does not necessarily result in the same level of expression of *F3'5'H* in flower tissues (Brugliera et al. 2013), or of *Cas9* in the female reproductive tissues (Ma et al. 2016; Tsutsui & Higashiyama 2017), which is the primary target of *A. tumefaciens* infection in the floral drip transformation method of *A. thaliana* (Desfeux et al. 2000).

Noda et al. (2013) and Brugliera et al. (2013) found that CaMV 35S was not suitable for expressing *F3'5'H* in ray florets of *C. x morifolum*, but CaMV 35S in combination with *F3'5'H* from *Viola* sp.

was successfully used to engineer blue flowers in *R. hybrida* (Katsumoto et al. 2007), indicating that the efficiency of the promoter to control expression of *F3'5'H* in floral tissues varies between plant species.

CaMV 35S has been shown to be able to sufficiently drive expression of *Cas9* in *A. thaliana* and many other plant species, but the reported mutation efficiencies have ranged from 1-85% in *A. thaliana* stably transformed with *Cas9* under the control of CaMV 35S (Bortesi & Fischer 2015; Kim et al. 2016; Ma et al. 2016; Osakabe et al. 2016; Song et al. 2016). However, the use of the stronger ubiquitin promoters produced higher editing efficiencies in many cases (Ma et al. 2016), and it has been found that the lower editing efficiency of the CRISPR/Cas9 system in *A. thaliana*, which can explain the absence of the predicted mutations in this thesis work, can be greatly improved by using promoters with high activity in germline cells or dividing tissues to control *Cas9* instead of constitutive promoters such as CaMV 35S (Ma et al. 2016; Tsutsui & Higashiyama 2017).

Using the most appropriate *F3'5'H* source is also of great importance when engineering blue flowers. *F3'5'H* first evolved from *F3'H* before the separation of angiosperms and gymnosperms (Seitz et al. 2006), but has independently evolved from *F3'H* at least four more times at later stages in dicotyledonous plants (Seitz et al. 2015). At the amino acid level, some *F3'5'H* alleles are more similar to *F3'H* than to other *F3'5'H*, indicating a substantial amount of variation between various F3'5'H coding sequences (Seitz et al. 2015).

Genes encoding F3'5'H were first isolated from *P. x hybrida* by Holton et al. (1993) and have now been cloned from many species (Tanaka & Brugliera 2013). When used as transgenes the *F3'5'H* alleles result in varying rates of accumulation of delphinidin-based anthocyanins under different promoters in different species, and sometimes even in different cultivars (Brugliera et al. 2013; Noda et al. 2013; Tanaka & Brugliera 2013). This, in part, can be explained by variation in the amino acid composition and/or the different transgene coding sequences, which may contain codons that are not as efficiently translated as more common endogenous codons (Plotkin & Kudla 2011). Furthermore, in some cases, introduction of several *F3'5'H* genes from different sources result in the highest rates of accumulation of delphinidin-based anthocyanins in petals (Tanaka & Brugliera 2013).

Finding the most appropriate promoter and transgene source seems to largely rely on trial and error, as the differences in gene expression between species is quite unpredictable, further exacerbated by the variance in gene expression levels caused by the fairly random integration of T-DNA into the host genome (Gelvin 2003; Kim et al. 2007). Even when using modern biotechnology, breeding

novel flower colours is a numbers game, in which testing a lot of different constructs and genotypes often is necessary to obtain the desired phenotypes (Tanaka & Brugliera 2013).

The *Cas9* coding sequence used in the CRISPR/Cas9 plasmids had been codon optimised for use in *A. thaliana* by Sigma-Aldrich, and is, to the best of our knowledge, the most efficient *Cas9* variant known to date for use in *A. thaliana*, but, as discussed above, CaMV 35S has been shown to not be the most efficient promoter for expression of *Cas9* in *A. thaliana* (Ma et al. 2016). Furthermore, low expression levels of sgRNA have been correlated with low mutation efficiencies (Ma et al. 2015), but we are unaware of any studies performed on the efficiency of various sgRNA promoters in *A. thaliana*. However, the nucleotide composition of the sgRNA has been found to have a large effect on mutation efficiency (Bortesi & Fischer 2015; Liang et al. 2016; Ma et al. 2016), as discussed below.

4.2 Disrupting *F3'H*

Competition for substrate between F3'H and F3'5'H has been shown to inhibit maximum accumulation of delphinidin-based anthocyanins in the flowers of several species, and thus prevent the desired shift towards bluer flowers in plants transformed with *F3'5'H* (Brugliera et al. 2013; Davies 2009; Tanaka & Brugliera 2013). To avoid this issue, it is possible to either select host material with low or no F3'H activity, as in the case of transgenic blue-hued *R. hybrida* flowers (Katsumoto et al. 2007), or disrupt the gene activity, as in the case of transgenic violet/blue *C. x morifolum* (Brugliera et al. 2013). Competition for substrate between DFR and F3'5'H can also inhibit maximum accumulation of delphinidin-based anthocyanins (Katsumoto et al. 2007), but as DFR is also essential for conversion of DHM to delphinidin, it is reasonable to first test whether disruption of *F3'H* results in a desired phenotype before, if necessary, replacing the endogenous *DFR* with a more suitable allele.

Because it was unfeasible to perform gene expression and flavonoid composition analyses within the constraints of this thesis, an alternative approach had to be used. It was decided to use CRISPR/Cas9 to disrupt *F3'H* in *B. tuberhybrida* 'Urban Bicolor Pink', as this technology is considered a precise and easy-to-use method for gene disruption (Doudna & Charpentier 2014). Even though the exact target sequence was unknown, we hypothesized that a sequence fully conserved in several related species would have a high probability of also being conserved in *B. tuberhybrida*. Furthermore, the technology has been predicted to have a revolutionary impact on

plant research (Bortesi & Fischer 2015; Ma et al. 2016), and as such it would be of great interest to study and try out the technology hands-on at the Norwegian University of Life Sciences.

To find possible sgRNA target sites likely to be present in *B. tuberhybrida*, *F3'H* sequences from *C. melo*, *C. sativus*, which belong to the same order (Cucurbitales) as *B. tuberhybrida*, and *A. thaliana*, which belong to the same clade (Rosids) as *B. tuberhybrida*, were aligned, and a single possible target conserved in all three species' was found. Because, to the best of our knowledge, this approach to designing sgRNAs has not been documented by anyone previously, it was decided that *A. thaliana* also was to be transformed with the same construct as a proof of concept.

It is common practise to test several sgRNAs targeting the same gene, since the mutation rates are lower than the transformation rates, and heavily depend on the sgRNA design, as well as several other known and unknown factors (Bortesi & Fischer 2015; Liang et al. 2016; Ma et al. 2016; Tsutsui & Higashiyama 2017). Hence, another construct containing sgRNA targeting another part of *F3'H* in *A. thaliana* was designed. Because only a single possible target was found when aligning the *F3'H* sequences from *C. melo*, *C. sativus*, and *A. thaliana*, the second sgRNA was designed to only target *F3'H* in *A. thaliana*.

There are many factors to consider when designing sgRNAs and because the technology is relatively new, not all of them are well understood. In essence, they impact cleavage efficiency and target specificity (Jinek et al. 2012; Liang et al. 2016; Ma et al. 2016). The system is generally working very similarly in all organisms, but it has recently been discovered that there seems to be differences between the efficiency of sgRNAs in plants and in other organisms (Liang et al. 2016; Ma et al. 2016).

Liang et al. (2016) found no nucleotide preference in any of the sgRNA-positions when used in plants, in contrast to research performed on animals (Doench et al. 2014; Wang et al. 2014; Xu et al. 2015). Even though not specifically mentioned by Liang et al. (2016), their research is implying that it is not even necessary to add an extra guanine (G) nucleotide at the 5' end of the sgRNA when the sgRNA does not begin with a G. Because the U6 RNA polymerase III promoter commonly used to express the sgRNA, such as AtU6-1 used in this thesis work, prefers a G as the first base of its transcript, earlier studies on CRISPR/Cas9 commonly restricted the target sites to $GN_{20}NGG$ (Jinek et al. 2013; Kim et al. 2016; Mali et al. 2013) or appended an extra G at the 5' side of the sgRNA (Ran et al. 2013). The total nucleotide composition does however seem to matter when designing sgRNAs for use in plants. A G/C content between 30% and 80% (Liang et al. 2016), preferably >50% (Ma et al. 2015), correlates with the highest editing efficiencies in plants.

However, Tsai et al. (2015) found that a G/C content of ≥70% is associated with more off-target effects, so the optimal G/C content is probably 50-70%.

To reduce the chance of any off-target effects it is important to select unique target sites, especially in the seed sequence 12 bp adjacent to PAM (Cong et al. 2013; Jinek et al. 2012). Furthermore, the length of the sgRNA also seem to influence the specificity. Fu et al. (2014) and Osakabe et al. (2016) found that truncated sgRNAs can decrease off-target effects without sacrificing on-target efficiency. For the CRISPR/Cas9 constructs used in this thesis work a sgRNA length of 19 nt was chosen, as the technicians at Sigma-Aldrich had determined this to be an optimal balance between specificity and efficiency (G Jackson 2016 pers. comm. 16 September).

Fortunately, software has been designed to find and analyse possible target sequences and the corresponding sgRNA. During the sgRNA design process CRISPR Design (Zhang Lab 2015), CRISPRdirect (Naito et al. 2015), and CRISPOR (Haeussler et al. 2016) were used to scan the genetic code for possible target sequences and estimate their specificity. Unfortunately, it was not discovered until after the CRISPR/Cas9 constructs had been produced that plant specific sgRNA design software had been developed (Lei et al. 2014; Liu et al. 2017). The use of CRISPR-P 2.0 (Liu et al. 2017) would have made the design process somewhat easier, but the end result would probably have been the same, as the scoring systems used in the various sgRNA design software are similar (Haeussler et al. 2016; Liu et al. 2017; Naito et al. 2015; Zhang Lab 2015).

Unfortunately, no *B. tuberhybrida* 'Urban Bicolor Pink' plantlets putatively transformed with CRISPR/Cas9 plasmid #2 could be analysed during the time frame of this thesis work because of unpredicted slow growth, and no predicted mutations could be detected in the five *A. thaliana* plants transformed with CRISPR/Cas9 plasmid #1 or #2. The absence of any predicted mutations in the five *A. thaliana* plants transformed with CRISPR/Cas9 plasmid #1 or #2 can probably mainly be attributed to the choice of *Cas9* promoter, as discussed above, unknown factors affecting sgRNA efficiency, and the fairly random integration of T-DNA and variable transgene copy number (Gelvin 2003; Kim et al. 2007), which can have a great effect on *Cas9* and sgRNA expression levels (Ma et al. 2016; Osakabe et al. 2016).

The choice of CaMV 35S as a promoter for *Cas9* was probably not the optimal choice, as discussed above, but the sgRNAs did fulfil all current established requirements for optimal efficiency with regards to nucleotide composition, length, and target specificity (Fu et al. 2014; Liang et al. 2016; Ma et al. 2015), except for the sgRNA used in CRISPR/Cas9 plasmid #2, which had a G/C content of 42%, which is lower than the >50% content suggested by Ma et al. (2015), but within the 30-80% range suggested by Liang et al. (2016). Furthermore, *Cas9* had been codon optimised for

translation in *A. thaliana*, and we are unaware of any studies reporting that there are better choices than the AtU6-1 promoter for control of sgRNA expression, although sgRNA expression driven by the AtU6-1 promoter has been shown to vary greatly (Osakabe et al. 2016). However, because the CRISPR/Cas9 system is a novel system, there are still many unknown factors affecting mutation efficiency, and it is hence recommended to test several sgRNAs targeting multiple sites in the same gene to ensure efficient editing (Liang et al. 2016).

4.3 pH and colour

Elevated pH levels are a prerequisite for formation of blue flowers, even though low accumulation rates of delphinidin-based anthocyanins, and not the very low pH levels of *B. tuberhybrida* 'Urban Bicolor Pink' sepals and petals, probably was the cause for the lack of significant change in phenotype towards blue flowers in the two transformed 'Urban Bicolor Pink' plants tested by measuring the colour of extracted sap. Anthocyanins are very unstable, and pH have a great effect on their stability. A vacuolar pH of >5.5 (Davies 2009), or at least ~4.0 (Tanaka et al. 2009), is considered necessary for formation of blue flowers, because the balance conversions of different anthocyanin tautomers favour the flavylium cation at low pH levels (pH <3), which are redder and more stable than the tautomers at elevated pH levels (Pina 2014; Tanaka et al. 2008; Zhao & Tao 2015).

Begonia spp. are characterized by a very high content of oxalic acid (Nordal & Resser 1966; Rose & Hard-Karrer 1927) and the pH of sap from *Begonia* spp. tissue have been reported to be as low as ~1 (Beerstecher 1964; Rose & Hard-Karrer 1927), dramatically lower than the typical vacuolar pH of 3-6 (Davies 2009). The studies on begonias mentioned did not specifically examine the pH of flower tissues, but sepal and petal sap of *B. tuberhybrida* 'Urban Bicolor Pink' was confirmed to be very acidic (pH=1.5) when measured as a part of this thesis work. However, to the best of our knowledge, it is not known whether the acid is mainly localized in specialized cells and/or specialized vacuoles or found in most cells, or how that would affect pigmentation.

No flowering 'Urban Bicolor Pink' plants were available at the beginning of this thesis work, as such it was not possible to measure the pH in the sap of 'Urban Bicolor Pink' sepals and petals until after all the transformation experiments had been performed. The very low pH of 1.5 indicates that true blue flowers are unlikely to materialise even with exclusive accumulation of delphinidin-based anthocyanins, unless the pigments are stored in separate vacuoles with an elevated pH environment,

but this is improbable. However, a definite change in flower colour is expected, even at very acidic pH levels, if the flavonoid composition is significantly altered.

It should however be possible to breed for a higher vacuolar pH using traditional breeding methods and/or modern genetic engineering techniques, although no articles on engineering significant changes in the vacuolar pH of ornamentals has been published (Davies 2009). Literature searches in 2017 still does not reveal any published work on this topic, but commercial companies have reported successful engineering of the vacuolar pH of ornamentals at Eucarpia conferences (T Hvoslef-Eide pers. comm. 2017). Several genes involved in the regulation of vacuolar pH have already been identified (Fukada-Tanaka et al. 2000; Mol et al. 1998; Momonoi et al. 2009; Spelt et al. 2002; Verweij et al. 2008) and could be potential targets for genetic engineering to elevate the vacuolar pH in sepals and petals of *B. tuberhybrida*. Furthermore, some anthocyanin transcription factors also seem to have an impact on vacuolar pH (Mol et al. 1998; Spelt et al. 2002), and could also possibly be targeted to increase the vacuolar pH in sepals and petals. However, as maintaining an appropriate cellular pH is very important for normal cell metabolism, it is likely that mechanisms exist which can counter changes induced by a single transporter gene or transcription factor (Davies 2009).

4.4 High copy numbers and gene silencing

Agrobacterium-mediated transformation is thought to result in a lower transgene copy number than direct DNA delivery methods, but still results in integration of variable transgene copy numbers in the plant genome and sometimes silencing of transgenes (Gelvin 2003; Jia et al. 2012; Slater, A. et al. 2008). Furthermore, it has been found that relatively high concentrations of selective agents can cause biased selection in favour of transformants with multiple copies of the transgenes (Dalton et al. 1995; Dekeyser et al. 1989; Hamill et al. 1987), and delaying transfer of explants to selection medium after co-cultivation or gradually increasing the concentration of the selective agent during regeneration has hence been used to avoid such biased selection of transformants in some plant species (Bhuiyan et al. 2011; Milojevic et al. 2012; Visser et al. 1989).

It is possible that a high *F3’5’H* and *Cas9* copy number could have caused gene silencing in some of the transformed plants analysed during this thesis work, but it is unlikely that this can explain the lack of a significant shift towards blue flowers in any of the *B. tuberhybrida* ‘Urban Bicolor Pink’ transformants, or the absence of predicted mutations in any of the *A. thaliana* transformants analysed. However, it might have been a contributing factor to low levels of transgene expression in

some of the cases. The copy numbers could have been detected by southern blotting or real-time quantitative PCR (Dugdale et al. 2014; Shepherd et al. 2009), but such analyses were not performed.

4.5 Regeneration and screening of transformants

4.5.1 Choice of *B. tuberhybrida* 'Urban Bicolor Pink' explant material and regeneration of transformants

The type and quality of the starting material used for transformation purposes is a very important factor affecting the transformation efficiency (Komari et al. 2004). For transformation of *B. tuberhybrida* 'Urban Bicolor Pink' with pIA291 and CRISPR/Cas9 plasmid #2 leaf explants from *in vitro* cultures were primarily used, in addition to some petiole explants, based on the studies performed by Kiyokawa et al. (1996), Nada et al. (2011), and Nakano et al. (1999).

Kiyokawa et al. (1996) reported obtaining a relatively large amount *B. tuberhybrida* transformants in a relatively short amount of time from, presumably, both leaf and petiole explants. It is unclear whether they mainly used explants from leaf or petioles, as only the number of leaf explants used is mentioned, but it is later written in the article that "Four to six weeks after co-cultivation, 48 shoots were formed at the edge of leaf or petiole cuttings", implicating that both tissue types were used and that the regeneration time was similar. Furthermore, Nada et al. (2011) found a higher frequency of adventitious shoot formation and maximum number of shoots in *B. tuberhybrida* leaf explants than in petiole explants, and Nakano et al. (1999) found no significant difference in adventitious shoot regeneration between *B. tuberhybrida* leaf and petiole explants. Leaf explants were also the tissue type of choice in previously reported transformation of *Begonia x cheimantha* (Einset & Kopperud 1995), *Begonia x hiemalis* (Kishimoto et al. 2002), and *Begonia* Rex (Ohki et al. 2009).

However, during the regeneration phase, it became evident that petiole explants had a higher survival rate, but at that point all transformation procedures were already finished. Unfortunately, only anecdotal evidence is available, as it was not feasible to record accurate individual data on the 1804 explants, which were routinely cut into smaller pieces during regeneration. The reason for the higher survival rate of petiole explants might be a result of the handling when performing the transformation protocol, as the leaves of *B. tuberhybrida* from *in vitro* cultures can easily desiccate while the petioles are more robust (A Sivertsen pers. comm. 2016).

Significant differences between petiole and leaf explants in the regeneration rate of wild type explants on various concentrations of glufosinate was detected ($p=0.0017$) when performing the dose-response experiments, which, in hindsight, should have been taken into consideration before progressing to performing the transformations. However, because of the aforementioned reports suggesting that leaf explants are more commonly used and should have similar regeneration rates as petiole explants, and because leaf tissue was available in larger quantities, it was decided to primarily use leaf explants.

Even though petiole explants might have been a better choice than leaf explants, 616 putatively transformed plantlets were regenerated and transplanted to potting mix from the 1579 leaf explants and 113 petiole explants treated, averaging 0.36 putatively transformed plantlets per explant (not counting the explants transformed with CRISPR/Cas9 plasmid #2). Given more time to grow *in vitro*, additional plantlets could have been transplanted. The total number of putatively transformed plantlets regenerated proved that the transformation protocol was working very well, especially considering the amount of explants lost due to contaminations, although some non-transformed escapes seemed to be present among the putative transformants, and the time needed to regenerate flowering plants from explants was much greater than anticipated, which made it impossible to obtain any results from the experiment involving transformation of *B. tuberhybrida* 'Urban Bicolor Pink' with CRISPR/Cas9 within the time frame of this thesis.

Kiyokawa et al. (1996) reported that shoots were formed four to six weeks after co-cultivation, after which they were individually excised and transferred to rooting medium without any phytohormones or antibiotics. However, during this thesis work, it was discovered that individual shoots could not be excised until at least 15 weeks after co-cultivation, usually requiring 17 to 23 weeks before any shoots had elongated to >2 mm. The slow regeneration of plantlets severely hampered this thesis work, as subsequent verification and analysis of transformants could not be performed as scheduled.

The discrepancy in regeneration time needed can probably be mainly attributed to the use of different cultivars, different selective agents, and different culture conditions. Kiyokawa et al. (1996) used *B. tuberhybrida* 'Perfecta', while 'Urban Bicolor Pink' was used in this thesis work. Unfortunately, it has not been possible to find any information about 'Perfecta'. Furthermore, Kiyokawa et al. (1996) used 100 mg/l kanamycin as a selective agent during regeneration of transformants, while 1 mg/l glufosinate was used in this thesis work. It is possible that regeneration of transformants might have been faster if kanamycin was used instead of glufosinate. However, the difference in how kanamycin and glufosinate affects the growth of resistant transformed plants is

probably species and genotype dependent, and we are not aware of any studies performed on the potential difference in regeneration efficiency between kanamycin and glufosinate in any begonia.

The difference in light intensity and temperature can probably explain the largest part of the discrepancy in regeneration time needed. In this thesis work a light intensity of approximately 14 µmol m^{-2} s^{-1}, an 18-hour photoperiod, and a temperature of 20°C was used, as per advice of technicians experienced with micropropagation of *B. tuberhybrida* (A Sivertsen & G Skjeseth pers. comm. 2016). However, Kiyokawa et al. (1996) used ~92 µmol m^{-2} s^{-1} (converted from 20 W/m^2 using a lighting radiation conversion calculator (Environmental Growth Chambers 2017)), a 16-hour photoperiod, and a temperature of 25°C. Other experiments on micropropagation of *B. tuberhybrida* have reported using 30-35 µmol m^{-2} s^{-1} and a temperature of ~25°C (Nada et al. 2011; Nakano et al. 1999). In hindsight, the light intensity should probably have been increased to at least 30 µmol m^{-2} s^{-1}, but it was decided not to change the growth parameters during the experiments. The temperature should possibly also have been increased to 25°C, but it was not possible to regulate the temperature since the plantlets were growing in a shared growth chamber.

Furthermore, it is possible that the use of other types and concentrations of phytohormones in the regeneration media could have resulted in faster regeneration of a larger amount of transformants; Nada et al. (2011) found that the combination of 1 mg/l NAA, a synthetic auxin, and 2 mg/l TDZ, a synthetic cytokinin, resulted in the largest number of excisable shoots, while Nakano et al. (1999) found that the combination of 0.1 mg/l NAA and 0.1 mg/l BAP, a synthetic cytokinin, resulted in the largest number of excisable shoots. For *Begonia* spp. it has been reported that it is possible to obtain a large number of adventitious shoots from a single explant, but most of them are very small and difficult to excise, especially when high concentrations of cytokinin is used in the medium (Peck & Cumming 1984; Simmonds & Werry 1987). This effect was demonstrated by Nakano et al. (1999) whom reported that in a "'rose-formed' strain" of *B. tuberhybrida*, use of ≥1 mg/l BAP and 0.1-1.0 mg/l NAA resulted the largest number of shoots per explant, but they were mostly small and difficult to excise. By lowering the concentration of BAP to 0.1 mg/l, fewer, but more elongated shoots were obtained. However, it was decided to follow the protocol developed by Kiyokawa et al. (1996), which reported regenerating a relatively large amount of *B. tuberhybrida* transformants in a relatively short period of time by using 0.1 mg/l NAA and 0.5 mg/l BAP, as the studies by Nada et al. (2011) and Nakano et al. (1999) reported very different results and did not involve the use of any selective agents or antibiotics.

4.5.2 Contaminations of *in vitro* cultures

Contamination by microorganisms are unfortunately a common issue when working with *in vitro* cultures. Although difficult to constantly achieve, maintaining aseptic conditions is of the utmost importance, as microorganisms will often grow faster than the plants, especially in medium containing sugar.

During this thesis work the main contamination issue was overgrowth by *A. tumefaciens*, although sporadic contamination by fungi and other bacteria also occurred. The first detected overgrowth of *A. tumefaciens* was verified by PCR, and subsequent contaminations of similar appearance were assumed to be *A. tumefaciens*, as *A. tumefaciens* growing on explants and on media has a characteristic cloudy brownish thick liquid appearance.

Although some *A. tumefaciens* overgrowth was expected, it was not expected to be occurring at the observed rate or that it could occur even after six months of growth on medium containing 500 mg/l cefotaxime. The *A. tumefaciens* overgrowth mainly, but not exclusively, seemed to occur on unhealthy explants and seedlings excreting phenolic compounds. Phenols are often produced in plant tissues exposed to stress, such as when non-transformed plants are growing on selective medium, and are toxic to most microorganisms, but *A. tumefaciens* have evolved to use phenols as a host recognition factor. Phenolic compounds such as acetosyringone and hydroxyacetosyringone excreted from plant wounds induce *vir* genes on the Ti plasmid in *A. tumefaciens*, and act as a chemoattractant together with several other phenolic compounds and sugars (Bhattacharya et al. 2010; Bolton et al. 1986). The excreted phenolic compounds, in combination with sugar components released from wound sites and sugar in the medium, might influence the *A. tumefaciens* to grow more rapidly and cause overgrowth of the explants and seedlings. As such, unhealthy explants, or parts of explants, visibly excreting phenolic compounds were removed when discovered. Moribund *A. thaliana* seedlings could unfortunately not be removed because of the small seedling size and large number of seedlings per Petri dish.

When regenerating *B. tuberhybrida* ‘Urban Bicolor Pink’ plantlets putatively transformed with pIA291 or CRISPR/Cas9 plasmid #2, several measures were taken to prevent the overgrowth of explants by *A. tumefaciens*; (1) a high concentration (500 mg/l) of cefotaxime was used, an antibiotic commonly used to prevent *A. tumefaciens* overgrowth. As cefotaxime is not very stable in solution over time (Sigma-Aldrich 2017) the antibiotic solution was prepared fresh every time and the medium was used within 14 days. (2) The explants were visually inspected at least once a week (usually 3-4 times a week) and, any contaminated explants were washed with sterile water containing 500 mg/l cefotaxime or discarded. (3) The explants were subcultured onto fresh medium every 14 days to maintain a high concentration of cefotaxime in the media, avoid build-up of

excreted phenols, and to remove any dead or unhealthy explants and excise dead tissue from living explants.

The main problem with overgrowth of *A. tumefaciens* on 'Urban Bicolor Pink' was that 72 out of 230 adventitious shoots excised and transferred to rooting medium was completely overgrown by what was presumed to be *A. tumefaciens* only six days after being transferred, and several more were contaminated to various degrees. Because of this issue, it was decided to transplant the shoots directly to potting mix, but only 163 out of 616 shoots survived, even though they were covered with a plastic sheet to maintain plant turgor. If the shoots had been allowed to elongate and develop roots in elongation and/or rooting medium before being transplanted to potting mix the survival rate would probably have been much greater. In hindsight, it would probably have been better to make new rooting medium containing antibiotics than to directly transplant the shoots to potting mix. However, because the low survival rate was not evident until several weeks after transplanting, and the amount of 'Urban Bicolor Pink' plantlets surviving was greater than what was feasible to screen by PCR, all subsequent excised shoots were also transplanted directly to potting mix.

Overgrowth by *A. tumefaciens* was also a big problem when screening for transformed *A. thaliana in vitro*. However, the overgrowth could probably mainly be explained by unsuccessful surface sterilization of the seeds. It was expected that the protocol for surface sterilization of the seeds would kill all microorganisms covering the seeds, as it has been used successfully for *in vitro* screening for transformed *A. thaliana* at the Plant Cell Lab for several years (T Melby pers. comm. 2017), and no contaminations were observed when performing the dose-response experiments. It is possible that decreasing the seed/wash solution ratio, adding NaOCl to the wash solution, and removal of sugar from the medium would have reduced the contamination rates. Addition of 500 mg/l cefotaxime to the medium did cause the contamination rate to drop, but did not eliminate the problem. However, it was decided to rather screen for transformed *A. thaliana* in potting mix than improving the protocol for screening of transformants *in vitro*, as screening of transformants in potting mix is considered to be easier (Glazebrook & Weigel 2002).

Using another strain of *A. tumefaciens* might have led to fewer instances of overgrowth, as it has been observed by other researchers that *A. tumefaciens* strain GV3101 (pMP90) (Koncz & Schell 1986), which was used for transformation of both *B. tuberhybrida* 'Urban Bicolor Pink' and *A. thaliana* in this thesis work, cause more overgrowth than other strains (Y Yau pers. comm. 16 April 2017), but no systematic studies have been performed on the subject. Reducing the density of the bacterial solution and the co-cultivation time, and in the case of transformation of 'Urban Bicolor Pink'; washing the explants with distilled water containing antibiotics after transformation, and in the case of transformation of *A. thaliana*; not repeating the transformation procedures, all could

possibly have reduced the rate of overgrowth by *A. tumefaciens*, but it would also probably have had a negative impact on transformation efficiency. Furthermore, using a combination of antibiotics in the regeneration medium, such as cefotaxime and timentin, reducing the number of explants and seeds per Petri dish, reducing the concentration of sugar in the regeneration medium, and reducing the interval between subculturing onto fresh medium, might also have reduced the amount of overgrowth. However, the time and effort spent on preventive measures must always be weighed against the necessity of avoiding the issues. In the case of transformation of 'Urban Bicolor Pink', the bacterial overgrowth first started appearing a month after co-cultivation, and because most explants were unaffected, it was not decided to make changes to the regeneration medium or the interval between subculturing. In the case of transformation of *A. thaliana*, it was decided to rather screen for transformants in potting mix.

4.5.3 Selection of transformed *A. thaliana*

Screening of transformed *A. thaliana in vitro* on medium containing glufosinate was severely hampered by the rate of contaminations, as discussed above, and the vigorous growth observed on medium containing 5 mg/l glufosinate. When performing dose-response experiments on the survival rate of wild type *A. thaliana* seeds it was discovered that all seeds germinated, but six days after sowing it was possible to distinguish the growth of the seedlings on medium containing ≥2.5 mg/l glufosinate from the more vigorous growth observed in medium without any glufosinate. Twelve days after sowing all seedlings had died on medium containing ≥5.0 mg/l glufosinate, while the growth of the surviving seedlings on medium containing 2.5 mg/l glufosinate was very stunted compared to the growth on medium without any glufosinate. Furthermore, no contaminations were observed during the dose-response experiments in any of the 35 Petri dishes (five Petri dishes per treatment) containing the combined total of 175 seeds (25 seeds per treatment).

However, when screening for transformed *A. thaliana* on medium containing 5.0 mg/l glufosinate, it was discovered that all seeds germinated and could not be distinguished from the seeds sown on medium without any glufosinate as controls ten days after sowing. Moreover, as discussed above, contaminations killed all seedlings, except for the wild type seedlings sown as positive and negative controls.

The reason for the more vigorous growth observed when screening transformants compared to when performing the dose-response experiments are unknown. The growth conditions were the same when performing the screening of transformants as when performing the dose-response

experiments. The genetic differences are minimal, as the seeds used in the dose-response experiments were from the same plant and vial as the seeds used for transformation purposes. It is however possible that the cause for the difference in vigour and glufosinate tolerance is that the seeds used for the dose-response and transformation experiments had been stored for approximately one year, while the seeds screened by PCR had only been stored for a few weeks, and/or that the seed-producing plants might have been grown under different conditions.

Screening of transformed *A. thaliana* in potting mix by spraying with 40 mg/l glufosinate was however simple and effective. After spraying the seedlings with a fine mist four times at three-day intervals it was easy to distinguish putative transformants from moribund seedlings, which could then be transferred to individual pots.

4.5.4 Verification of mutations in *F3'H* in transformed *A. thaliana* by enzyme mismatch cleavage assays and Sanger sequencing

All five *A. thaliana* plants chosen for screening by PCR were verified as transformed with CRISPR/Cas9 plasmid #1 or #2. However, introduction of the constructs does not necessarily mean that the desired mutations have been obtained. To achieve the desired mutations both *Cas9* and the sgRNA must be expressed at a sufficient level and the complex must be able to bind to the target sequence (Ma et al. 2016).

Enzyme mismatch cleavage assays are commonly used to detect predicted CRISPR/Cas9-induced mutations (Bortesi & Fischer 2015; Ma et al. 2016). Both the Surveyor and T7E1 mismatch cleavage assays use nucleases that cleave heteroduplex DNA at mismatches and extrahelical loops formed by single or multiple nucleotides resulting from hybridization of wild type and mutant DNA (Vouillot et al. 2015). The main differences are that the Surveyor nuclease is better at detecting single nucleotide changes and is more sensitive to PCR buffers, while T7E1 has a higher signal-to-noise ratio and is less sensitive to PCR buffers (Vouillot et al. 2015).

Unfortunately, it was not possible to get the IDT Surveyor Mutation Detection Kit to work as intended, even after numerous attempts. The surveyor nuclease is very sensitive to PCR buffers, and the IDT Surveyor Mutation Detection Kit has been developed for use with Transgenomic Optimase or Maximase DNA polymerases and the reaction buffers included with those enzymes (Appendix Ie, A Ling pers. comm. 1 March 2017). These reagents were not available, so substitutions had to be made. As it was not possible to get the control reactions to work, the problem is probably not

caused by issues with the DNA, primers, or nuclease; it is probable that the protocol had to be optimised for the substituted PCR buffers, as commercially available PCR buffers vary in content and the contents are often not defined by the suppliers. After contacting technical support at IDT, it was suggested that it would be easier to use the T7E1 mismatch cleavage assay instead, as troubleshooting the Surveyor mismatch cleavage assay was too difficult (A Ling 2017 pers. comm. 1 March).

When performing the T7E1 mismatch cleavage assay, the provided control worked as intended. However, no digested heteroduplexes could be observed when analysing the products from any of the transformed *A. thaliana* plants. Even though the control worked as intended, the chance of the results being false negatives could not be excluded, as T7E1 cannot reliably detect single nucleotide changes (Vouillot et al. 2015), which have been reported to make up about half of all CRISPR/Cas9-induced mutations in plants (Ma et al. 2016).

Finally, amplicons flanking the predicted cut sites for both CRISPR/Cas9 plasmid #1 and #2, from all five verified *A. thaliana* transformants, in case there had been a mix-up of plasmid #1 and #2 or plant labelling at some point, were sent to GATC Biotech for Sanger sequencing. However, no predicted mutations were found. The accuracy of the sequencing data for all predicted cut sites were of Phred20 quality, indicating 99% accuracy (Ewing & Green 1998; Ewing et al. 1998).

4.6 Recommendations for future work

Although no blue *B. tuberhybrida* flowers were obtained during this thesis work, it is probably achievable given enough time and resources.

The most important obstacle to overcome in future work is probably the extremely low pH of *B. tuberhybrida* sepals and petals. Unless there are some mechanism in which anthocyanins are stored in specialized vacuoles with an elevated pH, something that is unlikely, the breeding process should start with breeding for higher vacuolar pH levels in sepals and petals. Targeting some of the many genetic elements known to influence pH levels (Fukada-Tanaka et al. 2000; Mol et al. 1998; Momonoi et al. 2009; Spelt et al. 2002; Verweij et al. 2008), either by introduction of transgenes or by disrupting endogenous genes, could possibly lead to new cultivars with elevated vacuolar pH levels in sepals and petals. However, because very little is known about *B. tuberhybrida* at the molecular level, and it is likely that mechanisms exist which can counter changes induced by a single transporter gene or transcription factor (Davies 2009), it would probably be easier to use

mutation breeding methods or, if varieties with higher pH levels can be found in the gene pool, traditional selective breeding methods to achieve new cultivars with elevated pH levels in the sepals and petals.

When varieties with proper vacuolar pH levels in sepals and petals are engineered or found, it should be possible to engineer new cultivars which accumulate significant amounts of delphinidin-based anthocyanins. Several constructs containing various combinations of *F3'5'H* transgenes and promoters should then be tested on *B. tuberhybrida* to find the combination which results in the highest levels of delphinidin-based anthocyanins. If no combinations result in transgenic plants accumulating significant amounts of delphinidin-based anthocyanins, then analysis should be performed to find out if the issue is that F3'5'H is not efficiently catalysing the hydroxylation of DHK, or if DFR is not efficiently catalysing the conversion of DHM. If the issue is F3'5'H not efficiently catalysing the hydroxylation of DHK, then the probable cause is competition for substrate from F3'H and/or DFR, and *F3'H* should be disrupted and/or *DFR* should be replaced with a *DFR* allele exclusively catalysing the conversion of DHM. If DFR is not efficiently catalysing the conversion of DHM, then *DFR* should be replaced with a *DFR* allele exclusively catalysing the conversion of DHM. Sequencing of *F3'H* and *DFR* in *B. tuberhybrida* would provide valuable information for designing appropriate sgRNAs if CRISPR/Cas9 is to be used for gene disruption, gene deletion, or gene replacement.

If high levels of delphinidin-based anthocyanins is being accumulated in sepals and petals, and the vacuolar pH is sufficient for expression of blue colour, but a phenotype exhibiting blue flowers is still missing, then the probable reason is lack of appropriate co-pigments such as flavones and flavonols (Fukui et al. 2003; Katsumoto et al. 2007), and/or metal ions such as Mg^{2+}, Al^{3+}, and Fe^{3+} (Yoshida et al. 2009), which could also be altered by traditional breeding methods or modern genetic engineering or gene editing.

Further development of the CRISPR/Cas9 technology, especially improvement of site-specific insertion and gene replacement efficiencies, as well as development of more efficient Cas9 orthologs that can recognise other PAM sites, will make breeding of ornamentals exhibiting novel colouration much easier in the future. Currently, site-specific insertion and gene replacement is a major challenge in plants (Ma et al. 2016), but if the efficiencies can be improved it will be possible to insert transgenes into specific transcriptionally active regions, and more easily substitute genes with more suitable alleles.

5 Conclusions

F3’5’H was successfully introduced into the genome of *B. tuberhybrida* ‘Urban Bicolor Pink’, but only a slight change in flower colour towards blue could be measured with a colorimeter. No significant shift in flower colour was observed. It is likely that the very acidic sepals and petals of *B. tuberhybrida* inhibits exhibition of blue colour, and that genotypes with elevated pH levels must be found in the gene pool, or engineered, before significant progress towards blue flowers can be made. However, the refined protocol for *Agrobacterium*-mediated transformation of *B. tuberhybrida* used in this thesis work yielded excellent results; an average of 0.36 putatively transformed plantlets per explant. This result indicates that *B. tuberhybrida* is easily transformed using *Agrobacterium*-mediated transformation, and that it is a promising ornamental species for future genetic engineering or gene editing, as *Agrobacterium*-mediated transformation also is the most commonly used delivery system for CRISPR/Cas9.

Several *B. tuberhybrida* ‘Urban Bicolor Pink’ plantlets putatively transformed with CRISPR/Cas9 were growing *in vitro* at the time of conclusion, but could not be analysed during the time frame of this thesis.

At least five *A. thaliana* plants were successfully transformed with CRISPR/Cas9, but no mutations could be detected near the predicted cut sites, even though the sgRNAs fulfilled all current established requirements for optimal efficiency. This result corresponds to previous studies showing that the mutation efficiency of the CRISPR/Cas9 system is much lower in *A. thaliana* than in other species, especially when constitutive promoters are used to control the expression of *Cas9*.

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Appendix I – Protocols

Appendix Ia – Genomed JETquick Plasmid Miniprep Spin Kit Protocol

Protocol / Plasmid Miniprep

Very important!

- **Before starting the procedure, make sure that buffers GX and G4 are reconstituted, as indicated on the bottle´s label.**
- **Solution G1 contains RNase and should be stored at 4°C.**
- **All centrifugation steps are carried out at ≥ 12,000 x g in a conventional table-top microfuge.**
- **Do not overload the spin column. The use of 1-3 ml *E.coli* cultures is recommended. The maximum of culture used should be 5 ml. With higher culture volumes DNA yield and quality may decrease rapidly.**

1. Harvesting Bacterial Cells ***E. coli*** cells are pelleted by centrifugation. Remove all traces of medium carefully. Make sure that culture medium back-draining from the tube´s wall is removed.

2. Cell Resuspending Add **250 µl of solution G1** to the pellet and resuspend the cells (by vortexing or with a pipette) until the suspension is **homogeneous**.

3. Cell Lysis Add **250 µl of solution G2** and mix gently, but thoroughly, by inverting the tube several times. **Do not vortex!** Incubate at **room temperature** for **5 min**.

4. Neutralization Add **350 µl of solution G3** and mix **gently** but **thoroughly**, by inverting the tube until a **homogeneous** suspension is obtained. **Do not vortex!** Centrifuge the mixture at **room temp.** and at **maximum speed** for **10 min**.

Protocol / Plasmid Miniprep

5. Column Loading Place a **JETQUICK spin column** into a 2 ml receiver tube (provided). Load the supernatant from step 4 into the spin column. Centrifuge at **>12.000 x g** for **1 min**. Discard the flowthrough.

6. (Optional) This additional column wash is recommended, if nuclease-rich bacteria (e.g. *endA*⁺ strains of *E. coli*) are processed:
After having emptied the receiver tube re-insert the micro-spin column into it. Add **500 µl** of **reconstituted buffer GX** into the spin column and centrifuge **at >12.000 x g for 1 min**. Discard flowthrough and place the **JETQUICK** column back into the same receiver tube. Proceed with step 7.

7. Column Washing Empty the receiver tube, and re-insert the spin column into the receiver tube. Add **500 µl of reconstituted buffer G4** and centrifuge at **>12.000 x g** for **1 min**. Discard flowthrough and place the spin column back into the same receiver tube. Centrifuge **again** at **maximum speed** for **1 min**.
Important: *Residual solution G4 will not be completely removed unless the flowthrough is discarded before this additional centrifugation.*

8. Plasmid Elution Place the **JETQUICK spin column** into a new 1.5 ml microfuge tube and add **75 µl** of **sterile water** (or **TE buffer** or **10 mM Tris/HCl, pH 8**) directly onto the center of the silica matrix of the spin column. Centrifuge at **>12.000 x g** for **2 min**.
Important: *Higher DNA concentrations can be obtained if the elution is carried out in only 50 µl elution buffer volume. In this case, preheat your elution buffer to 65-70°C, add the buffer onto the center of the silica matrix of the spin column and let stand for 1 min before centrifugation. Preheated elution buffer is generally recommended when plasmids >5 kb are eluted. DNA eluted in water should be stored at -20°C.*

Appendix Ib – Novagen NovaBlue *E. coli* Transformation Protocol for Experienced Users

Procedure

1. Remove the appropriate number of competent cell tubes from the freezer (include one extra sample for the Test Plasmid positive control, if desired). Immediately place the tubes on ice, so that all but the cap is immersed in ice. Allow the cells to thaw on ice for 2–5 min.
2. Visually examine the cells to see that they have thawed and gently finger-flick the tube 1–2 times to evenly resuspend the cells. The cells are then ready for removal of an aliquot (Standard Kits), or for the addition of the DNA (Singles Kits).
3. | *Standard Kits:* | *Singles Kits:* |
   |---|---|
   | Place the required number of 1.5-ml snap-cap polypropylene tubes on ice to pre-chill. Pipet 20 µl aliquots of cells into the pre-chilled tubes. | Proceed to Step 4 or 5, depending on whether a Test Plasmid sample is included as a positive control. |
4. (Optional) To determine transformation efficiency, add 1 µl (0.2 ng) Test Plasmid to one of the tubes containing cells. Stir gently to mix and return the tube to the ice.
5. Add 1 µl of a ligation reaction or purified plasmid DNA directly to the cells. Stir gently to mix and return the tube to the ice, making sure that the tube is immersed in ice except for the cap. Repeat for additional samples.

Note: *Transformation efficiencies can be increased several fold by diluting the ligation reaction 5-fold with TE or water prior to adding the DNA to the cells, or by extracting the ligation reaction twice with 1:1 TE-buffered phenol:CIAA (24:1 chloroform:isoamyl alcohol), once with CIAA, precipitating in the presence of sodium acetate, and resuspending in TE or water before adding the DNA to the cells.*

6. Incubate the tubes on ice for 5 min.
7. Heat the tubes for exactly 30 s in a 42°C water bath; do not shake.

Note: *This "heat shock" step is most easily accomplished if the tubes are in a rack that leaves the lower halves of the tubes exposed. Hold the rack in the water bath so that the lower halves of the tubes are submerged for 30 s, and then replace the rack on ice.*

8. Place the tubes on ice for 2 min.
9. | *Standard Kits:* | *Singles Kits:* |
   |---|---|
   | Add **80 µl** of room temperature SOC medium to each tube. Keep the tubes on ice until all have received SOC. | Add **250 µl** of room temperature SOC medium to each tube. Keep the tubes on ice until all have received SOC. |

Selection for transformants is accomplished by plating on medium containing antibiotic(s) for the plasmid-encoded drug resistance(s). Additional host-specific antibiotics also may be appropriate to insure maintenance of the host encoded feature(s) (see chart on page 3).

When using NovaBlue: if selecting for ampicillin or chloramphenicol resistance, plate 5–50 µl cells directly on selective media (plus IPTG/X-gal for plasmids which permit blue/white screening). If selecting for kanamycin or streptomycin/spectinomycin resistance, shake at 37°C (250 rpm) for 30 min prior to plating on selective media.
When using strains other than NovaBlue: incubate at 37°C while shaking at 250 rpm for 60 min prior to plating on selective media.

Notes: *The outgrowth incubation is conveniently performed in a shaking incubator using a test tube rack anchored to the shaking platform. Place each transformation tube in an empty 13 mm × 100 mm glass test tube in the rack. The snap-caps on the transformation tubes prevent them from falling to the bottom of the test tubes, and all transformation tubes remain vertical.*

During the outgrowth (or earlier if omitting outgrowth), place the plates at 37°C. If the plates contain a lot of moisture, place them cover-side up and open the cover ~1/3 of the way to allow the plates to dry for 30–45 min. If the plates do not need drying, keep them closed and place them cover-side down in the 37°C incubator for ~20 min prior to plating.

10. Refer to "Plating Technique" in the subsequent section for specific instructions. Spread 5–50 µl of each transformation on LB agar plates containing the appropriate antibiotic(s) for the plasmid and host strain (see page 3). When plating less than 25 µl, first pipet a "pool" of SOC onto the plate and then pipet the cells into the SOC. Please see the next section for additional details on plating technique.

Important: *The appropriate amount of transformation mixture to plate varies with the efficiency of both the ligation and the competent cells. For recombinants in NovaBlue, expect 10^5–10^7 transformants/µg plasmid, depending on the particular insert and the ligation efficiency. Transformations with the pETcoco™ plasmid require a plating volume of 50 µl to obtain sufficient colonies because the pETcoco plasmid is large (12,272 bp).*

***When using the Test Plasmid**, plate no more than 5 µl of the final NovaBlue transformation mix or plate 10 µl of any strain with a 2×10^8 efficiency in a pool of SOC on an LB agar plate containing 50 µg/ml carbenicillin or ampicillin (because the Test Plasmid carries the ampicillin resistance gene, bla).*

***For blue/white screening of recombinants**, also include IPTG and X-gal in the LB agar. These can be pre-spread on the plates and allowed to soak in for about 30 min prior to plating. Use 35 µl of 50 mg/ml X-gal in dimethyl formamide and 20 µl 100 mM IPTG (in water) per 82 mm plate. Alternatively, X-gal and IPTG can be added to the LB agar at a final concentration of 70 µg/ml and 80 µM, respectively, just prior to pouring the plates.*

11. Set the plates on the bench for several min to allow excess liquid to be absorbed, and then invert and incubate overnight at 37°C.

Appendix Ic – Sigma-Aldrich Extract-N-Amp Plant Tissue PCR Kit Protocol

Procedure
All steps are carried out at room temperature unless otherwise noted.

A. DNA extraction

1. Rinse the paper punch and forceps in 70% ethanol prior to use and between the handling of different samples.
2. Punch a 0.5 to 0.7 cm disk of leaf tissue into a 2 ml collection tube or suitable vessel using a standard one-hole paper punch. If frozen plant tissue is used, keep the leaves on ice while punching disks.
3. Add 100 µL of the Extraction Solution to the collection tube. Close the tube and vortex briefly. Make sure the disk is covered by the Extraction Solution.
4. Incubate at 95 °C for 10 minutes. Note that leaf tissues usually do not appear to be degraded after this treatment.
5. Add 100 µL of the Dilution Solution and vortex to mix.
6. Store the diluted leaf disk at 2-8 °C. It is not necessary to remove the leaf disk before storage.

B. PCR amplification

The Extract-N-Amp PCR ReadyMix contains JumpStart Taq antibody for specific hot start amplification. Therefore, PCR reactions can be assembled at room temperature without premature Taq DNA polymerase activity.

Typical final primer concentrations are ~0.4 µM each. The optimal primer concentration and cycling parameters will depend on the system being used.

1. Add the following reagents to a thin-walled PCR microcentrifuge tube or plate:

Reagent	Volume
Water, PCR reagent	x µL
Extract-N-Amp PCR ReadyMix	10 µL:
Forward primer	y µL
Reverse primer	y µL
Leaf disk extract	4 µL*
Total volume	20 µL

*Note: The Extract-N-Amp PCR ReadyMix is formulated to compensate for components in the Extraction and Dilution Solutions. If less than 4 µL of leaf disk extract is added to the PCR reaction volume, use a 50:50 mixture of Extraction:Dilution Solutions to bring the volume of leaf disk extract up to 4 µL.

2. Mix gently and briefly centrifuge to collect all the components at the bottom of the tube.
3. For thermal cyclers without a heated lid, add 20 µL of mineral oil to the top of each tube to prevent evaporation.
4. The amplification parameters should be optimized for individual primers, template, and thermal cycler.

Common cycling parameters:

Step	Temperature	Time	Cycles
Initial Denaturation	94 °C	3 minutes	1
Denaturation	94 °C	0.5-1 minutes	30-35
Annealing	45 to 68 °C	0.5-1 minutes	
Extension	72 °C	1-2 minutes (~ 1 kb/min)	
Final Extension	72 °C	10 minutes	1
Hold	4 °C	Indefinitely	

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Appendix Id – Qiagen MinElute Reaction Cleanup Kit Protocol

1. Add 300 µl Buffer ERC to the enzymatic reaction (sample volume 20–100 µl) and mix. If the enzymatic reaction is in a volume of < 20 µl, adjust the volume to 20 µl. If the enzymatic reaction exceeds 100 µl, split your reaction, add 300 µl Buffer ERC to each aliquot, and use the appropriate number of MinElute columns.
2. Check that the color of the mixture is yellow (similar to Buffer ERC without the enzymatic reaction). If the color of the mixture is orange or violet, add 10 µl 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.
3. Place a MinElute column ● in a provided 2 ml collection tube or ▲ into a vacuum manifold. See the *MinElute Handbook* for details on how to set up a vacuum manifold.
4. Apply sample to the MinElute column and ● centrifuge for 1 min or ▲ apply vacuum to the manifold until all samples have passed through the column. ● Discard flow-through and place the MinElute column back into the same collection tube.
5. Add 750 µl Buffer PE to the MinElute column and ● centrifuge for 1 min or ▲ apply vacuum. ● Discard flow-through and place the MinElute column back into the same collection tube.
6. Centrifuge the column in a 2 ml collection tube (provided) for 1 min. Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
7. Place each MinElute column into a clean 1.5 ml microcentrifuge tube.
8. To elute DNA, add 10 µl Buffer EB (10 mM Tris-Cl, pH 8.5) or water to the center of the MinElute membrane. (Ensure that the elution buffer is dispensed directly onto the membrane for complete elution of bound DNA.) Let the column stand for 1 min, and then centrifuge the column for 1 min.
9. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

Appendix Ie – Integrated DNA Technologies Surveyor Mutation Detection Kit Protocol

Step-by-step instructions—Detecting mutations with Surveyor Nuclease

This section provides detailed instructions for the detection of mutations using the Surveyor Mutation Detection Kit for standard agarose or PAGE.

In general, processing of samples should be carried out from start to finish as described in this User Guide. If processing of a sample is stopped before completion of all steps, the DNA should be stored at –20°C until the next step is carried out. Exposure of any frozen sample to repeated freeze-thaw cycles should be avoided and storage at –20°C of PCR amplified DNA or Surveyor Nuclease digestion products for extended periods (>1 week) should be avoided.

Step 1—PCR amplification of reference and test samples

⚠ **This step is critical to the success of the surveyor nuclease digestion. Do not proceed until:**

- **Your PCR yield is sufficiently high (>25 ng/µL).**
- **Your PCR product has low background (a single species of the correct size).**
- **Your PCR product is free of primer-dimer artifacts.**

Several factors must be considered carefully in preparing PCR amplified DNA to be used as substrate for Surveyor Nuclease analysis. **Primer placement and amplified product quality and yield are crucial to obtaining good results**.

- **Amplicon size**

Fragment size (bp)	Primer placement
<1000	Primers should be placed ≥50 bp outside the region of interest. Place primers so that resulting amplicon cleavage products are ≥70 bp.
1000–4000	Primers should be placed ≥10% of the length of the substrate from the region of interest, ensuring that large digestion products can be separated from undigested substrate and small digestion products produce visible bands.

- **Optimize your PCR conditions carefully.**

  The PCR amplicon should appear as a single sharp peak or band of the expected size when analyzed by microfluidic DNA fragment analyzer or agarose gel electrophoresis. Mispriming during PCR amplification can result in the formation of spurious DNA fragments that produce increased background during Surveyor Nuclease digestion. Use primers that are at least 20 nucleotides long (oligomers 25–35 nucleotides in length are preferred), and have a G-C content of 45–60%. If possible, use a high fidelity DNA polymerase to minimize the introduction of errors that will result in higher background. If after careful design of primers and optimization of PCR conditions non-specific PCR products persist, consider using a hot-start DNA polymerase, a touchdown PCR protocol and/or a second amplification with nested primers.

- **Amount and concentration of DNA used**

  Both the amount and concentration of DNA in a Surveyor Nuclease reaction mixture influence the efficiency and specificity of Surveyor Nuclease digestion. For the amount of enzyme recommended for use in a reaction mixture (0.5–2 µL of Surveyor Nuclease S), 200–400 ng of substrate at 50 ng/µL

is optimal. If DNA product yield is <25 ng/µL, consider a second amplification with nested primers. Alternatively concentrate the DNA by ethanol precipitation and dissolve the DNA pellet in a smaller volume of 1X PCR buffer to increase DNA concentration.

Amplification of homogeneous DNA populations

In order to detect a homogeneous mutation in a test sample the PCR product must be hybridized with a wild-type reference PCR product to generate mismatches for Surveyor Nuclease cleavage. Both test sample and wild-type reference DNA are amplified separately with the same PCR primers. Test sample and wild-type reference PCR products are then mixed in a 1:1 ratio to maximize the formation of heteroduplexes during hybridization.

Amplification of heterogeneous DNA populations

A DNA sample can be heterogeneous either because it is derived from a heterozygous source or because it contains a pool of fragments derived from genetically different homozygous or heterozygous sources. Such heterogeneous samples can be PCR amplified and hybridized without mixing them with a wild-type reference DNA. The proportion of mutant to wild-type DNA in the population should be above 5–10% for analysis by gel electrophoresis. After hybridization, retain some of the hybridized PCR product as an undigested reference (control).

Sample preparation by PCR

This kit is compatible with standard DNA isolation protocols. Before PCR amplification, make sure you have enough high quality DNA starting material to amplify your gene of interest.

For PCR products <2500 bp in length, we recommend using a proofreading enzyme that is compatible with SNP detection and mutation discovery applications (e.g., Optimase Polymerase; Transgenomic Inc.).

For PCR products >2500 bp in length, use a DNA polymerase blend containing Taq DNA polymerase supplemented with a proofreading DNA polymerase (e.g., Maximase Polymerase; Transgenomic Inc.).

Perform the PCR amplification of test sample and wild-type (reference) DNA using a high fidelity, thermostable DNA polymerase according to the manufacturer's instructions, and follow the steps below.

1. Verify quality and quantity of the amplified DNA by gel electrophoresis, or using a microfluidic DNA fragment analyzer. If a single band matching the predicted amplicon size is visible in each sample proceed to the next step. If multiple bands or primer dimers are visible, do not proceed further, but optimize your PCR reaction condition or change PCR primers.
2. Use a mass DNA ladder, such as a 100 bp DNA Ladder (New England BioLabs, Beverly, MA) as a reference to estimate the concentration of the amplified DNA by visual inspection. The DNA concentration is ideally ~50 ng/µL, but should be in the range of 25 to 80 ng/µL.
3. As long as the DNA polymerase buffer is compatible with Surveyor Nuclease, the amplified DNA can be used without further purification. If the buffer conditions are not compatible, or obtained results are not optimal, purify using a PCR cleanup kit, enzymatic cleanup kit, or ethanol precipitation.

Preparing PCR products:

1. Isolate test sample and wild-type (reference) DNA by standard methods. For the G and C controls, amplify separately in 2 tubes.
2. Add the following components in the order listed to each of the two 0.2-mL tubes (keet on ice). One tube will be used for test sample DNA and the other for reference DNA:
   - Sterile, deionized water sufficient to bring the final volume to 50 µL
   - 5 µL 10X Polymerase Buffer
   - Test sample or reference DNA (10 ng plasmid DNA or 100 ng genomic DNA)
   - 4 µL dNTPs (2.5 mM each of dTTP, dATP, dCTP and dGTP; final concentration of each dNTP is 0.2 mM)
   - 15 picomoles sense primer (~120 ng of a 25-mer)
   - 15 picomoles antisense primer
   - 1 µL Polymerase (2.5 units) (See Appendix A for recommendations)
3. Determine the annealing temperature (Ta) by calculating the Tm for each primer using the OligoAnalyzer Tool on the IDT webpage: www.idtdna.com/calc/analyzer. We recommend that you adjust the settings to account for the magnesium and dNTPs present in the PCR buffer (i.e., 3 mM Mg^{++} and 0.8 mM dNTPs).
4. Use the following program for a heated-lid thermal cycler:
   - Initial Denaturation: x1 Cycle 94°C 2 minutes
   - Amplification (x30 cycles):
   - 94°C 30 seconds
   - T_a °C 30 seconds
   - 72 °C 30 seconds/250 bp
   - Final Extension: 1 cycle 72°C 5 minutes
   - ≤4 °C Hold ∞
5. Analyze 2- to 5-µL aliquots of each product by electrophoresis in a 2% (w/v) agarose gel, prepared with high-resolution agarose and cast in 1X TBE [89 mM Tris-Borate (pH 8.3), 1 mM EDTA] + 0.2 µg/mL ethidium bromide. Add 1/6 volume of a 6X loading dye buffer [10 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), 50% (w/v) sucrose, 0.15% (w/v) bromophenol blue] or your loading dye buffer of choice to the aliquot and mix. Run the gel in 1X TBE at 5 V/cm until the bromophenol blue has run 2/3 of the length of the gel. Run several different amounts of a mass DNA ladder, such as a 100-bp DNA Ladder (New England BioLabs, Beverly, MA), as a reference.
6. Visualize the DNA bands using a UV transilluminator at 250 to 300 nm and photograph the gel.
7. Use the ladder to estimate the concentration of the amplified DNA by visual inspection. If a single band is visible in each sample, proceed; if not, consider optimizing the PCR further as already described in *Step 1 – PCR amplification of reference and test samples*. The DNA concentration is ideally ~50 ng/µL, but should be in the range of 25 to 80 ng/µL. The amplified DNA can be used without further purification. Alternatively, the DNA can be purified by ethanol precipitation or spin column.

Step 2—DNA duplex formation

In this step, sample and reference DNA are mixed and hybridized to form heteroduplexes. It is important to include sample alone and/or reference DNA alone in separate tubes to serve as negative controls. The use of a heated-lid thermal cycler is recommended—it is important to carefully follow the protocol shown here.

Note: If your thermal cycler cannot be programmed appropriately for hybridization or if it lacks a heated lid, go to the *Performing heteroduplex formation without a thermal cycler* section below.

Performing heteroduplex formation using a thermal cycler

1. Mix equal amounts of test sample and reference PCR products in a 0.2 mL tube. Place reference DNA alone in a separate 0.2 mL tube. For efficient annealing, the final volume should be at least 10 µL.

   Note the following:

   - The concentration of test sample DNA and wild-type reference DNA should be in the range 25–80 ng/µL (**ideally 50 ng/µL**). About 200–400 ng of hybridized DNA is recommended for treatment with Surveyor Nuclease S, so that **each tube should contain ≥200 ng total DNA**. For some applications, increasing the DNA input to 800ng can help to resolve more difficult bands.
   - Heterogeneous test sample DNA that does not require the addition of wild-type, or reference DNA should still undergo the following hybridization procedure to ensure the formation of cleavable mismatches.

2. Place the tube in a thermal cycler and run the following program:

Temperature	Time	Temperature ramp
95°C	10 min	
95°C to 85°C		(–2.0°C/sec)
85°C	1 min	
85°C to 75°C		(–0.3°C/sec)
75°C	1 min	
75°C to 65°C		(–0.3°C/sec)
65°C	1 min	
65°C to 55°C		(–0.3°C/sec)
55°C	1 min	
55°C to 45°C		(–0.3°C/sec)
45°C	1 min	
45°C to 35°C		(–0.3°C/sec)
35°C	1 min	
35°C to 25°C		(–0.3°C/sec)
25°C	1 min	
4°C	Hold ∞	

The product is now ready to be treated with Surveyor Nuclease for heteroduplex analysis. Continue with ***Step 3—Treatment with Surveyor Nuclease***.

Performing heteroduplex formation without a thermal cycler

To perform heteroduplex formation without a thermal cycler:

1. Mix in equal quantities of the two PCR products to generate the heteroduplex. Set up reference DNA in a separate tube as above.
2. Incubate the mixture at 95°C for 5 min in a 1-liter beaker filled with 800 mL of water and then allow the water to come to <30°C.

   Note:

   Because of evaporation of liquid at the tube bottom and condensation under the tube lid, the volume in a tube should be ≥20 µL so that sufficient volume is present to prevent the concentrations of constituents in the mixture from changing substantially during the hybridization step.
3. Spin the tube contents to the bottom of the tube and mix.

The product is now ready to be treated with Surveyor Nuclease for heteroduplex analysis.

Continue with *Step 3—Treatment with Surveyor Nuclease*.

Step 3—Treatment with Surveyor Nuclease

In this step, the heteroduplex test sample DNA from Step 2 is cleaved by the Surveyor Nuclease, along with necessary reference DNA and controls.

Optimized Mg^{++} concentration is essential to Surveyor Nuclease function. Consult the table in Appendix A for appropriate Surveyor Nuclease reaction conditions specific to the polymerase buffer being used for digestion of the DNA. If the DNA polymerase you are using is not listed in the Appendix A table, consult the instructions in Appendix A to select the appropriate reaction conditions.

Digest the hetero/homoduplex DNA experimental samples and any reference DNA in separate tubes.

Set up Surveyor Nuclease reactions for experimental sample DNA as follows:

1. The volumes of 0.15 M $MgCl_2$ Solution required are calculated based upon the volume (V) of the PCR product used.
2. For each digestion, add the following components in the order shown to a nuclease-free 0.2 mL tube (keep on ice):

Component	Amount
Hybridized DNA*	200–400 ng
0.15 M $MgCl_2$ Solution†	1/10th volume
Surveyor Enhancer S	1 µL
Surveyor Nuclease S	1 µL

* DNA volume should be 8–40 µL

† This additional magnesium is required even if PCR buffer also contains some magnesium

3. Mix by vortexing gently, by agitation or by aspiration/expulsion in a pipette tip using a micro-pipetter.
4. Incubate at 42°C for 60 min.
   - We recommend staring with a 60 min incubation time at 42°C. In some cases, reaction time can be reduced to 20 minutes. Increasing reaction time beyond 60 min will increase DNA degradation due to exonuclease activity of the Surveyor enzyme
5. Add 1/10th volume of Stop Solution and mix. Store the digestion products at –20°C if not analyzed immediately.

Note:

- DNA prepared with some DNA polymerase/buffer formulations requires double the amount of Surveyor Nuclease S (2 µL; see APPENDIX A table). The amount of Enhancer S used should be kept at 1 µL in all cases.
- To reduce the number of manipulations, $MgCl_2$, Surveyor Enhancer S and Nuclease S can be mixed and a single pipetting can be done. This mixture should be used immediately after preparation and should not be stored, since reducing agent in the Enhancer storage buffer will inactivate Surveyor Nuclease over time.

- When a heterogeneous DNA sample is analyzed, a portion of the hybridized heterogeneous DNA is NOT digested with Surveyor Nuclease and is run as a control in *Step 4—Analysis of DNA fragments*.

Continue with *Step 4—Analysis of DNA fragments*

Step 4—Analysis of DNA fragments

Test samples

Run your Surveyor digestion on an agarose or polyacrylamide gel. For gel electrophoresis instructions, see *Appendix B: Agarose gel electrophoresis* or *Appendix C: Polyacrylamide gel electrophoresis*.

Control samples

Surveyor Nuclease digestion of hybridized Control G/C PCR products gives rise **to two cleavage products**, 217 and 416 bp in size, which are clearly distinguishable by agarose gel as shown in **Figure 1**. Analysis of different amounts of substrate digested with the recommended amount of Surveyor Nuclease S provides an opportunity to establish the optimal ratio of enzyme to DNA with DNA amplified with your PCR enzyme. The optimal conditions produce the maximum amount of cleavage products while maintaining low background. Keep in mind that PCR amplification of a plasmid template such as Control G or C will result in higher yields of DNA product than amplification of a comparable amount of genomic DNA template. Using 5 to 10 times more genomic DNA (50–100 ng) will give comparable yields of PCR product. This should be taken into consideration in preparing and digesting DNA amplified from genomic DNA (Figure 1).

Appendix If – Integrated DNA Technologies Alt-R Genome Editing Detection Kit Protocol

B. Amplify genomic DNA and detect mutations.

1. Set up the PCR using template, primers, and components of the Alt-R Genome Editing Detection Kit and KAPA HiFi HotStart PCR Kit as follows:

Component	Amount: sample	Amount: Alt-R™ Control A	Amount: Alt-R™ Control B
Genomic DNA (Step A6)	4 µL (~40 ng)	—	—
Forward primer	300 nM	—	—
Reverse primer	300 nM	—	—
Alt-R™ Control A (template/primer mix)	—	2 µL	—
Alt-R™ Control B (template/primer mix)	—	—	2 µL
KAPA HiFi Fidelity Buffer (5X)*	5 µL (1X)	5 µL (1X)	5 µL (1X)
dNTPs	1.2 mM (0.3 mM each)	1.2 mM (0.3 mM each)	1.2 mM (0.3 mM each)
KAPA HiFi HotStart DNA Polymerase (1 U/µL)*	0.5 U	0.5 U	0.5 U
Total volume	**25 µL**	**25 µL**	**25 µL**

*Note: the reagents in the Alt-R Genome Editing Detection Kit are also compatible with other polymerases and buffers.

2. Run the PCR using the following cycling conditions:

Step	Temperature (°C)	Time (min:sec)	Cycles
Denature	95	5:00	1
Denature	98	0:20	30
Anneal	Variable (primer specific)* 64–67 for Alt-R™ Control A and B	0:15	
Extend	72	0:30	
Extend	72	2:00	1

*Optimal annealing temperatures have been determined using KAPA HiFi HotStart DNA Polymerase. You may need to optimize for other polymerases. Annealing temperature for additional Alt-R controls: Alt-R CRISPR-Cas9 *HPRT* Primers, Human and Mouse = 67°C and Alt-R CRISPR-Cas9 *HPRT* Primers, Rat = 64°C.

C. Form heteroduplexes for T7EI digestion.

1. Combine the following in an appropriate PCR tube:

Component	Amount: sample	Amount: Homoduplex control*	Amount: Heteroduplex control*
PCR (from Step B2)	10 µL experimental target or Alt-R™ HPRT control	10 µL Control A	5 µL Control A 5 µL Control B
T7EI Reaction Buffer (10X)	2 µL	2 µL	2 µL
Nuclease-Free Water	6 µL	6 µL	6 µL
Total volume	18 µL	18 µL	18 µL

* Alt-R Controls A and B are positive controls that each contain template and PCR primers for monitoring the function of the T7EI assay. Full length PCR products from Controls A and B contain the same sequences, except for a 6 bp deletion in Control B.

2. Heat and cool PCR products in a thermal cycler as follows:

Step	Temperature (°C)	Time
Denature	95	10 min
Ramp 1	95–85	Ramp rate –2°C/sec
Ramp 2	85–25	Ramp rate –0.3°C/sec

3. Combine the following in a microcentrifuge tube for the T7EI digestion:

Component	Amount (µL)
PCR heteroduplexes (from Step C2)	18
T7 endonuclease I (1 U/µL)	2
Total volume	20

4. Incubate the T7EI reaction at 37°C for 60 min.

D. Visualize T7EI mismatch detection results.

Visualize the digestion using one of the following methods:

- Use agarose gels.
- Dilute digestion with 150 µL of 0.1X IDTE, and run on a Fragment Analyzer™ system with the Mutation Discovery Kit. See Figure 1 for representative results.

The expected amplicon and digested product sizes for Alt-R Controls A/B and the Alt-R CRISPR-Cas9 HPRT Positive Controls are listed in Figures 1 and 2, respectively.

References

1. Mean RJ, Pierides A, Pollet N. (2004) Modification of the enzyme mismatch cleavage method using T7 endonuclease I and silver staining. Biotechniques, 36(5):758–760.
2. Vouillot L, Thélie A, et al. (2015) Comparison of T7EI and Surveyor mismatch cleavage assays to detect mutations triggered by engineered nucleases. G3: Genes|Genomes|Genetics, 5(3):407–415.

Appendix Ig – PCR Protocol for New England BioLabs' One*Taq* DNA Polymerase

Protocol taken from New England BioLabs' web page (New England BioLabs 2016a).

Reaction setup:

We recommend assembling all reaction components on ice and quickly transferring the reactions to a thermocycler preheated to the denaturation temperature (94°C).

Add to a sterile thin-walled PCR tube:

Component	25 µl reaction	50 µl reaction	Final Concentration
5X One*Taq* Standard Reaction Buffer*	5 µl	10 µl	1X
10 mM dNTPs (#N0447)	0.5 µl	1 µl	200 µM
10 µM Forward Primer	0.5 µl	1 µl	0.2 µM
10 µM Reverse Primer	0.5 µl	1 µl	0.2 µM
One*Taq* DNA Polymerase	0.125 µl	0.25 µl	1.25 units/50 µl PCR**
Template DNA	variable	variable	< 1,000 ng
Nuclease-free water	to 25 µl	to 50 µl	

*One*Taq* GC Reaction Buffer and High GC Enhancer can be used for difficult amplicons

**For amplicons between 3–6 kb, use 2.5–5 units/50 µl rxn

Notes: Gently mix the reaction. Collect all liquid to the bottom of the tube by a quick spin if necessary. Overlay the sample with mineral oil if using a PCR machine without a heated lid.

Transfer PCR tubes to a PCR machine and begin thermocycling.

Appendix Ih – PCR Protocol for New England BioLabs' *Taq* DNA Polymerase with Standard *Taq* Buffer

Protocol taken from New England BioLabs' web page (New England BioLabs 2016b).

Reaction setup:

We recommend assembling all reaction components on ice and quickly transferring the reactions to a thermocycler preheated to the denaturation temperature (95°C).

Component	25 µl reaction	50 µl reaction	Final Concentration
10X Standard *Taq* Reaction Buffer	2.5 µl	5 µl	1X
10 mM dNTPs	0.5 µl	1 µl	200 µM
10 µM Forward Primer	0.5 µl	1 µl	0.2 µM (0.05–1 µM)
10 µM Reverse Primer	0.5 µl	1 µl	0.2 µM (0.05–1 µM)
Template DNA	variable	variable	<1,000 ng
Taq DNA Polymerase	0.125 µl	0.25 µl	1.25 units/50 µl PCR
Nuclease-free water	to 25 µl	to 50 µl	

Notes: Gently mix the reaction. Collect all liquid to the bottom of the tube by a quick spin if necessary. Overlay the sample with mineral oil if using a PCR machine without a heated lid.

Transfer PCR tubes from ice to a PCR machine with the block preheated to 95°C and begin thermocycling.

Appendix Ii – PCR Protocol for New England BioLabs' Q5 High-Fidelity DNA Polymerase

Protocol taken from New England BioLabs' web page (New England BioLabs 2017)

Reaction Setup:
We recommend assembling all reaction components on ice and quickly transferring the reactions to a thermocycler preheated to the denaturation temperature (98°C). All components should be mixed prior to use. Q5 High-Fidelity DNA Polymerase may be diluted in 1X Q5 Reaction Buffer just prior to use in order to reduce pipetting errors.

COMPONENT	25 µl REACTION	50 µl REACTION	FINAL CONCENTRATION
5X Q5 Reaction Buffer	5 µl	10 µl	1X
10 mM dNTPs	0.5 µl	1 µl	200 µM
10 µM Forward Primer	1.25 µl	2.5 µl	0.5 µM
10 µM Reverse Primer	1.25 µl	2.5 µl	0.5 µM
Template DNA	variable	variable	< 1,000 ng
Q5 High-Fidelity DNA Polymerase	0.25 µl	0.5 µl	0.02 U/µl
5X Q5 High GC Enhancer (optional)	(5 µl)	(10 µl)	(1X)
Nuclease-Free Water	to 25 µl	to 50 µl	

Notes: Gently mix the reaction. Collect all liquid to the bottom of the tube by a quick spin if necessary.

Overlay the sample with mineral oil if using a PCR machine without a heated lid.

Transfer PCR tubes to a PCR machine and begin thermocycling.

Appendix II – Growth media and other solutions

Lysogeny Broth (LB) medium

LB medium (Bertani 1951) was used for cultivation of *E. coli* and *A. tumefaciens*.

Ingredients for one litre LB medium:

- Tryptone: 10 g
- NaCl: 10 g
- Yeast extract: 5 g
- Final volume adjusted to 1l with Milli-Q H_2O.

* 15 g/l bacteriological agar was added to make solid LB medium for Petri dishes.

The pH was adjusted to 7.5 and the medium sterilised by autoclaving.

Any antibiotics used were filter sterilised and added to the medium after autoclaving.

Murashige and Skoog (MS) medium

MS medium (Murashige & Skoog 1962) was used for *in vitro* cultivation of *B. tuberhybrida* 'Urban Bicolor Pink' plants, plantlets, and explants, *in vitro* cultivation of *A. thaliana* seedlings, washing of *A. tumefaciens* before co-cultivation with 'Urban Bicolor Pink' explants, and in the infiltration medium used for *Agrobacterium*-mediated transformation of *A. thaliana* by "floral drip".

1x jar of pre-mixed MS medium powder including vitamins (4.4 g, M0222.0001) from Duchefa Biochemie was mixed with 1l Milli-Q H_2O.

* 0.35 g/l Gelrite was added to make semi-solid MS medium for Petri dishes.

** ½ MS indicates that half a jar (2.2 g) of MS medium powder was used per litre.

*** The number after MS indicates g/l sucrose added (example: MS3 means that 3g/l of sucrose was added to the medium).

The pH was adjusted to 5.8 and the medium sterilised by autoclaving.

Antibiotics, phytohormones, and glufosinate was filter sterilised and added to the medium after autoclaving.

Potting mix

Potting mix was used to grow *A. thaliana* and putatively transformed *B. tuberhybrida* 'Urban Bicolor Pink' to flower.

The potting mix consisted of approximately 80% (v/v) peat (*Sphagnum* "Go' torv" from Degernes Veksttorv) and 20% (v/v) perlite.

TAE buffer

1x TAE buffer was used to make agarose gels for gel electrophoresis.

Ingredients for 1 litre 50x stock:

- Tris-base: 242 g
- Acetate (100% acetic acid): 57.1 ml
- EDTA: 100 ml 0.5M sodium actetate
- Final volume adjusted to 1l with Milli-Q H_2O.

To make 1x TAE from 50x TAE stock, 20 ml was diluted in 980 ml Milli-Q H_2O.

Agarose gels for electrophoresis

To make agarose gels for electrophoresis 1x TAE buffer and electrophoresis grade agarose (1-1.5% w/v)) was mixed and boiled until the agarose had dissolved. 1 µl GelRed was added per 50 ml 1x TAE buffer used when the solution had cooled some, the solution mixed, and finally poured in a casting tray.

Appendix III – Statistical analyses

Statistical analyses of the *B. tuberhybrida* 'Urban Bicolor Pink' dose-response to glufosinate

1. ANOVA table; testing if the more complex model with interaction effect (Model 1) is significantly different from the simpler model without interaction effect (Model 2).

```
Analysis of Deviance Table

Model 1: Status ~ Tissue * Dose
Model 2: Status ~ Tissue + Dose
  Resid. Df Resid. Dev Df Deviance Pr(>Chi)
1       288     205.79
2       293     208.17 -5   -2.379   0.7946
```

2. ANOVA table; testing the effect of explant type (Tissue) and concentration of glufosinate (Dose).

```
Analysis of Deviance Table (Type II tests)

Response: Status
       LR Chisq Df Pr(>Chisq)
Tissue    9.853  1   0.001696 **
Dose    143.506  5  < 2.2e-16 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Statistical analyses of the *A. thaliana* dose-response to glufosinate

1. ANOVA table; testing the effect of concentration of glufosinate (Dose).

```
Analysis of Deviance Table (Type II tests)

Response: Status
     LR Chisq Df Pr(>Chisq)
Dose    174.5  6  < 2.2e-16 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

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