Norwegian University of Life Sciences
Faculty of Veterinary medicine and Biosciences
Department of Chemistry, Biotechnology and
Food Science

Master Thesis 2015
60 credits

Genetic Transformation of Poinsettia (*Euphórbia pulchérrima*)

Comparing *Agrobacterium*-mediated transformation and transformation by electrophoresis

Genetisk transformasjon av julestjerne (*Euphórbia pulchérrima*)

Sammenligning av *Agrobacterium*-mediert transformasjon og transformasjon ved elektroforese

Anders K.B. Sagvaag

Acknowledgements

Firstly, I would like to thank my supervisor, Trine Hvoslef-Eide, for introducing me to research in plants and applied biotechnology. The project of creating a purple poinsettia, as well as refining transformation by electrophoresis, have been long time interests of the department, and I feel truly honoured to be a part of this. I would also like to show my gratitude for all the occasions where you shared your knowledge and lent me crucial support, which truly made a world of difference.

I am also grateful for the opportunity I had to go to the John Innes Centre, Norwich, England, where I got a glimpse of the world of research outside NMBU. I would like to thank my host, Ingo Appelhagen, for all his help during my stay.

I would also like to thank my co-supervisor Tone Melby for all her support in the lab. This project would be impossible without your helpful guidance in the laboratory! The same goes for Ida Hagen, who took care of all my plants in the greenhouse. I am sure most would have drowned or dried out without you! I also appreciate the way I was accepted into the group at the Plant Cell Lab, where you all made me feel at home. A special thanks to Micael Wendell for your friendship, and for sharing both joys and frustration during my project!

Special thanks is also in order to my mom and dad for all their support, and especially my sister Tonje, who always knew when a glass of anthocyanin rich wine was needed.

Lastly, I would like to show my deepest gratitude to my beautiful girlfriend, Elena, for all her motivation, help and patience! You pushed me to continue, and made me work harder than I would ever be able to on my own. Thank you for being the best, you mean the world to me!

Anders K.B. Sagvaag

Ås, 13th of August 2015

Abstract

Agrobacterium-mediated transformation and transformation by electrophoresis were used in an attempt to produce poinsettias (*Euphórbia Pulchérrima*) with blue or purple bracts. The two methods were compared in order to determine whether one method is better suited for further transformations of poinsettia. The red poinsettia is one of the most popular Christmas plants in Norway, and creating a purple poinsettia would be of great commercial interest, as it could extend sales to the Advent season.

To achieve the desired colour change, the gene coding for flavonoid 3'5'hydroxylase (F3'5'H) derived from petunia (*Petunia x hybrida*) was introduced. This would modify the anthocyanin pathway, potentially causing an accumulation of delphinidin, a plant pigment responsible for blueish colour in several ornamentals.

The *Agrobacterium*-mediated transformations was tried on roughly 1500 explants. The explants were used to produce tissue cultures following the transformation, with new plants regenerated through somatic embryogenesis. Transformation by electrophoresis was used in an attempt to transform 42 shoots from 13 different plants *in vivo*. New shoots were derived from the putatively transformed ones, and grown in the greenhouse until bract colour developed.

The *Agrobacterium*-mediated transformation resulted in only one completely regenerated plant within the time available for this project. Screening by PCR gave a negative result. However, several somatic embryos and shoots were still in development at the time of conclusion, and may be positive if allowed to regenerate into new plants. Transformations by electrophoresis did not result in any observed visual difference in the putatively transformed plants compared to the control plants, indicating that the transformations were so far unsuccessful.

At the time of conclusion, neither method had produced poinsettias with blue or purple coloured bracts. Based on the observations made in this project, as well as previous experiments, *Agrobacterium*-mediated transformation seems to be the safer choice when transforming poinsettia. However, transformation by electrophoresis may be an equally efficient method for transforming poinsettia if developed further.

Sammendrag

Agrobacterium-mediert tranformasjon og transformasjon ved elektroforese ble brukt i et forsøk på å produsere julestjerner (*Euphórbia Pulchérrima*) med lilla eller blå høyblader. De to metodene ble sammenlignet, i et forsøk på å avgjøre hvorvidt en av metode er bedre egnet for videre transformasjon av julestjerne. Den røde julestjernen er en av de mest populære juleplantene i Norge, og produksjon av lilla julestjerner ville være av stor kommersiell interesse, da dette vil kunne utvide salget av julestjerne til adventstiden.

For å oppnå fargeendring, ble genet som koder for flavonoid 3'5'-hydroxylase (F3'5'H) i petunia (*Petuna x hybrida*) introdusert til julestjerne av sorten 'Early Prestige'. Dette kan føre til en endring i antocyaninsynteseveien, noe som gir mer delphinidin, et pigment som fører til blålig farge i mange blomster.

Agrobacterium-mediert transformasjon ble forsøkt på omtrent 1500 stilkskiver. Disse ble behandlet i vevskultur, og nye planter ble dyrket frem via somatisk embryogenese. Transformasjon ved elektroforese ble benyttet i et forsøk på å transformere 42 sideskudd fra totalt 13 forskjellige planter *in vivo.* Nye skudd fra disse antatt transformerte skuddene ble dyrket i veksthus, frem til høybladene utviklet farge.

Agrobacterium-mediert transformasjon resulterte kun i en regenerert plante i løpet av dette prosjektet. Screening ved PCR gav et negativt resultat. Likevel er det flere somatiske embryo og planteskudd som fremdeles utvikler seg, og det er muligheter for at disse vil vise seg å være positive transformasjoner, gitt at de regenereres til nye planter. Transformasjon ved elektroforese førte ikke til noen synlig fargeendring i de antatt transformerte plantene, sammenlignet med kontrollplanter. Dette indikerer at transformasjonene foreløpig ikke er vellykkede.

Ved oppgavens avslutning, hadde ingen av metodene ført til julestjerner med blå eller lilla høyblader. Basert på observasjoner gjort i både dette og andre eksperimenter, ser *Agrobacterium*-mediert transformasjon ut til å være det tryggeste valget for å transformere julestjerne. Transformasjon ved elektroforese har likevel stort potensiale, og kan vise seg å være en like effektiv metode for å transformere julestjerner dersom den blir utviklet videre.

Index

1. Introduction

Euphórbia pulchérrima, commonly called poinsettia, is one of the most popular potted plants in many parts of the world, particularly in Norway, where almost five million plants are sold annually (Ladstein pers. comm.). However, due to the red colour of the bracts, and the short day requirements for flowering (Kristoffersen 1968), the poinsettia has become a symbol of Christmas in the Northern Hemisphere, limiting the demand to the Christmas holidays.

One way to increase the demand for Poinsettias could be to increase the colour range of the bracts, as colour is one of the most important traits of poinsettia cultivars (Catanzaro & Bhatti 2006). Today's cultivars are red, pink, marble pink/white and white. These colour variants are chimera plants with the L1-layer colourless (pink), L1 and L2 colourless (marbled) or all three cell layers colourless (white) (Preil, W 1994). A light pink variety of poinsettia, 'Princettia', is already promoted as an autumn plant (Ladstein, pers. comm.). As poinsettia is a traditional Christmas plant, extending the holiday demand might be a more natural approach. As purple is the colour of Advent, this may be achieved by creating poinsettias with purple bracts.

While the purple poinsettia has a lot of potential, the problem is that the colour in poinsettia comes from the plant pigment cyanidin-3-glucoside (in short: cyanidin), a type of anthocyanin (Tanaka et al. 2008). Cyanidin produces a red colour, and different amounts of cyanidin will determine the shade of red. If the amount of cyanidin is reduced, one might produce a pink plant, as is the case with 'Princettia'. However, to produce a blue or purple colour, a different anthocyanin, delphinidin-3-glucoside (in short: delphinidin), is possibly required. This pigment has been reported in poinsettia, but in low quantities (Slatnar et al. 2013). There have been attempts of artificially colouring the bracts to create purple, but the colour turned out to be a dirty brownish purple, which was not very attractive (Hvoslef-Eide pers. comm.). A possible solution is to introduce a gene coding for the enzyme Flavonoid 3'5' Hydroxylase (F3'5'H) through genetic transformation. This enzyme causes a shift in the anthocyanin biosynthesis, producing delphinidin instead of cyanidin (Figure 1).

Agrobacterium-mediated transformation is well understood, with protocols described for a number of plants, including poinsettia (Clarke et al. 2008). The major obstacle with this method is the relatively low frequency of positive transformations, or the number of plants successfully transformed of the total start material. This generally low frequency means a lot of plant material goes to waste (Clough & Bent 1998; Gelvin 2003). In poinsettia, there is

another obstacle, namely that the self-branching habit of modern cultivars is due to a pathogenic phytoplasma (Lee et al. 1997). Poinsettias derived from tissue culture through somatic embryogenesis will lose this important self-branching ability (Preil 1991) and the plants have to be re-infected with the phytoplasma (Clarke et al. 2011) to obtain this desired self-branching characteristic.

Agrobacterium-mediated transformation is the most common way of transforming plants, and is based on a natural gene transfer system found in *Agrobacterium tftumefaciens* (Bevan 1984). In nature, *A. tumefaciens* will infect dicotyledonous plants to produce crown-gall disease by transferring genes coding for crown-gall into the plant (Hoekema et al. 1983; Stachel & Nester 1986). This is done by a virulence region (*vir*-region) located in a tumour inducing plasmid (Ti-plasmid) in the *Agrobacterium*. During infections, this region transfers DNA (t-DNA) to the plant, which randomly incorporates into the plants nuclear DNA, causing the formation of tumours (De Groot et al. 1998). This is exploited in genetic transformations, by replacing the genes coding for crown-gall disease with genes coding for the trait(s) of interest, while leaving the *vir*-region intact (Gelvin 2003). The gene(s) of interest can either be inserted directly into the Ti-plasmid or placed in a separate plasmid (binary vector) (Hoekema et al. 1983). As the *Agrobacterium* is responsible for the gene transfer, this is considered an indirect method of plant transformation.

There are also direct methods of transforming plants, meaning that naked DNA is introduced directly into the plant. Of the direct transformation techniques, the particle gun is the most common. However, this method has traditionally had the same problem as *Agrobacterium*-mediated transformation: Low transformation efficiency (Finer & McMullen 1991; Travella et al. 2005), though this has improved a lot in later years (Bhattacharyya et al. 2015). In addition, there is often a problem with high copy numbers (Cheng et al. 2000; Travella et al. 2005), which can cause unintentional gene silencing (Reddy et al. 2003).

Another, less used method for direct transformations is transformation by electrophoresis. The method was originally described as a method of transformation on germinating barley (*Hordeum vulgare*) seeds (Ahokas 1989). It has later been utilised for transformations in several different plants, resulting inter alia in transient gene expression in ornamentals (Burchi et al. 1995) and stable gene expression in orchids (Griesbach & Hammond 1992; Griesbach 1994). In poinsettia, electrophoresis has resulted in a strong transient expression, however a stable expression verified by Southern Blot has yet to be achieved (Clarke et al. 2006; Vik et al. 2000; Vik 2003).

Electrophoresis utilises a low current electricity to create a flow through the plant cell walls and cell membranes, allowing DNA to enter the plant (Dekeyser et al. 1990). The transient transformation rates when using this method has been reported to be as high as 25% to 35% (Vik et al. 2000; Vik 2003), making the success rate far superior to that of both *Agrobacterium*-mediated transformation and the particle gun. However, this method is still to be verified using Southern blotting, and thus more experimental than the other techniques.

This project will utilise both *Agrobacterium*-mediated transformation and transformation by electrophoresis to transform poinsettia. The study questions are:

1. Is it possible to transform poinsettia using *Agrobacterium*-mediated transformation or transformation by electrophoresis to produce plants with blue or purple bracts?
2. Given that transformations are successful in both methods, which method is better suited for further transformations in poinsettia?

2. Methods

2.1 Plant material

The plant material used in transformations was *Euphórbia pulchérrima*, poinsettia 'Early Prestige'. Established mother plants were used to produce cuttings. Both cuttings and mother plants were kept under long day conditions (23°C, 18 h of light). The cuttings were transferred to larger pots after rooting (approximately 3-5 weeks), and kept until side shoots were 1-2 cm.

When cultivating poinsettia, day length is of great concern (Kristoffersen 1968). Under long day conditions, the plants will stay in a vegetative state. This will allow the plants to grow and produce shoots. However, they will stay green. If transferred to short day conditions (23°C, 12 hours of light), they will be initiated to start flowering instead. This will first lead to a colour change in the bracts while the buds are emerging.

2.2 Gene of interest

The gene of interest is a cDNA clone derived from petunia (*Petunia x hybrida*) and codes for the enzyme flavonoid 3'5'-hydroxylase (F3'5'H), and corresponds to the *hf1* loci. This enzyme is responsible for shifting the anthocyanin biosynthesis pathway from dihydroquercetin and dihydrokaempferol towards dihydromyricetin (Holton & Tanaka 1994). This leads to a synthesis of a Delphinidin-3-glucoside instead of the normally produced Cyanidin-3-glucoside (Figure 1). Both delphinidin and cyanidin are anthocyanins responsible for colour in plants (Tanaka et al. 2008). However, cyanidin produces a red to pink colour, while delphinidin produces a blue to purple colour (Tanaka et al. 1998). Insertion of the *F'3'5'H* gene should therefore shift the colour of the bracts towards a blueish colour, as the precursors to "red" are shifted towards "blue".

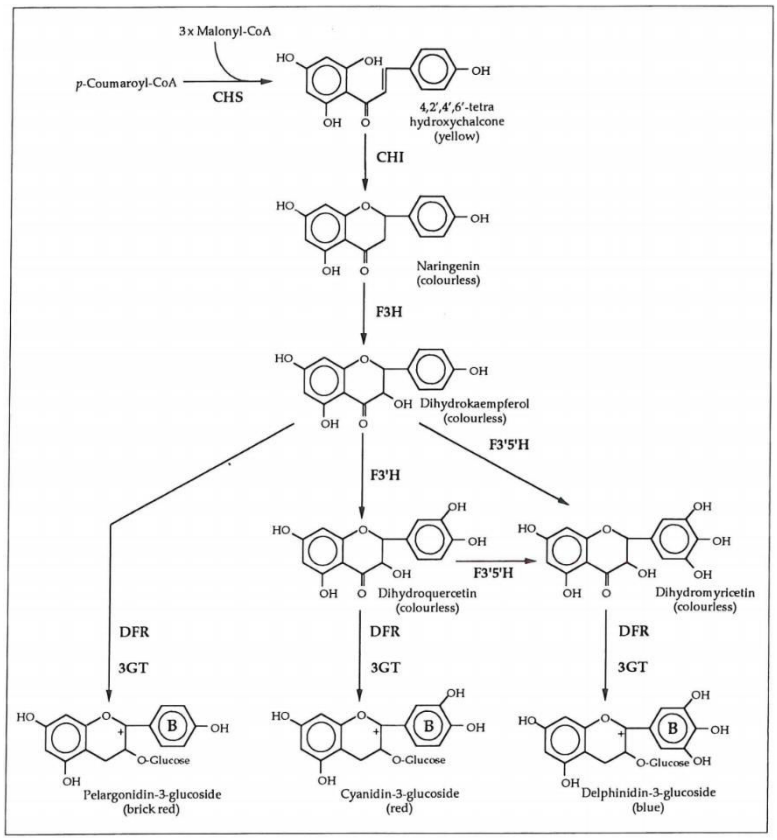


***Figure 1**: The anthocyanin biosynthesis pathway. Enzymes involved in the synthesis of anthocyanin 3-glucosides are: CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3',5'-hydroxylase; DFR, dihydroflavonol 4-reductase; 3GT, UDP-glucose: flavonoid 3-O-glycosyltransferase. Taken from Holton and Tanaka (1994).*

Agrobacterium tumefaciens strain GV3101 and *Escherichia coli* strain DH5α were kindly provided by Ingo Appelhagen in Cathy Martin's lab (John Innes Centre, Norwich, England). Both strains included the plasmid pJAM1983. The pJAM1983 plasmid contains the petunia *F3'5'H* gene and two times cauliflower mosaic virus (CaMV) 35S promotor in a pBIN19 backbone (Figure 2).

The plasmid contains a *NPTII* gene coding for kanamycin resistance (de Vries & Wackernagel 1998; Mazodier et al. 1985), which allows both strains of bacteria to be grown on a lysogeny broth (LB) medium (Bertani 1951)(Appendix II) containing kanamycin.

The additional plasmids will normally be a disadvantage for the bacterium, as it will use additional resources to replicate the plasmid (Patrick 2014). Adding kanamycin resistance to the plasmid, as well as adding kanamycin to the nutrient media, creates a selection pressure, where the kanamycin works as a selection agent. This prevents loss of plasmid mutations

(Valvekens et al. 1988), as the bacterium will die without the kanamycin resistance in a kanamycin-containing medium.

In addition to pJAM1983, the *A.tumefaciens* strain contains a Ti-plasmid (Appelhagen, Pers.Comm). This contains *vir*-genes responsible for transformations, as well as resistance to the antibiotic gentamycin. As such, the LB medium used for *A.tumefaciens* also contained gentamycin.

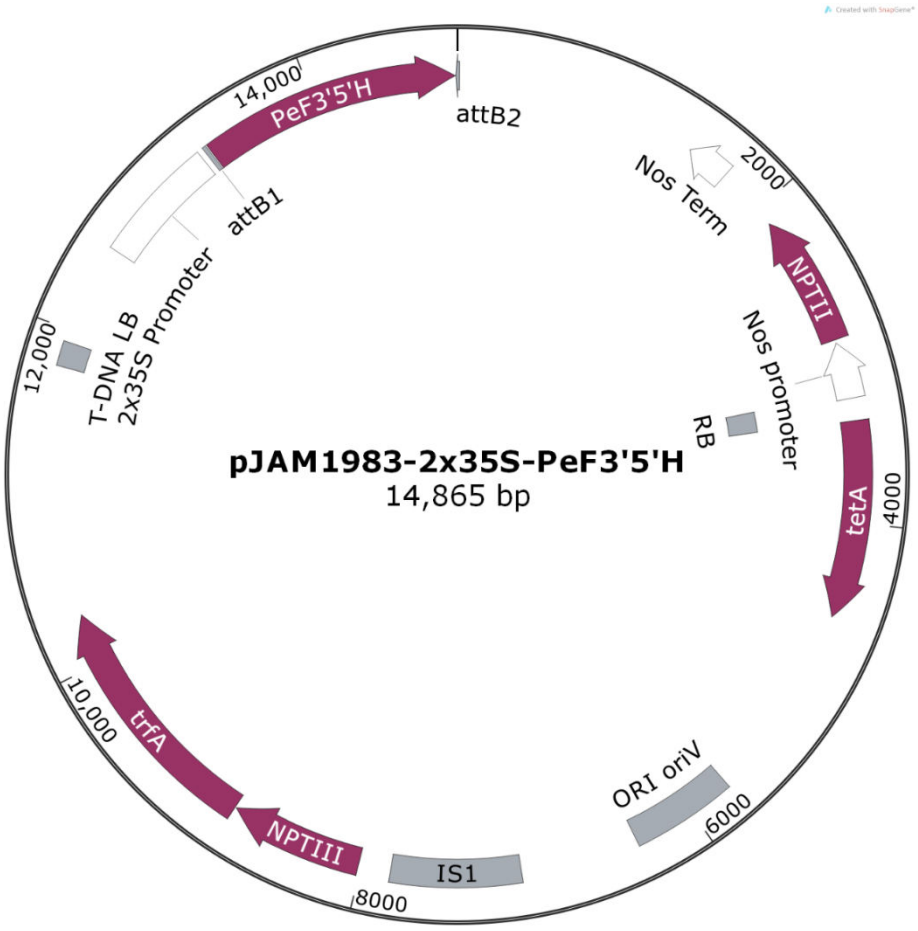


Figure 2: *The plasmid pJAM1983 containing 2x CaMV 35s promotor and gene coding for* F3'5'H *derived from petunia. The plasmid also contains a resistance for kanamycin (NPTII). This plasmid is present in both the* E.coli *strain and the* A.tumefaciens *strain, and is used as a vector for both transformation techniques.*

2.3 Regenerating bacteria from stab cultures

Stab cultures of both bacterial strains were brought from England. These were used to make new liquid cultures, which were transferred to agar plates to produce fresh, single colonies. Fresh colonies were used to produce glycerol stocks for backup, stored at - 80°C.

E.coli strains were grown on agar plates to produce single colonies. Single colonies were used for starter cultures in 5 ml LB medium and incubated at 37°C and 300 rpm for 6-8 hours. The starter cultures were then diluted 1/100 or 1/200 into 100 ml LB medium and grown for 16 – 18 hours. Kanamycin (50µg/ml) was used for selection in every stage.

A.tumefaciens was grown on agar plates to produce single colonies. These were used to produce starter cultures in 5 ml LB medium, and incubated at 28°C and approximately 200 rpm for 6-8 hours. Starter cultures were diluted 1/100 or 1/200 depending on growth. Cultures of 100 ml were then grown for 36-48 hours, until the optical density (OD_{600}) was between 0.6 and 0.9. This OD corresponds to the bacterial logarithmical phase, when the growth rate and availability of "healthy" or good quality bacteria is highest. Kanamycin (25µg/ml) and gentamycin (50µg/ml) was used for selection in every stage.

2.4 Transformation by electrophoresis

2.4.1 Plasmid isolation

Plasmid DNA was isolated from *E.coli* using Qiagen plasmid midi kit, Qiafilter maxi kit and Genomed JetQuick plasmid miniprep spin kit. The plasmid isolation was performed according to the kit handbooks (Appendix Ia-c), where GG buffer (Appendix II) was chosen as the final dilutant. The plasmid yield was then determined by using Nanodrop ND-1000 Spectrometer.

The plasmid DNA precipitated due to poor DNA yield from the plasmid isolation. Sodium acetate was added to the DNA samples to adjust the salt concentration. Isopropanol was then added and mixed, before centrifuging at 15000 x g for 20 minutes at 4°C. A pellet was formed, and the supernatant was decanted. The pellet was then washed in 70% ethanol before a new centrifugation at 15000 for 10 minutes at 4°C. After air-drying for a few minutes, the pellet was re-dissolved in $1/10^{th}$ of the original amount of GG-buffer. The new yield was again determined by Nanodrop ND-1000 Spectrometer.

2.4.2 Casting of pipette tips

DNA from plasmid isolation was used to make pipette tips prior to electrophoresis. Agarose (1%) was added to GG buffer, and used to make a layer in the lower end of the pipette. DNA was then added as a second layer (Table 1), and a larger amount of GG buffer with agarose was added as a third layer. There was some mixing of the layers, but the DNA was located towards the lower half of the pipette.

2.4.3 Electrophoresis transformation

New cuttings were rooted and transferred to larger pots, before being pinched to produce side shoots suitable for electrophoresis transformation. Transformations were carried out when the plant had three to five healthy shoots of approximately 1-2 cm. Poinsettia meristems were exposed under a binocular, removing the small leaves surrounding the meristem carefully with a needle. A pipette tip containing plasmid DNA and a silver thread was placed over the top of the exposed meristem with great care to ensure good contact and no damage to the meristem. The silver thread was connected to the negative electrode of a power supply, while the positive electrode was placed in the soil, close to the plant stem (Figure 3). Both electrodes were connected to a power supply set to 10 minutes, 50V and 1W.

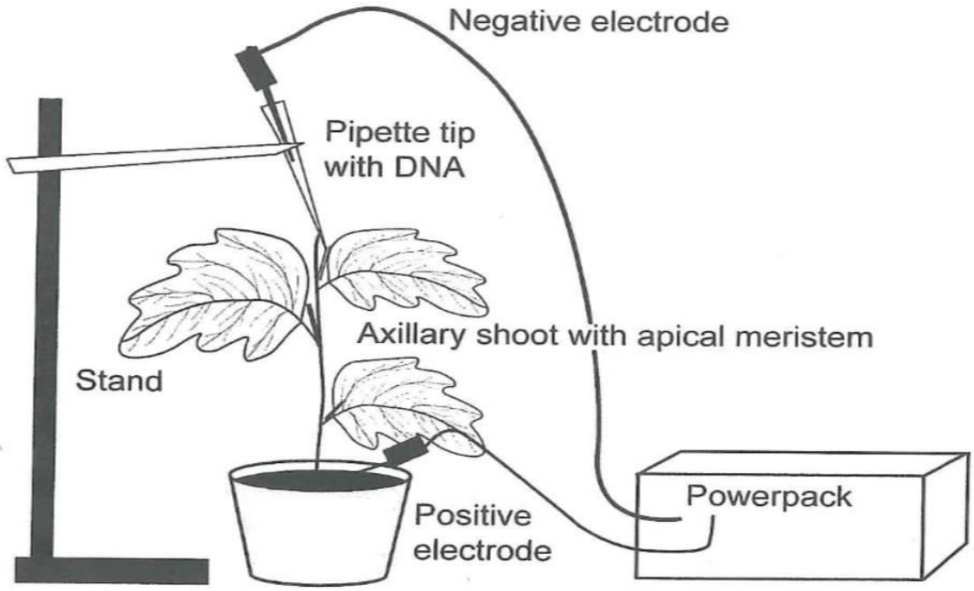


***Figure 3**: Diagram of the electrophoresis transformation system for in vivo DNA transfer. From Vik (2003)*

An electrical current will open the pores in the cell walls and membranes (Dekeyser et al. 1990), allowing the negatively charged DNA to travel towards the positive pole, into the plant cells. If successful, this will facilitate incorporation of the foreign DNA into the nuclear DNA, leading to positive transformation of new shoots.

When using electrophoresis, there is a risk of completely frying the cells of the exposed area of the plants, thereby killing the material. To prevent this, the electrical current is kept within a range of 40 to 70 mA, with a preferred value of 50 mA (Bakke & Gjerde 1998). As it is

impossible to set the power supply to a specific amperage level, the voltage was adjusted instead, and the current is kept for 8 to 10 minutes (Appendix IV). In this project, 42 shoots from 13 different plants were transformed using four different pipette tips (A-D) with DNA (Table 1).

Table 1: *Overview of number of shoots on each plant and pipette tip used for transformation by electrophoresis, as well as the concentration of plasmid DNA in the pipette tips.*

Pipette tip used	Concentration of DNA in DNA layer (ng/µl)	Plant	Shoots per plant
A	221	1	4
		2	3
		3	2
		4	2
B	241	5	2
		6	3
		7	2
C	256	8	2
		9	4
D	245	10	4
		11	5
		12	4
		13	5
Total		13	42

As a control, seven shoots were “transformed” using pipette tips with GG-buffer and agarose, but without DNA. In addition, three other shoots had their meristems carefully exposed, but were not “transformed” as further negative controls.

2.4.4 Greenhouse conditions for putative transformants

Transformed plants were kept under a plastic tent under long day conditions for 2-4 days to prevent the exposed meristems from drying out. When the shoots were strong enough (i.e. had grown to a suitable size), they were used as new cuttings. These were rooted, and some were transferred to short day conditions and kept until the colour of the bracts developed. In an attempt to prevent chimeras, the remaining rooted cuttings were grown until new shoots were large enough to produce new cuttings. The hope was that these new cuttings would consist

exclusively of transformed plant cells. These new cuttings were rooted and transferred to the short day conditions until coloured bracts developed.

Bract colour developed after approximately 6-8 weeks. The colours were planned to be determined by a ColorStriker True Color machine. However, the new device was dead on arrival and was not returned in time from the supplier to be used for this project. As such, the colours had to be visually determined instead.

2.5 *Agrobacterium*-mediated transformation

The *A.tumefaciens* strain GV3101 was used for transformations (Appelhagen, Pers. Comm). This strain contains a binary plasmid containing the T-DNA region, as well as a Ti-plasmid containing the *vir*-region (Koncz & Schell 1986). Transformations were performed on six different occasions, marked experiment A-F (Table 2).

2.5.1 Sterilization of plant material

Fresh shoots were collected and sterilized prior to transformation, to avoid infections. This was done in three steps: First, the shoots were put in 70% ethanol for 1 minute, then 1% Sodium hypochlorite (NaOCl) (with 3 drops of Tween20) for 5 minutes, before finally washing in autoclaved RO-water for 3, 10 and 20 minutes (based on Bakke and Gjerde (1998); Østerud (2013)). There were slight variations to the washing in different experiments (Table 2). Cutting the segments in half was an attempt to obtain cultures free from endogenous microorganisms

Following the sterilization, the meristem was excised and each segment was cut into small discs of around 1.5- 2 mm width.

Table 2: *Sterilization techniques and number of shoot discs used in each of the experiments for Agrobacterium-mediated transformation. One wash consists of five steps: 1. 70% ethanol for 1 minute 2. 1% NaOCL (+ 3 drops of Tween20) 3.-5. Autoclaved RO-water for 3, 10 or 20 minutes respectively. Discs used as a control are not included in the table.*

Experiment	Method of disinfection	Transformed discs
A	Single wash	113
B	Single wash	210
C	Single wash	192
D	Segments cut in half, then single wash	480
E	Single wash, then cut in half and another wash	380
F	Double wash	192
Total		1567

2.5.2 Agrobacterium-*mediated transformation*

The *A.tumefaciens* grown to the correct OD was transferred to 50 ml centrifuge tubes. These were centrifuged at 18°C and 2700 RPM for 10 minutes. The supernatant was disposed of, and the pellet re-suspended in 20 ml MS-II. This step was followed by another centrifugation at 18°C and 2850 RPM for 5 minutes. The supernatant was once again disposed of, and the pellet re-suspended in 8 ml MS-II.

The bacteria suspensions (16ml) were transferred to 5 cm petri dishes. These were then filled with the sterilized shoot discs, and placed on a shaker for five minutes. After five minutes of infections, the segments were dried on sterile filter paper, placed on petri dishes containing callus induction (CI) medium (Appendix II) and incubated in a dark growth chamber at 23°C for three days for co-cultivation. This medium did not contain any selection, and allowed *A.tumefaciens* to continue its infections.

As a control, approximately 20-40 stem discs were collected from each experiment following sterilisation (not included in Table 2). These were not transformed by *A.tumefaciens*, but rather put in petri dishes with MS-II medium for five minutes. The nutrient media used for controls did not contain selection, but the controls were otherwise treated the same way as the transformed shoots discs.

2.5.3 Callus formation, maintenance and somatic embryogenesis

After three days of incubation, the putatively transformed poinsettia discs were transferred to new petri dishes containing CI medium with selection to prevent growth of negatively

transformed plants (kanamycin 50 mg/ml) and overgrowth of *A.tumefaciens* (Cefotaxim 500 mg/l). The dishes were placed in a growth chamber with 25°C and a light intensity of 27 $mMs^{-1}m^{-2}$. The discs were to be transferred to fresh agar plates every three weeks. However, because of massive amounts of infection, the discs were inspected as often as every day and infections were removed upon sight. This was a necessity, as an infection would cover most of a plate within a week. The infections were mainly caused by fungi, but bacterial infections also occurred.

The shoot discs were transferred to somatic embryo induction (SEI) medium (Appendix II) after callus appeared. The growth room conditions were the same as earlier, and the discs were moved to fresh plates every three weeks.

Cultures were moved to somatic embryo maturation (SEM) medium (Appendix II) when early stage somatic embryos were visible, and kept there until small leaves were clearly visible. Shoots were then moved to a rooting induction (RI) medium (Appendix II).

2.6 Verification of stable genetic variants

2.6.1 Primers

Primers were designed using Primer3 software (http://www.bioinformatics.nl/cgi-bin/primer3/primer3_www.cgi). Different primers were designed to verify insertion of plasmid DNA (Table 3).

Table 3: *Primers used for PCR to verify stable transformants. Two of the primers cover the transition from promotor to gene, while the other two promotors only cover the gene area. These primer sets were used to verify putatively transformed plants from both* Agrobacterium*-mediated transformation and transformation by electrophoresis.*

Primer number	Primer region	Left primer	Right primer	Fragment length (BP)
1	35'S + Ph*F3'5'H*	cgcacaatcccactatcctt	ctagctcattggcacgaaca	599
2	35S + Ph*F3'5'H*	ttcgcaagacccttcctcta	aggcttttccccctagcata	545
3	Ph*F3'5'H*	caaatgttcgtgccaatgag	tcaaaatggcagggttcttc	537
4	Ph*F3'5'H*	acctaatgcaggtgccactc	ctggtttccccttacgttca	503

2.6.2 Verification by PCR

Transgenic lines were verified by PCR. Young, inner leaves from six transformed poinsettia plants showing slight colour differences, one green transformed poinsettia that had not yet developed any colour, and one control plant, were collected.

The leaves were put in 2 ml tubes containing a tungsten bead, and immediately frozen in liquid nitrogen. Leaves were crushed to a fine powder using Retsch MM301 TissueLyser set to 25 hz for 40 seconds. DNA was then isolated using DNEasy plant mini kit from Qiagen (Appendix Id). Final dilutions were either 2x 100 µl (as stated in the kit) or 40µl for a significantly higher DNA concentration in the final samples.

Isolated DNA (1µl) was added to 1X PCR master mix (Appendix II), to a total volume of 10µl. The program used for PCR is in Table 4.

Table 4: *The steps used for the PCR for verification of putatively transformed poinsettia after both Agrobacterium-mediated transformation and transformation by electrophoresis.*

Step	Temperature	Time (min:sec)
Initial denaturation	94°C	10:00
Denaturation	94°C	00:10
Primer annealing	60°C	00:20
Extension	72°C	01:00
Cycle to step 2 for 34 times	-	-
Final extension	72°C	10:00
Cooling	4°C	Forever

PCR products were analysed by gel electrophoresis on a 1% agarose gel containing GelRed (Appendix II). The electrophoresis was run for approximately 40 minutes at 90 volt. The DNA was visualised using ImageLab version 5.0 and BioRad ChemiDoc MP.

2.6.3 pH in poinsettia

The pH in poinsettia was determined in order to decide if this influenced the visual readings of the bracts. First, the white sap from a leaf was tested using litmus paper. Second, bract and stem was crushed to a fine powder (as in 2.6.2), and a few drops of tap water (pH 6.3) was added to make a liquid. The pH was determined using a Thermo Electron Corporation Orion 420A+ pH-meter.

3. Results

3.1 Rate of transformations

Unfortunately, no positive transformations were found during the time scale of this master project. Some plants in the greenhouse seemed to have a slightly darker red colour than the control plants upon visual inspection. However, screening by PCR gave negative results on every sample except the positive control (plasmid DNA). This was true for all primer sets, as shown in Figure 4.

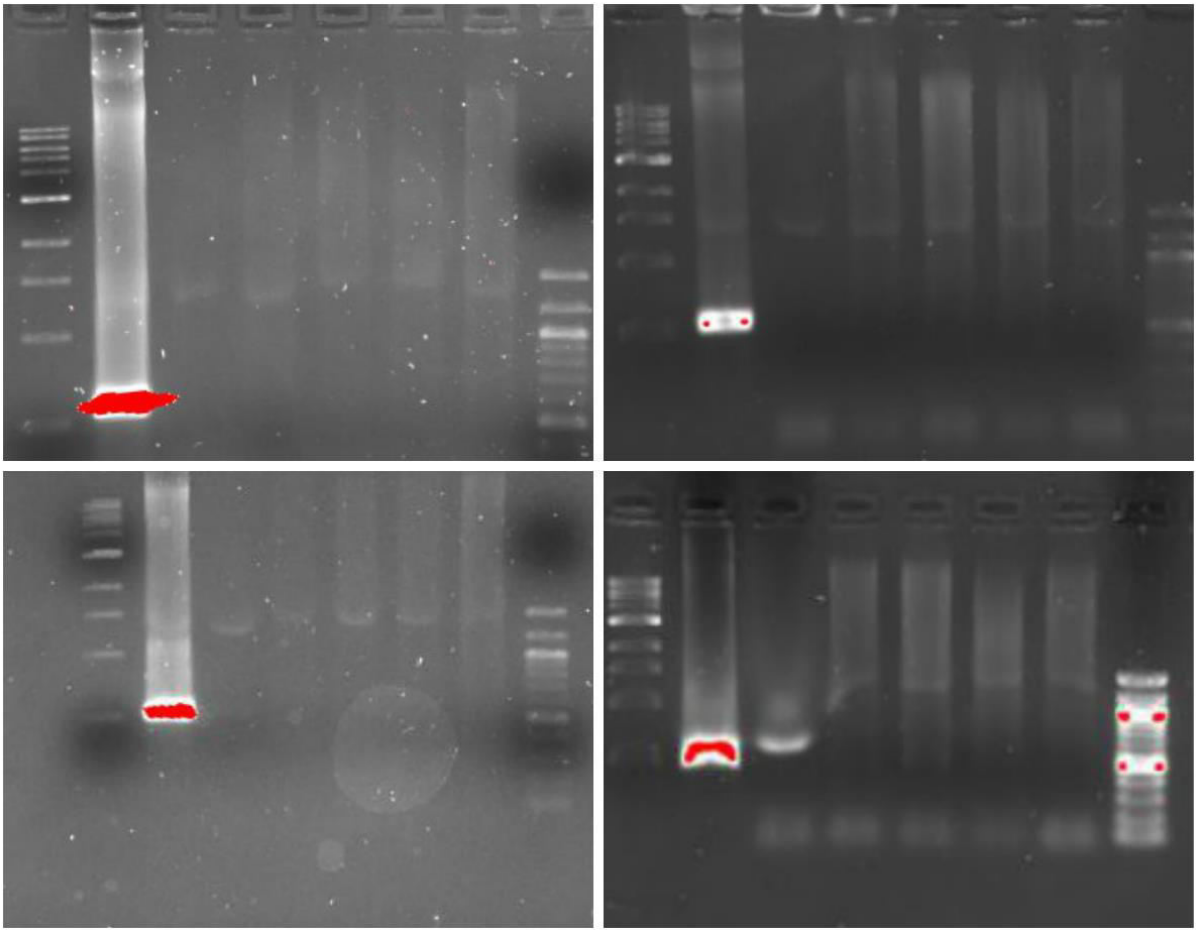


Figure 4: *Results after gel electrophoresis of PCR products from poinsettia. Top left: Primer 1 Top right: Primer 2 Bottom Left. Primer 3 Bottom Right: Primer 4. Samples from left to right: Primer 1+3: 1kb ladder, positive control, negative control, shoot 5.1, 4.3.1, 1.1, Control plant 1 and 100 bp ladder. Primer 2+4: 1kb ladder, positive control, negative control, shoot E1, 10.1, 2.1.1, 4.11 and 100 bp ladder. E1 is a regenerated plant from* Agrobacterium*-mediated transformation, Control 1 is an untransformed plant, while the remaining shoots are from transformation by electrophoresis.*

3.2 Infection rates in tissue cultures

Different sterilization methods were used in each experiment (Table 2) prior to *Agrobacterium*-mediated transformations to determine the optimal conditions for removing the infections, without killing the plant tissue. However, every experiment had several infections, including both fungi and bacteria, on the nutrient media (Figure 5). This resulted in a major loss of potentially transformed plant material, and happened prior to callus formation. The rate of infection, as well as the amount of plant segments developing callus is shown in Table 5. All these infections were found during the establishment of cultures and not after callus had formed, indicating proper sterile working habits and conditions.

Table 5: *Number of shoot discs infected by bacteria or fungi, as well as number of callus derived from shoot discs after* Agrobacterium*-mediated transformations, for all six transformation occasions (experiment A-F). The percentage infected is calculated from total transformed shoot discs in each experiment. The percentage callus is estimated from the surviving (non-infected) shoot discs in each experiment. The sterilization varied between experiments; A-C= single wash, D= Segments cut in half, then single wash, E = Single wash, then cut in half and another wash, F = Double wash*

Experiment	Transformed	Infected	Percentage infected	Callus	Percentage callus
A	113	110	97%	0	0
B	210	130	62%	19	38%
C	192	144	75%	0	0
D	480	256	53%	89	40%
E	380	280	74%	33	33%
F	192	163	85%	0	0
Total	1567	1083	69%	141	29%

The control segments had approximately the same amounts of infection (data not included), but seemed to develop faster than the transformed shoots, and developed into a brownish callus. However, the development seemed to stop after approximately two months.

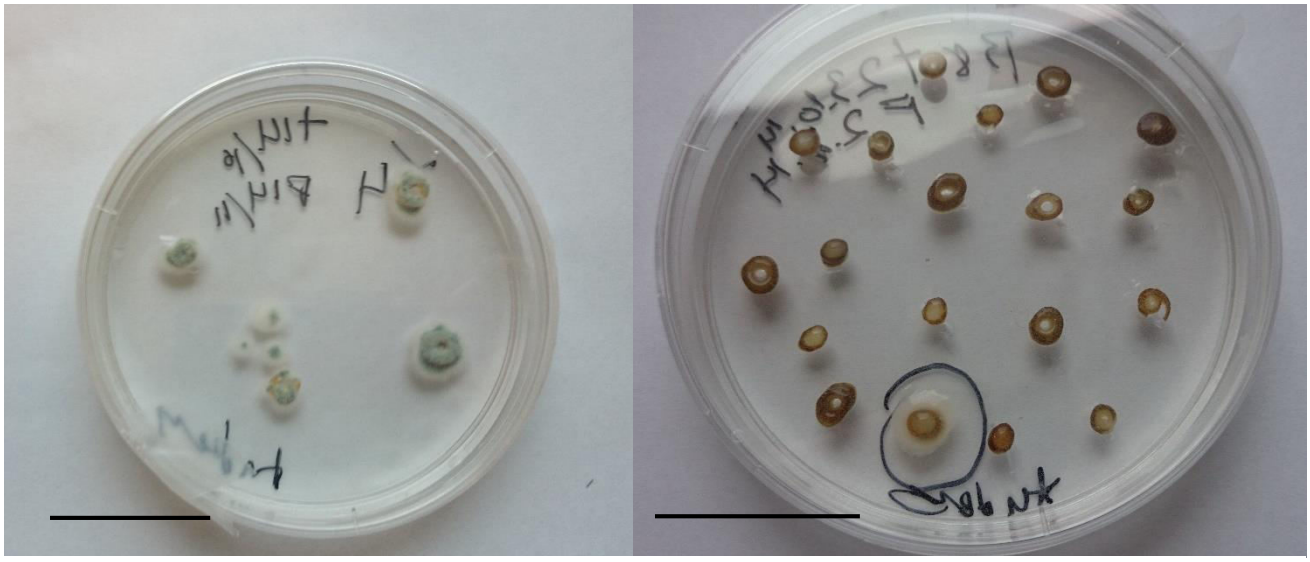


Figure 5: *Infections on stem discs after* Agrobacterium*-mediated transformation. Left: Fungal overgrowth after four days on a fresh nutrient (CI) medium (1 month after transformation) (scale bar 3 cm) Right. A single stem disc (circled) covered by a bacterial overgrowth (9 days after transformation) (scale bar 3 cm).*

3.3 Somatic embryos

Shoots and somatic embryos developed from different plant segments (Figure 6), resulting in different clones (Table 6). Only one plant was completely regenerated during the timeframe of this project. Unfortunately, the PCR results of this plant were negative (Figure 4), indicating an escape.

Table 6: *After Agrobacterium-mediated transformations, only three of the six experiments produced somatic embryos (SE). The table shows the amount of callus producing SE, number of SE observed and the number of SE developing further into plantlets. Results from the different sterilisation methods have been pooled.*

Experiment	Somatic embryos	Callus producing somatic embryos	Plantlets placed on rooting medium
B	7	4	3
D	10	4	6
E	13	5	5
Total	30	13	14

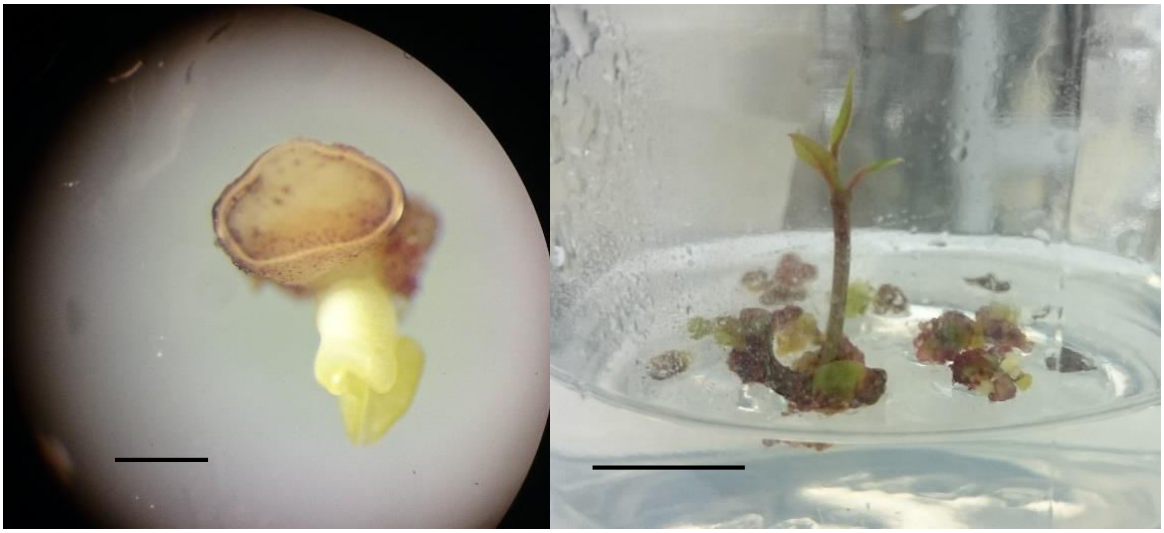


Figure 6: *Left: The first developed shoot after* Agrobacterium-*mediated transformation, as seen through a binocular (Four months after transformation) (bar 0.1mm) Right: A regenerated shoot on rooting medium, almost nine months after* Agrobacterium-*mediated transformation (bar 1 cm)*

3.4 DNA yield from plasmid isolations

The DNA used in the electrophoresis experiments originated from *E.coli* containing the plasmid of interest. Due to low DNA yields, different kits had to be tested to extract DNA, with major differences in yield compared to the stated maximum yield of each kit (Appendix III). Qiafilter maxi kit gave the worst results, with a yield of approximately 2.8% of the theoretical maximum. Qiagen plasmid midi kit and Genomed JetQuick plasmid miniprep spin kit, provided slightly better yields compared to the specified amount, at 3.4% and 3.2% respectively.

3.5 Visual differences after Electrophoresis

Putatively transformed poinsettias started flowering and developed bract colour after 6-8 weeks under short day condition, and can be achieved in roughly three months after transformation (Figure 7). Some of the plants transformed by electrophoresis appeared to have a slightly different colour when compared to control plants. This was mainly apparent on the smallest, still expanding inner bracts, which had a slightly deeper shade of red than the equivalent bracts on the control plants. However, the differences were so small that a visual reading would be subjective and will therefore be considered as a lack of colour change. An objective difference in colour could have been obtained by using a ColorStricker instrument. However, this was not possible as there were no functioning instrument available at the time.

Figure 7: *A selection of poinsettias after transformation by electrophoresis. Both putatively transformed plants and control plants developed a red bract colour.*

3.6 pH in poinsettia

The sap did not succeed in changing the colour of the litmus paper. The liquid mixture of crushed poinsettia leaf and shoot and tap water gave a pH of approximately 5.5-5.6, slightly lower than the tap water (pH 6.3).

4. Discussion

An ideal transformation system needs to be extremely efficient, simple to perform, inexpensive, genotype-independent, and give the required expression of the transgene (Harwood 2012). Considerable progress has been achieved for many transformation systems. However, genotype dependency often slows down progress in recalcitrant species. Being able to avoid cell- and tissue cultures is therefore potentially an advantage in transformation systems. *Agrobacterium*-mediated transformation is a well-established patented method, but requires cell- or tissue culture for obtaining putative transformants. Involving cell- and tissue culture can have its advantages and disadvantages. The main disadvantage is that regeneration protocols are frequently very genotype specific (Harwood 2012; Somers et al. 2003). Secondly, cell-and tissue culture protocols are lengthy processes with many steps, and it frequently takes from 6-12 months to regenerate putative transformants. Often, the easiest genotype to transform and regenerate is one that is of no commercial interest whatsoever. The advantage of using *Agrobacterium* and a cell- and tissue culture protocol is 1) the regenerated plant can be a solid transformant, depending on regeneration method; 2) the selection process *in vitro* is the most efficient selection method there is (Hvoslef-Eide & Vik 2000).

The transformation by electrophoresis, on the other hand, requires no cell-or tissue culture, as the buds can be transformed on the plant. This obviously makes the method genotype independent, a much sought-after feature. However, the large drawback is that the selection process is obscured since the plant produced will have buds with independent transformation events and is a very complicated chimera. These chimera plants needs sorting out, using well-known horticultural methods, unfamiliar to the molecular biologists. Ornamental breeders have been doing this for centuries, carefully taking care of novel colours appearing in side shoots and cultivating the plants to obtain side shoots from that particular section of the plant.

Both methods mentioned above were used in this master project in an attempt to produce poinsettias with purple or blueish bracts by the introduction of the *F3'5'H* gene from petunia. The attempt was to alter the anthocyanin pathway, which may cause an accumulation of the pigment delphinidin while reducing the amount of cyanidin. This would introduce more blue pigments in the bracts.

Agrobacterium-mediated transformations were performed on roughly 1500 explants, divided into six different experiments labelled A-F (Table 2). Transformation by electrophoresis was

performed on a total of 42 meristems from 13 different poinsettia plants of the same cultivar 'Early Prestige' (Appendix IV).

Poinsettia has previously been transformed using both methods, in which *Agrobacterium*-mediated transformations resulted in more compact poinsettias (Islam et al. 2013) and resistance to *Poinsettia mosaic virus* (Clarke et al. 2008), while the transformations by electrophoresis resulted in both GUS (Vik et al. 2000) and GFP expression (Clarke et al. 2008; Hvoslef-Eide et al. unpublished). There has even been an attempt to produce a poinsettia with purple bracts at an earlier occasion, by the insertion of *F3'5'H* from Petunia using electrophoresis (Vik 2003). This is very similar to what has been attempted in this master project, although the plasmid and the subsequent DNA concentration used varied. Contrary to the plasmid used in my project, Vik's plasmid lacked a promotor. This is likely the reason why Vik's attempt did not produce poinsettias with the desired colour, even though some plants showed a slight tone variation in the bract colour when compared to a control plant. A positive transformation was confirmed by Vik (2003) using PCR analysis. These results were of great encouragement during my project, as a transformation with a promotor is likely to give larger differences than observed by Vik (2003).

Actual change of colour due to F'3'5H has been observed in a number of other plants, including roses (*Rosa hybrid*), carnations (*Dianthus caryophyllus*) and chrysanthemum (*Chrysanthemum (Dendranthema)x morifolium*) (Sasaki & Nakayama 2015). All of these plants were transformed by *Agrobacterium*-mediated transformation. The success in previous projects further improved the likelihood of success in this master project, as the manipulation of the anthocyanin pathway actually seem to be effective.

In this project, neither *Agrobacterium*-mediated transformation nor transformation by electrophoresis produced positively transformed poinsettia plants within the timeframe of the project. Possible reasons for this will be discussed in the following sections.

4.1 Cell culture infections during *Agrobacterium* transformations

Both bacterial and fungal infections were observed after *Agrobacterium*-mediated transformation. This dramatically reduced the amount of plant material, which affected the potential for positively transformed plants.

The observed infections in experiment A led to an adjustment of the sterilisation protocol from Bakke and Gjerde (1998) and Østerud (2013). Different sterilization steps were

attempted (Table 2) to overcome the problem of infections. Still, roughly 70% of the transformed plants had to be discarded due to severe infections (Table 5). This infection rate is significantly higher than expected, although a similar number was reported in the first two experiments done by Clarke et al. (2008). Other experiments using poinsettias cell cultures have also reported both bacterial (Bakke & Gjerde 1998) and fungal infections (Østerud 2013).

One possible explanation for the infections is that bacteria and fungi have survived the mild surface sterilisation. The original protocol for sterilising poinsettia segments uses 3% NaOCL for 10 minutes (Preil, Walter 1994), whereas in this project, the plant material was sterilized in 1% NaOCL for 5 minutes, with some modifications between experiments (Table 2). This reduction in time and concentration was done based on the works of Bakke and Gjerde (1998). They experimented with different combinations of concentration and time-intervals, to find the right balance between plant-stress and plant-infections. Since plants are exposed to additional stress during *Agrobacterium*-mediated transformations, stress caused by sterilization should be kept at a minimum, while still preventing infections. As damage to the plant material reduces its ability to regenerate and form cell cultures, this is crucial when performing *Agrobacterium*-mediated transformations. Bakke and Gjerde (1998) found the rate of infection when using 1% NaOCL for 3 minutes to be roughly equal to that obtained when using 3% NaOCL for 10 minutes, indicating that a more gentle sterilization can be equally effective. Islam et al. (2013) used 1% NaOCL for 10 minutes when sterilising poinsettia, and did not report any infections at all. Based on this, the sterilization methods used in this project should have been sufficient to keep the rate of infection to a minimum. However, the condition under which the mother plants are grown and how they are watered have been shown to greatly influence both *in vitro* cultures and daughter plants (Hvoslef-Eide 1991a; Hvoslef-Eide 1991b). This could cause higher infection potential and may explain some of the increased infection rates. While plants are normally watered from above in a greenhouse production, mother plants for *in vitro* cultures should be watered carefully by hand, directly into the pot. There has been a change in personnel in the greenhouses and they have possibly not been told the importance of careful watering to mother plants. Still, it is likely that at least some of the infections were not caused by poor surface sterilization, but rather by internal microbes. These infections were likely those which appeared later in the initiation process.

Internal microbes, or endophytes (Strobel & Daisy 2003), are known to be present in woody plants, possibly due to a mutually beneficial relationship with the host plant (Carroll 1988; Carroll & Carroll 1978). There have even been reports of endophytes promoting adventitious root formation of poinsettia cuttings (Druege et al. 2007). As such, some of the observed infections may be due to endophytes that originally were neutral, beneficial or symbiotic with the poinsettia. When the shoot discs where placed on rich nutrient media, this symbiosis may have become redundant for the endophytes, as the media provided easy access to nutrients. This could cause endophytes to break with the symbiosis and expand faster than normal, leading to infection.

Endophytic fungi seem to be the cause of most of the fungal infections observed. Had the infections originated from external sources, one would expect infections to establish on either the nutrient media or along the stem, and spread to exposed plant cells. This was not the case here, as most fungal infections appeared as single colonies isolated at the cut sites (Figure 8), indicating an internal origin. Of course, this may have been the result of surviving spores, though this is less likely, as this phenomenon was observed on several different shoot discs.

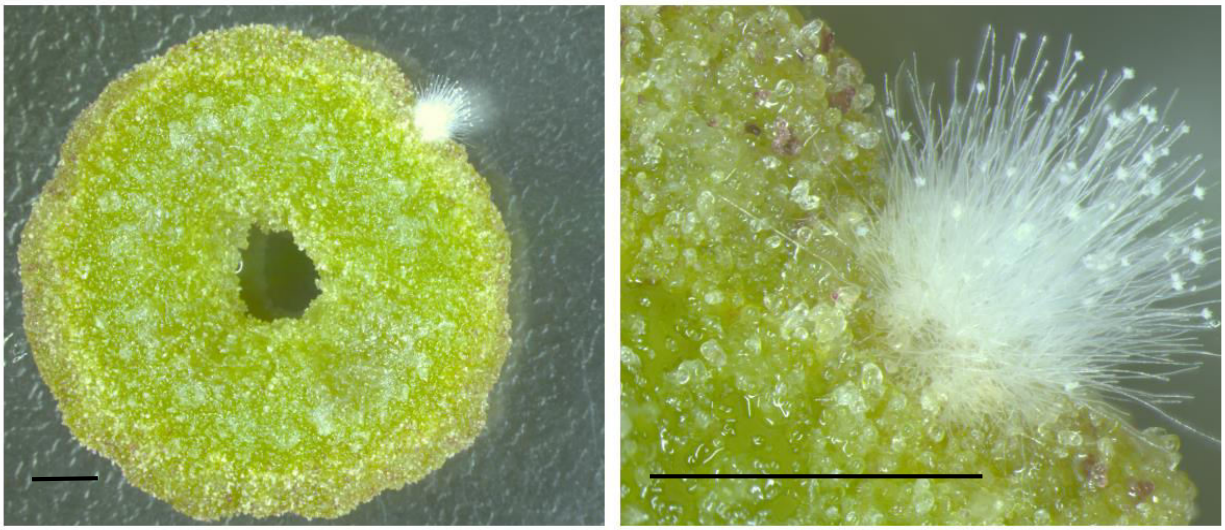


Figure 8: *Fungi infection on shoot disc after* Agrobacterium*-mediated transformation. Left: Infection compared to the disc (*bar *2mm). Right: Close up of infection (*bar 2mm*). Photos by Tone Melby.*

Some of the bacterial infections observed appeared shortly after the shoot discs were moved to CI media with cefotaxim as selection against *A.tumefaciens*. The occurrence of infections on media with selection may indicate that some of the *A.tumefaciens* survived *in refugia* on the plant material, avoiding the selection agent. This seems likely, as most of the bacterial infections on media with selection seemed to originate from parts of the plant material not in

contact with the nutrient media. The media would then provide the plant material with nutrients, indirectly feeding the surviving *A.tumefaciens* and causing an overgrowth. However, the selection used should have been able to kill all *A.tumefaciens* (Okkels & Pedersen 1987), indicating that some of the bacterial infections may have been of endophytic origin. Several endophytic bacterial strains have been isolated from poinsettia (Zheng et al. 2008), some of which may also be present in our 'Early Prestige' mother plants.

None of the infections were analysed any further, mostly because it was beyond the scope of the investigation. However, this could be an interesting project for further studies, as lowering the infection rate will provide a greater amount of usable plant material. Still, the most efficient is way to prevent infections is probably to apply the measures of watering mother plants directly into the pots and not from above. Watering from above may cause a drainage of infections onto the buds used as explants, as suggested by Hvoslef-Eide (1991a; 1991b).

4.2 Transformation by Electrophoresis

When we had to close this project, no blue or purple bracts could yet be observed. The lack of positive results from the electrophoresis transformations may have been caused by a number of factors. One possible explanation is that the amounts of DNA used during the transformations were simply too low. Other electrophoresis experiments have used a DNA concentration of 1 mg/ml in the pipette tips (Bakke & Gjerde 1998) (Vik 2003). This is roughly 4 to 5 times the concentration used in this project (Table 1). Experiments on transformation by electroporation, a similar method to electrophoresis utilizing a much higher voltage, have found that the DNA concentration linearly increases the transformation efficiency (Klöti et al. 1993). Most likely, this relation also exists when using electrophoresis. The low DNA concentrations used in this project was due to extremely low plasmid yields from *E.coli,* probably caused by the plasmids low copy number. When the bacteria strains (*E.coli* and *A.tumefaciens*) were provided from the John Innes Centre, both strains were said to include the plasmid pJAM1983. Due to a misunderstanding, we believed that pJAM1983 was based on a pDONR207 vector. pDONR207 has a high copy number in *E.coli* due to its pUC origin of replication (Invitrogen 2003; Qiagen), and would have been well suited to produce high amounts of the gene of interest prior to electrophoresis. However, multiple DNA extraction kits resulted in extremely low DNA yields. In an effort to explain this, a BLAST (Zhang et al. 2000) comparison of pJAM1983 with several commonly used binary vectors in *Agrobacterium*-mediated transformations (Komori et al. 2007) was performed. The

comparison revealed a 100% match with a 79% cover with pBIN19 (Appendix V), confirming this as the actual backbone for pJAM1983. pBIN19 would be a logical backbone in pJAM1983, as the provided *A.tumefaciens* strain used in this project was previously utilized to transform tobacco (*Nicotiana tabacum*) plants at the John Innes Centre (Appelhagen Pers. Comm.).

The BLAST results imposed that the pDONR207 was actually used as an entry vector to transfer the gene of interest into a pBIN19 backbone, where the 21% not covered by pBIN19 is the insert (2x35S promotor and the *F3'5'H* gene). However, pBIN19 is known to be unstable (McBride & Summerfelt 1990) and has very low plasmid yields in *E.coli* (van Engelen et al. 1995). This is likely the reason for the low yields when compared to theoretical kit values, resulting in a low concentration of plasmid DNA in the pipette tips used for electrophoresis. This discovery was made too late to repeat these experiments in the time available for this master project.

4.3 The source of *F3'5'H*

The use of genetic engineering to obtain a bluish petal colour has been successful in a number of plants, including carnations, roses and chrysanthemums (Sasaki & Nakayama 2015; Tanaka et al. 1998). The blueish colour is obtained by inserting a gene coding for F3'5'H, causing an accumulation of delphinidin when expressed. The source of the *F3'5'H* gene has varied between experiments.

In this project *F3'5'H* derived from petunia was used in transformations of poinsettia. This is the same source as was used to create a mauve coloured carnation 'Moondust'. As mauve is a very light purple, it might be that this gene source is not optimal in creating the desired deep purple colour of Advent. As such, pansy (*Viola wittrockiana*) may have been a better-suited source for transforming poinsettia. *F3'5'H* derived from pansy was used to obtain a darker, more purple coloured carnation, 'Moonshadow', as well as blue varieties of rose and chrysanthemum (summarized in Sasaki and Nakayama (2015)), indicating its ability to create the desired blue/purple colour.

Even though several different sources of the *F3'5'H* gene have proven successful in transforming plants, this has not always been the case. As an example, a transgenic chrysanthemum failed to produce a blue colour, due to a lack of accumulation of delphinidin in the petals (He et al. 2013) A possible explanation for the lack of desired colour change is

said to be due to the unstable and unpredictable expression levels of delphinidin (Noda et al. 2013). As such, low accumulation of delphinidin may cause putatively transformed poinsettias to appear untransformed. In addition, some gene sources appear to be more compatible with certain host plants than others. *F3'5'H* derived from petunia was successful when creating 'Moondust', while it did not provide the desired colour in chrysanthemum (Seo et al. 2007). This indicates that different combinations of gene source and host plant may have an effect on transformation success to obtain certain shades.

4.4 Anthocyanin expression and pH

Although all our poinsettias appear to have red bracts, there is still a possibility that some of the plants are actually transformed. A possible explanation for this is that that the colours produced by anthocyanin is largely connected to pH, as suggested by Willstätter and Everest as early as 1913 (as referenced by Sasaki and Nakayama (2015)). Anthocyanin will appear to be bluer at an alkaline pH, red at an acidic pH, while neutral or weakly acidic solutions will be purple (Asen 1976; Yoshida et al. 2009). Studies on hydrangea (*Hydrangea macrophylla*) has shown that a vascular pH of around 3 will give a red colour, while a pH around 4 will give a blue colour (Yoshida et al. 2003; Yoshida et al. 2009). When creating the blue rose, several hundred rose cultivars were screened, and cultivars with a higher vacuole pH was selected for transformation (Katsumoto et al. 2007). This was done as the pH of the vacuoles proved to be of a great concern for the final colour (Katsumoto et al. 2007; Tanaka et al. 2009). This indicates that the pH could also be of great importance in poinsettia, possibly causing transformed plants to appear red instead of blue. As such, the pH has to be taken into consideration when transforming plants.

When the researchers discovered that the expression of the blue colour in roses were very unstable and was pH-dependant (Yoshida et al. 2009), they needed to add genes for alteration of pH to the constructs to obtain the desired results. We checked the pH of the poinsettia sap to ensure that we did not run into the same problem. The sap of poinsettia turned out to have a pH between 5.5-5.6 and hence no bearing on the expression of delphinidin in poinsettia.

4.5 Comparison of methods

No transformed plants were verified, from neither *Agrobacterium*-mediated transformation nor transformation by electrophoresis, making it difficult to decide on a preferred method

based on transformation efficiency. Still, both methods have major advantages and flaws when used to transform poinsettia.

The main reason to use *Agrobacterium*-mediated transformation is that the method is well established, and has been used successfully in poinsettia. Previously, the method has been used to produce more compact plants (Islam et al. 2013) and to introduce hairpin RNA constructs to poinsettia (Clarke et al. 2008).Still, the method relies on tissue cultures, which requires a regeneration protocol and often is highly genotype dependant (Harwood 2012). In this master project, the cultivar used for transformations differs from the cultivars previously used (Clarke et al. 2008; Vik 2003). As such, part of the reason for not obtaining positive transformants within the timeframe could be due to the cultivar, as cultivars differ in genotype. Tissue cultures also entails a risk of infections and loss of explants. Infections were a major problem in this project. In addition, time-consuming development of tissue cultures and somatic embryogenesis is needed to regenerate transformed plants. The timeframe for an *Agrobacterium*-mediated transformation proved to be much longer than the published time (Clarke et al. 2008). In our hands, the timeframe is more likely to be 18 months, rather than the 6 months indicated. A technician working on Clarkes projects later said that it took closer to two years before positive plants were regenerated (Haugslien Pers. Comm.)

Transformation by electrophoresis is a far less established and more experimental method. One reason for this could be that the method is not patented and hence no commercial company is promoting it the same way as *Agrobacterium* and the particle gun has been promoted (Hvoslef-Eide, Pers. Comm.) There are few protocols available, and experiments will feature more trial and error than using a published protocol for poinsettia transformation with *Agrobacterium*. Still, the method uses naked DNA, with no need for neither sterile conditions upon transformations nor tissue culture for regenerating of plants, which means that the method is completely genotype independent. However, selection for transformed plants is more difficult when using electrophoresis. In *Agrobacterium*-mediated transformation, selection is used to prevent the development of cell cultures of untransformed plants. Since electrophoresis does not rely on cell cultures, there is no easy way to select for transformed shoots. As a result, one will either have to screen every transformed plant or develop a good method for selecting the shoots in the greenhouse. A protocol for selection would have to be developed, and could possibly be achieved by including a herbicide resistance to the t-DNA. This way, several cuttings from an experiment could be sprayed and selected at the same time. The ability to make cuttings and produce new shoots without the

use of tissue cultures is also less time consuming. Poinsettias transformed by electrophoresis can start flowering roughly three months after transformation, while it is estimated that a poinsettia from *Agrobacterium*-mediated transformation will need at least a year due to the tissue culture.

In this project, *Agrobacterium*-mediated transformation had one major advantage over electrophoresis. The plasmid present in both *A.tumefaciens* and *E.coli*, pJAM1983, contains the gene of interest in a pBIN19 backbone (Figure 2). This is not surprising, as pBIN19 is one of the most commonly used binary vectors for *Agrobacterium*-mediated transformations (Bevan 1984; van Engelen et al. 1995) . However, pBIN19 has a very low copy number in *E.coli* (Lee & Gelvin 2008). This leads to few plasmids per bacterium cell, and inefficient isolation of plasmids (Goldsborough et al. 1998). However, this also results in to the electrophoresis being performed with a lot less plasmid DNA than intended. This may account for the negative results using electrophoresis, as previously mentioned.

At the time of concluding this project, neither method seems to provide positive results. However, the *Agrobacterium*-mediated transformation may still prove successful, as only one plant developed fast enough to regenerate within the timeframe of the project. Several somatic embryos and shoots are still developing, and may still provide positive results given enough time.

When comparing the methods, *Agrobacterium*-mediated transformation is the safer choice when transforming poinsettia. Still, electrophoresis shows a lot of potential, and may be a superior option if the method is further developed and optimized.

5. Future work

Though no stable transformants were observed within the timeframe of this master project, several problems were uncovered and overcome, increasing the chances of future success. In the following section possible solutions will be discussed, both in terms of future work on this particular project, and in hopes of providing insight to other similar projects.

5.1 *Agrobacterium*-mediated transformations

As made clear by Table 5, the greatest challenge when working with *Agrobacterium*-mediated transformation and tissue culture, has been to keep infections from spreading. The amount of infections may be lowered by careful watering of mother plants, and possibly by choice of cultivar. A different cultivar may also influence the time needed for regeneration of putatively transformed plants. Future projects should consider this when deciding on the amount and type of start material. The choice of cultivar for this project was chosen on the basis of the most used cultivar at the time, not with regards to regeneration efficiency.

In addition, regeneration of transformed plants has taken longer than expected, and this should be planned for in future projects. We based our decision on published protocols and did not engage in a discussion with the authors until later. In this project, positively transformed plants may still appear, as several somatic embryos and shoots are developing. As such, the current *Agrobacterium*-mediated transformation experiments should be finished by regenerating as many plants as possible.

5.2 Transformation by Electrophoresis

The discussion proved that there might be several reasons why the plants transformed by electrophoresis have not obtained the desired results, with a visual colour difference of the bracts. There is a possibility that some plants are putative transformants without this being visible. As such, a large screening by PCR is necessary to determine whether this is the case.

If the screening does not provide any positive transformants, then the possibilities for further transformation experiments are many:

1. A higher concentration of plasmid DNA in the pipette tips.
   Previous studies ((Bakke & Gjerde 1998; Vik 2003) used a concentration close to five times what is used in this project (Table 1) . Genetic transformations largely rely on chance, and a higher concentration of DNA should increase the chances of a successful transformation.

2. Linearize the plasmid prior to transformation using restriction enzymes.
   Plasmids are circular, while the nuclear genome of a plant appears linear. A linearization of the plasmid prior to transformation may prevent the promotor or gene of interest from being damaged upon transformation and also make it easier for the linearized plasmid to enter the cells.

3. Use restriction enzymes to isolate the gene of interest with promotor, while excluding the rest of the plasmid.
   This would be a time consuming task, however, it might increase the likelihood of a successful transformation, as the amount of DNA expected to be taken up by the plant is much smaller. Transformation rates in bacteria have been shown to be higher when using smaller plasmids compared to larger plasmids (Hanahan 1983). This approach would also remove the selection marker (kanamycin resistance) prior to transformations, which would be a major advantage, as kanamycin cannot be used for selection after electrophoresis transformation in any case. In addition, resistance to antibiotics is a major concern in Norway and in the European Union, and producing a genetically modified plant without antibiotic resistance would have a higher chance of being approved by the Competent Authorities.

4. Use a marker gene, like GUS or GFP, to explore the method has already been done, but the verifications failed at that time too, even if the transformants were clearly visible. Still, transforming poinsettias with a marker gene is most likely easier than altering the colour of the bracts. As such, it could be used again to better understand the techniques, prior to further experiments with gene(s) of interest.

Considering the amount of efforts put into Agrobacterium and the particle gun, more efforts should be put in future experiments to develop better protocols for transformation by electrophoresis, thereby making the method more reliable.

5.3 Plasmid

During the project, it was discovered that the plasmid present in both *E.coli* and *A.tumefaciens*, pJAM1983, was based on a pBIN19 backbone. If we were to start again today, we would change the backbone, and move the insert to a new plasmid.

In *Agrobacterium*-mediated transformations, changing to a more modern plasmid like pGreen (Hellens et al. 2000) would probably increase the transformation efficiency due to its smaller size.

In transformation by electrophoresis, changing the plasmid would most likely have an even greater effect. In the current vector, the copy number in *E.coli* is very low, with only 7-10 copies per bacterial cell. As such, large amounts of *E.coli* is needed to isolate the necessary amounts of DNA. With a high copy plasmid, such as a pUC vector (copy number 500-700 in *E.coli*) (Qiagen), one would only need to grow 2% of the current amount of *E.coli* to obtain the desired DNA level. This could have a major impact, as more time can be used to perform actual transformations. It would also be possible to achieve DNA concentrations of 1mg/ml in the pipette tips used for electrophoresis, as was used in previous experiments. Higher concentrations are shown to be extremely beneficial for transformation efficiency (Klöti et al. 1993).

6. Conclusions

Neither *Agrobacterium*-mediated transformation nor transformation by electrophoresis produced poinsettias with blue/purple coloured bracts within the timeframe of this project. Consequently, it was not possible to decide on a more effective or preferred method, although several advantages and flaws were uncovered. At present time, *Agrobacterium*-mediated transformation, being the best-tested method, may seem to have the largest potential. However, with further trials and development of proper protocols, transformation by electrophoresis has the potential to be a much more efficient method of transforming poinsettias.

Literature

Ahokas, H. (1989). Transfection of germinating barley seed electrophoretically with exogenous DNA. *Theoretical and applied genetics*, 77 (4): 469-472.

Asen, S. (1976). *Known factors responsible for infinite flower color variations*. I International Symposium on Floriculture Plant Breeding and Genetics 63. 217-224 pp.

Bakke, K. & Gjerde, H. (1998). *Transformering av julestjerne (Euphórbia pulchérrima Wild ex Klotzsch)*. Ås, Norway: Agriculture University of Norway, Department of Horticulture and Crop Sciences. 105 pp.

Bertani, G. (1951). STUDIES ON LYSOGENESIS I.: The Mode of Phage Liberation by Lysogenic Escherichia coli1. *Journal of bacteriology*, 62 (3): 293.

Bevan, M. (1984). Binary Agrobacterium vectors for plant transformation. *Nucleic acids research*, 12 (22): 8711-8721.

Bhattacharyya, J., Chakraborty, A., Roy, S., Pradhan, S., Mitra, J., Chakraborty, M., Manna, A., Sikdar, N., Chakraborty, S. & Sen, S. (2015). Genetic transformation of cultivated jute (Corchorus capsularis L.) by particle bombardment using apical meristem tissue and development of stable transgenic plant. *Plant Cell, Tissue and Organ Culture (PCTOC)*, 121 (2): 311-324.

Burchi, G., Griesbach, R., Mercuri, A. & De Benedetti, L. (1995). In vivo electrotransfection: transient GUS expression in ornamentals. *JOURNAL OF GENETICS AND BREEDING*, 49: 163-163.

Carroll, G. (1988). Fungal endophytes in stems and leaves: from latent pathogen to mutualistic symbiont. *Ecology*: 2-9.

Carroll, G. C. & Carroll, F. E. (1978). Studies on the incidence of coniferous needle endophytes in the Pacific Northwest. *Canadian Journal of Botany*, 56 (24): 3034-3043.

Catanzaro, C. & Bhatti, S. (2006). (309) Consumer Survey Reveals Poinsettia Cultivar Preferences for 2005. *HortScience*, 41 (4): 1061-1061.

Cheng, Z., Huang, X. & Ray, W. (2000). Comparison of biolistic and Agrobacterium-mediated transformation methods on transgene copy number and rearrangement frequency in rice. *Acta Botanica Sinica*, 43 (8): 826-833.

Clarke, J., Klemsdal, S., Floistad, E., Hvoslef-Eide, A., Haugslien, S., Moe, R. & Blystad, D. (2006). Genetic engineering of poinsettia with the aim of enhancing its resistance to Poinsettia Mosaic Virus. *Acta Horticulturae*, 722: 321-325.

Clarke, J. L., Spetz, C., Haugslien, S., Xing, S., Dees, M. W., Moe, R. & Blystad, D.-R. (2008). Agrobacterium tumefaciens-mediated transformation of poinsettia, Euphorbia pulcherrima, with virus-derived hairpin RNA constructs confers resistance to Poinsettia mosaic virus. *Plant cell reports*, 27 (6): 1027-1038.

Clarke, J. L., Spetz, C., Haugslien, S., Dees, M. W., Moe, R. & Blystad, D. R. (2011). *PRODUCTION OF TRANSGENIC POINSETTIA WITH RESISTANCE AGAINST POINSETTIA MOSAIC VIRUS (PNMV) USING AGROBACTERIUM-MEDIATED TRANSFORMATION*: International Society for Horticultural Science (ISHS), Leuven, Belgium. 87-93 pp.

Clough, S. J. & Bent, A. F. (1998). Floral dip: a simplified method forAgrobacterium-mediated transformation ofArabidopsis thaliana. *The Plant Journal*, 16 (6): 735-743.

De Groot, M. J., Bundock, P., Hooykaas, P. J. & Beijersbergen, A. (1998). Agrobacterium tumefaciens-mediated transformation of filamentous fungi. *Nature biotechnology*, 16.

de Vries, J. & Wackernagel, W. (1998). Detection of nptII (kanamycin resistance) genes in genomes of transgenic plants by marker-rescue transformation. *Molecular and General Genetics MGG*, 257 (6): 606-613.

Dekeyser, R. A., Claes, B., De Rycke, R. M., Habets, M. E., Van Montagu, M. C. & Caplan, A. B. (1990). Transient gene expression in intact and organized rice tissues. *The plant cell*, 2 (7): 591-602.

Druege, U., Baltruschat, H. & Franken, P. (2007). Piriformospora indica promotes adventitious root formation in cuttings. *Scientia Horticulturae*, 112 (4): 422-426.

Finer, J. & McMullen, M. (1991). Transformation of soybean via particle bombardment of embryogenic suspension culture tissue. *In Vitro Cellular & Developmental Biology - Plant*, 27 (4): 175-182.

Gelvin, S. B. (2003). Agrobacterium-mediated plant transformation: the biology behind the "gene-jockeying" tool. *Microbiology and molecular biology reviews*, 67 (1): 16-37.

Goldsborough, M. D., Evans, K., Xu, L. & Young, A. (1998). High purity plasmid DNA from anion exchange chromatography. *Focus*, 20 (3): 68-69.

Griesbach, R. & Hammond, J. (1992). *Incorporation of the GUS gene into orchids through embryo electrophoresis*. II International Symposium on In Vitro Culture and Horticultural Breeding 336. 165-170 pp.

Griesbach, R. (1994). An improved method for transforming plants through electrophoresis. *Plant Science*, 102 (1): 81-89.

Hanahan, D. (1983). Studies on transformation of Escherichia coli with plasmids. *Journal of molecular biology*, 166 (4): 557-580.

Harwood, W. A. (2012). Advances and remaining challenges in the transformation of barley and wheat. *Journal of experimental botany*, 63 (5): 1791-1798.

He, H., Ke, H., Keting, H., Qiaoyan, X. & Silan, D. (2013). Flower colour modification of chrysanthemum by suppression of F3'H and overexpression of the exogenous Senecio cruentus F3'5'H gene.

Hellens, R., Edwards, E. A., Leyland, N., Bean, S. & Mullineaux, P. (2000). pGreen: a versatile and flexible binary Ti vector for Agrobacterium-mediated plant transformation. *Plant Molecular Biology*, 42 (6): 819-832.

Hoekema, A., Hirsch, P. R., Hooykaas, P. J. J. & Schilperoort, R. A. (1983). A binary plant vector strategy based on separation of vir- and T-region of the Agrobacterium tumefaciens Ti-plasmid. *Nature*, 303 (5913): 179-180.

Holton, T. A. & Tanaka, Y. (1994). Blue roses—a pigment of our imagination? *Trends in biotechnology*, 12 (2): 40-42.

Hvoslef-Eide, A. K. (1991a). The effect of temperature, daylength and irradiance on the growth of mother plants of Nephrolepis exaltata (L.) Schott and on the subsequent growth in vitro of runner tip explants. *Scientia Horticulturae*, 47 (1): 137-147.

Hvoslef-Eide, A. K. (1991b). Mother plant temperature effects on growth of in vitro propagated daughter plants of Nephrolepis exaltata (L.) Schott. *Scientia Horticulturae*, 47 (1): 149-156.

Hvoslef-Eide, T. & Vik, N. (2000). Modern Methods for Breeding Ornamentals. . In Strømme, E. (ed.) *Advances in Floriculture Research*, pp. pp118-131. Ås, Norway: Agricultural University of Norway.

Invitrogen. (2003). *Gateway® pDONR™ Vectors*. Version B ed. Technologies, L. (ed.).

Islam, M. A., Lütken, H., Haugslien, S., Blystad, D.-R., Torre, S., Rolcik, J., Rasmussen, S. K., Olsen, J. E. & Clarke, J. L. (2013). Overexpression of the AtSHI gene in poinsettia, Euphorbia pulcherrima, results in compact plants. *PloS one*, 8 (1): e53377.

Katsumoto, Y., Fukuchi-Mizutani, M., Fukui, Y., Brugliera, F., Holton, T. A., Karan, M., Nakamura, N., Yonekura-Sakakibara, K., Togami, J. & Pigeaire, A. (2007). Engineering of the rose flavonoid biosynthetic pathway successfully generated blue-hued flowers accumulating delphinidin. *Plant and Cell Physiology*, 48 (11): 1589-1600.

Klöti, A., Iglesias, V. A., Wünn, J., Burkhardt, P. K., Datta, S. K. & Potrykus, I. (1993). Gene transfer by electroporation into intact scutellum cells of wheat embryos. *Plant Cell Reports*, 12 (12): 671-675.

Komori, T., Imayama, T., Kato, N., Ishida, Y., Ueki, J. & Komari, T. (2007). Current status of binary vectors and superbinary vectors. *Plant physiology*, 145 (4): 1155-1160.

Koncz, C. & Schell, J. (1986). The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of Agrobacterium binary vector. *Molecular and General Genetics MGG*, 204 (3): 383-396.

Kristoffersen, T. (1968). *Influence of daylength and temperature on growth and development in poinsettia (Euphorbia pulcherrima Willd.)*. Symposium on Flower Regulation in Florist Crops 14. 79-90 pp.

Lee, L.-Y. & Gelvin, S. B. (2008). T-DNA binary vectors and systems. *Plant physiology*, 146 (2): 325-332.

Lee, M., Klopmeyer, M., Bartoszyk, I. M., Gundersen-Rindal, D. E., Chou, T.-S., Thomson, K. L. & Eisenreich, R. (1997). Phytoplasma induced free-branching in commercial poinsettia cultivars. *Nature biotechnology*, 15 (2): 178-182.

Mazodier, P., Cossart, P., Giraud, E. & Gasser, F. (1985). Completion of the nucleotide sequence of the central region of Tn5 confirms the presence of three resistance genes. *Nucleic acids research*, 13 (1): 195-205.

McBride, K. & Summerfelt, K. (1990). Improved binary vectors for Agrobacterium-mediated plant transformation. *Plant Molecular Biology*, 14 (2): 269-276.

Murashige, T. & Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia plantarum*, 15 (3): 473-497.

Noda, N., Aida, R., Kishimoto, S., Ishiguro, K., Fukuchi-Mizutani, M., Tanaka, Y. & Ohmiya, A. (2013). Genetic engineering of novel bluer-colored chrysanthemums produced by accumulation of delphinidin-based anthocyanins. *Plant and Cell Physiology*, 54 (10): 1684-1695.

Okkels, F. & Pedersen, M. (1987). The toxicity to plant tissue and to Agrobacterium tumefaciens of some antibiotics. *Bacterial and Bacteria-like Contaminants of Plant Tissue Cultures 225*: 199-208.

Østerud, C. D. (2013). *Videreutvikling av transformasjonsmetoder i julestjerne for bedre kvalitet og produksjonsvilkår*. Ås, Norway: Norwegian University of Life Sciences, Department of Plant and Environmental Sciences.

Patrick, M. (2014). *Plasmids 101: Antibiotic Resistance Genes*. Available at: http://blog.addgene.org/plasmids-101-everything-you-need-to-know-about-antibiotic-resistance-genes (accessed: 22/07).

Preil, W. (1991). Application of bioreactors in plant propagation. In *Micropropagation*, pp. 425-445: Springer.

Preil, W. (1994). In vitro culture of poinsettia. *The scientific basis of poinsettia production. Agricultural University of Norway*: 49-55.

Preil, W. (1994). The study of chimerism, elimination of virus, and the induction of mutagenesis in poinsettia. *The Scientific basis of poinsettia production*: 57-63.

Qiagen. *Growth Of Bacterial Cultures*: Qiagen. Available at: https://www.qiagen.com/gb/resources/technologies/plasmid-resource-center/growth%20of%20bacterial%20cultures/#Plasmid copy number (accessed: 11.08.2015).

Reddy, M. S., Dinkins, R. D. & Collins, G. B. (2003). Gene silencing in transgenic soybean plants transformed via particle bombardment. *Plant cell reports*, 21 (7): 676-683.

Sasaki, N. & Nakayama, T. (2015). Achievements and Perspectives in Biochemistry Concerning Anthocyanin Modification for Blue Flower Coloration. *Plant and Cell Physiology*, 56 (1): 28-40.

Seo, J., Kim, S., Kim, J., Cha, H. & Liu, J. (2007). Co-expression offlavonoid 3' 5'-hydroxylase andflavonoid 3'-hydroxylase Accelerates Decolorization in Transgenic Chrysanthemum Petals. *Journal of Plant Biology*, 50 (6): 626-631.

Slatnar, A., Mikulic-Petkovsek, M., Veberic, R., Stampar, F. & Schmitzer, V. (2013). Anthocyanin and chlorophyll content during poinsettia bract development. *Scientia Horticulturae*, 150: 142-145.

Somers, D. A., Samac, D. A. & Olhoft, P. M. (2003). Recent advances in legume transformation. *Plant Physiology*, 131 (3): 892-899.

Stachel, S. E. & Nester, E. W. (1986). The genetic and transcriptional organization of the vir region of the A6 Ti plasmid of Agrobacterium tumefaciens. *The EMBO Journal*, 5 (7): 1445-1454.

Strobel, G. & Daisy, B. (2003). Bioprospecting for microbial endophytes and their natural products. *Microbiology and molecular biology reviews*, 67 (4): 491-502.
Tanaka, Y., Tsuda, S. & Kusumi, T. (1998). Metabolic Engineering to Modify Flower Color. *Plant and Cell Physiology*, 39 (11): 1119-1126.
Tanaka, Y., Sasaki, N. & Ohmiya, A. (2008). Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids. *The Plant Journal*, 54 (4): 733-749.
Tanaka, Y., Brugliera, F. & Chandler, S. (2009). Recent progress of flower colour modification by biotechnology. *International journal of molecular sciences*, 10 (12): 5350-5369.
Travella, S., Ross, S., Harden, J., Everett, C., Snape, J. & Harwood, W. (2005). A comparison of transgenic barley lines produced by particle bombardment and Agrobacterium-mediated techniques. *Plant cell reports*, 23 (12): 780-789.
Valvekens, D., Montagu, M. V. & Lijsebettens, M. V. (1988). Agrobacterium tumefaciens-mediated transformation of Arabidopsis thaliana root explants by using kanamycin selection. *Proceedings of the National Academy of Sciences*, 85 (15): 5536-5540.
van Engelen, F. A., Molthoff, J. W., Conner, A. J., Nap, J.-P., Pereira, A. & Stiekema, W. J. (1995). pBINPLUS: an improved plant transformation vector based on pBIN19. *Transgenic research*, 4 (4): 288-290.
Vik, N., Hvoslef-Eide, A., Gjerde, H. & Bakke, K. (2000). *STABLE TRANSFORMATION OF PIONSETTIA VIA ELECTROPHORESIS*. IV International Symposium on In Vitro Culture and Horticultural Breeding 560. 101-103 pp.
Vik, N. I. (2003). *Genetic Transformation of Poinsettia (Euphorbia pulcherrima)*. Ås: Agricultural University of Norway, Department of Horticulture and Crop Sciences.
Willstätter, R. & Everest, A. E. (1913). Untersuchungen über die Anthocyane. I. Über den Farbstoff der Kornblume. *Justus Liebigs Annalen der Chemie*, 401 (2): 189-232.
Yoshida, K., Toyama-Kato, Y., Kameda, K. & Kondo, T. (2003). Sepal color variation of Hydrangea macrophylla and vacuolar pH measured with a proton-selective microelectrode. *Plant and cell physiology*, 44 (3): 262-268.
Yoshida, K., Mori, M. & Kondo, T. (2009). Blue flower color development by anthocyanins: from chemical structure to cell physiology. *Natural product reports*, 26 (7): 884-915.
Zhang, Z., Schwartz, S., Wagner, L. & Miller, W. (2000). A greedy algorithm for aligning DNA sequences. *J Comput Biol*, 7 (1-2): 203-14.
Zheng, L., Li, G., Wang, X., Pan, W., Li, L., Hua, L., Liu, F., Dang, L., Mo, M. & Zhang, K. (2008). Nematicidal endophytic bacteria obtained from plants. *Annals of microbiology*, 58 (4): 569-572.

Online software

BLAST, Version BLASTN 2.2.32+, accessed 30/7.15, http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome

Primer 3, accessed 15.01.15, http://www.bioinformatics.nl/cgi-bin/primer3/primer3_www.cgi

Personal communications

Ingo Appelhagen, John Innes Centre

Sissel Hauglien, Bioforsk

Tove Ladstein, Opplysningskontoret for Blomster og Planter

Trine Hvoslef-Eide, Norwegian University of Life Sciences

Appendix I – Protocols

Plasmid DNA containing the gene of interest had to be harvested prior to transformation by electrophoresis. Three different kits (appendix 1a-c) was used in order to determine if poor plasmid yields were due to the kits or the plasmid itself. GG-buffer was used for the final DNA elution step, and volumes varied from 50µl (Genomed Jetquick Plasmid Miniprep), 100µl (Qiagen Plasmid Midi Kit) and 500µl (QIAfilter Plasmid Maxi Kit)

DNeasy plant mini kit (Appendix 1d) was used in order to isolate DNA from putatively transformed plants from both methods, prior to screening for transformation by PCR.

Appendix Ia – Genomed Jetquick Plasmid Miniprep

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**.

- 5 -

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.*

- 6 -

Appendix Ib – QIAGEN Plasmid Midi Kit

Protocol: Plasmid or Cosmid DNA Purification using QIAGEN Plasmid Midi and Maxi Kits

This protocol is designed for preparation of up to 100 µg of high- or low-copy plasmid or cosmid DNA using the QIAGEN Plasmid Midi Kit, or up to 500 µg using the QIAGEN Plasmid Maxi Kit. For additional protocols, such as for purification of very low-copy plasmids or cosmids of less than 10 copies per cell, see page 29 or visit www.qiagen.com/goto/plasmidinfo.

Low-copy plasmids that have been amplified in the presence of chloramphenicol should be treated as high-copy plasmids when choosing the appropriate culture volume.

Table 3. Maximum recommended culture volumes*

	QIAGEN-tip 100	QIAGEN-tip 500
High-copy plasmids	25 ml	100 ml
Low-copy plasmids	100 ml	500 ml

* For the QIAGEN-tip 100, the expected yields are 75–100 µg for high-copy plasmids and 20–100 µg for low-copy plasmids. For the QIAGEN-tip 500, the expected yields are 300–500 µg for high-copy plasmids and 100–500 µg for low-copy plasmids.

Important points before starting

- New users are advised to familiarize themselves with the detailed protocol provided in this handbook. In addition, extensive background information is provided on our plasmid resource page www.qiagen.com/goto/plasmidinfo.
- If working with low-copy vectors, it may be beneficial to increase the lysis buffer volumes to increase the efficiency of alkaline lysis, and thereby the DNA yield. In case additional Buffers P1, P2, and P3 are needed, their compositions are provided in Appendix B: Composition of Buffers, on page 44. Alternatively, the buffers may be purchased separately (see page 49).
- **Optional**: Remove samples at the indicated steps to monitor the procedure on an analytical gel (see page 41).
- Blue (marked with a ▲) denotes values for QIAGEN-tip 100 using the QIAGEN Plasmid Midi Kit; red (marked with a ●) denotes values for QIAGEN-tip 500 using the QIAGEN Plasmid Maxi Kit.

Things to do before starting

- Add the provided RNase A solution to Buffer P1 before use. Use 1 vial RNase A (centrifuge briefly before use) per bottle Buffer P1 for final concentration of 100 µg/ml.
- Check Buffer P2 for SDS precipitation due to low storage temperatures. If necessary, dissolve the SDS by warming to 37°C.
- Pre-chill Buffer P3 at 4°C.
- **Optional**: Add the provided LyseBlue reagent to Buffer P1 and mix before use. Use 1 vial LyseBlue reagent per bottle Buffer P1 for a final dilution of 1:1000 (e.g., 10 µl LyseBlue into 10 ml Buffer P1). LyseBlue provides visual identification of optimum buffer mixing thereby preventing the common handling errors that lead to inefficient cell lysis and incomplete precipitation of SDS, genomic DNA, and cell debris. For more details see "Using LyseBlue reagent" on page 14.

Procedure

1. **Pick a single colony from a freshly streaked selective plate and inoculate a starter culture of 2–5 ml LB medium containing the appropriate selective antibiotic. Incubate for approx. 8 h at 37°C with vigorous shaking (approx. 300 rpm).**

   Use a tube or flask with a volume of at least 4 times the volume of the culture.

2. **Dilute the starter culture 1/500 to 1/1000 into selective LB medium. For high-copy plasmids, inoculate ▲ 25 ml or ● 100 ml medium with ▲ 25–50 µl or ● 100–200 µl of starter culture. For low-copy plasmids, inoculate ▲ 100 ml or ● 500 ml medium with ▲ 100–200 µl or ● 250–500 µl of starter culture. Grow at 37°C for 12–16 h with vigorous shaking (approx. 300 rpm).**

   Use a flask or vessel with a volume of at least 4 times the volume of the culture. The culture should reach a cell density of approximately 3–4 x 10^9 cells per milliliter, which typically corresponds to a pellet wet weight of approximately 3 g/liter medium.

3. **Harvest the bacterial cells by centrifugation at 6000 x *g* for 15 min at 4°C.**

   ⊗ If you wish to stop the protocol and continue later, freeze the cell pellets at –20°C.

4. **Resuspend the bacterial pellet in ▲ 4 ml or ● 10 ml Buffer P1.**

   For efficient lysis, it is important to use a vessel that is large enough to allow complete mixing of the lysis buffers. Ensure that RNase A has been added to Buffer P1.

   If LyseBlue reagent has been added to Buffer P1, vigorously shake the buffer bottle before use to ensure LyseBlue particles are completely resuspended. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.

5. **Add ▲ 4 ml or ● 10 ml Buffer P2, mix thoroughly by vigorously inverting the sealed**

tube 4–6 times, and incubate at room temperature (15–25°C) for 5 min.

Do not vortex, as this will result in shearing of genomic DNA. The lysate should appear viscous. Do not allow the lysis reaction to proceed for more than 5 min. After use, the bottle containing Buffer P2 should be closed immediately to avoid acidification from CO_2 in the air.

If LyseBlue has been added to Buffer P1, the cell suspension will turn blue after addition of Buffer P2. Mixing should result in a homogeneously colored suspension. If the suspension contains localized colorless regions or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colored suspension is achieved.

6. **Add ▲ 4 ml or ● 10 ml of chilled Buffer P3, mix immediately and thoroughly by vigorously inverting 4–6 times, and incubate on ice for ▲ 15 min or ● 20 min.**

Precipitation is enhanced by using chilled Buffer P3 and incubating on ice. After addition of Buffer P3, a fluffy white material forms and the lysate becomes less viscous. The precipitated material contains genomic DNA, proteins, cell debris, and KDS. The lysate should be mixed thoroughly to ensure even potassium dodecyl sulfate precipitation. If the mixture still appears viscous, more mixing is required to completely neutralize the solution.

If LyseBlue reagent has been used, the suspension should be mixed until all trace of blue has gone and the suspension is colorless. A homogeneous colorless suspension indicates that the SDS has been effectively precipitated.

7. **Centrifuge at ≥20,000 x *g* for 30 min at 4°C. Remove supernatant containing plasmid DNA promptly.**

Before loading the centrifuge, the sample should be mixed again. Centrifugation should be performed in non-glass tubes (e.g., polypropylene). After centrifugation the supernatant should be clear.

Note: Instead of centrifugation steps 7 and 8, the lysate can be efficiently cleared by filtration using a QIAfilter Kits or Cartridges (see www.qiagen.com/products/plasmid/LargeScaleKits).

8. **Centrifuge the supernatant again at ≥20,000 x *g* for 15 min at 4°C. Remove supernatant containing plasmid DNA promptly.**

   This second centrifugation step should be carried out to avoid applying suspended or particulate material to the QIAGEN-tip. Suspended material (causing the sample to appear turbid) can clog the QIAGEN-tip and reduce or eliminate gravity flow.

   Optional: Remove a ▲ 240 µl or ● 120 µl sample from the cleared lysate supernatant and save for an analytical gel (sample 1) to determine whether growth and lysis conditions were optimal.

9. **Equilibrate a ▲ QIAGEN-tip 100 or ● QIAGEN-tip 500 by applying ▲ 4 ml or ● 10 ml Buffer QBT, and allow the column to empty by gravity flow.**

   Flow of buffer will begin automatically by reduction in surface tension due to the presence of detergent in the equilibration buffer. Allow the QIAGEN-tip to drain completely. QIAGEN-tips can be left unattended, since the flow of buffer will stop when the meniscus reaches the upper frit in the column.

10. **Apply the supernatant from step 8 to the QIAGEN-tip and allow it to enter the resin by gravity flow.**

    The supernatant should be loaded onto the QIAGEN-tip promptly. If it is left too long and becomes cloudy due to further precipitation of protein, it must be centrifuged again or filtered before loading to prevent clogging of the QIAGEN-tip.

    Optional: Remove a ▲ 240 µl or ● 120 µl sample from the flow-through and save for an analytical gel (sample 2) to determine the efficiency of DNA binding to the QIAGEN resin.

11. **Wash the QIAGEN-tip with ▲ 2 x 10 ml or ● 2 x 30 ml Buffer QC.**

    Allow Buffer QC to move through the QIAGEN-tip by gravity flow. The first wash is sufficient to remove contaminants in the majority of plasmid DNA preparations. The second wash is especially necessary when large culture volumes or bacterial strains producing large amounts of carbohydrates are used.

    Optional: Remove a ▲ 400 µl or ● 240 µl sample from the combined wash fractions and save for an analytical gel (sample 3).

12. **Elute DNA with ▲ 5 ml or ● 15 ml Buffer QF.**

    Collect the eluate in a 15 ml or 50 ml tube (not supplied). Use of polycarbonate centrifuge tubes is not recommended as polycarbonate is not resistant to the alcohol used in subsequent steps.

    Note: For constructs larger than 45–50 kb, prewarming the elution buffer to 65°C may help to increase yield.

    Optional: Remove a ▲ 100 µl or ● 60 µl sample of the eluate and save for an analytical gel (sample 4).

    ⊗ If you wish to stop the protocol and continue later, store the eluate at 4°C. Storage periods longer than overnight are not recommended.

13. **Precipitate DNA by adding ▲ 3.5 ml or ● 10.5 ml (0.7 volumes) room-temperature isopropanol to the eluted DNA. Mix and centrifuge immediately at ≥15,000 x *g* for 30 min at 4°C. Carefully decant the supernatant.**

    All solutions should be at room temperature to minimize salt precipitation, although centrifugation is carried out at 4°C to prevent overheating of the sample. Alternatively, disposable conical bottom centrifuge tubes can be used for centrifugation at 5000 x *g* for 60 min at 4°C. Isopropanol pellets have a glassy appearance and may be more difficult to see than the fluffy, salt-containing pellets that result from ethanol precipitation. Marking the outside of the tube before centrifugation allows the pellet to be more easily located. Isopropanol pellets are also more loosely attached to the side of the tube, and care should be taken when removing the supernatant.

14. **Wash DNA pellet with ▲ 2 ml or ● 5 ml of room-temperature 70% ethanol, and centrifuge at ≥15,000 x *g* for 10 min. Carefully decant the supernatant without disturbing the pellet.**

    Alternatively, disposable conical-bottom centrifuge tubes can be used for centrifugation at 5000 x *g* for 60 min at 4°C. The 70% ethanol removes precipitated salt and replaces isopropanol with the more volatile ethanol, making the DNA easier to redissolve.

15. **Air-dry the pellet for 5–10 min, and redissolve the DNA in a suitable volume of buffer (e.g., TE buffer, pH 8.0, or 10 mM Tris·Cl, pH 8.5).**

    Redissolve the DNA pellet by rinsing the walls to recover the DNA, especially if glass tubes have been used. Pipetting the DNA up and down to promote resuspension may cause shearing and should be avoided. Overdrying the pellet will make the DNA difficult to redissolve. DNA dissolves best under slightly alkaline conditions; it does not easily dissolve in acidic buffers.

Determination of yield

To determine the yield, DNA concentration should be determined by both UV spectrophotometry at 260 nm and quantitative analysis on an agarose gel. For reliable spectrophotometric DNA quantification, A_{260} readings should lie between 0.1 and 1.0.

Agarose gel analysis

We recommend removing and saving aliquots during the purification procedure (samples 1–4). If the plasmid DNA is of low yield or quality, the samples can be analyzed by agarose gel electrophoresis to determine the stage of the purification procedure where the problem occurred (see page 41).

Appendix Ic – QIAfilter Plasmid Maxi Kit

Protocol: Plasmid or Cosmid DNA Purification using QIAfilter Plasmid Midi and Maxi Kits

This protocol is designed for preparation of up to 100 µg of high- or low-copy plasmid or cosmid DNA using the QIAfilter Plasmid Midi Kit, or up to 500 µg using the QIAfilter Plasmid Maxi Kit. In this protocol, QIAfilter Cartridges are used instead of conventional centrifugation to clear bacterial lysates. For purification of double-stranded M13 replicative-form DNA, see the recommendations at www.qiagen.com/goto/ plasmidinfo.

Low-copy plasmids which have been amplified in the presence of chloramphenicol should be treated as high-copy plasmids when choosing the appropriate culture volume.

Table 3. Maximum recommended culture volumes*

	QIAfilter Midi	QIAfilter Maxi
High-copy plasmids	25 ml	100 ml
Low-copy plasmids	50–100 ml	250 ml†

* For high-copy plasmids, expected yields are 75–100 µg for the QIAfilter Plasmid Midi Kit and 300–500 µg for the QIAfilter Plasmid Maxi Kit. For low-copy plasmids, expected yields are 20–100 µg for the QIAfilter Plasmid Midi Kit and 50–250 µg for the QIAfilter Plasmid Maxi Kit using these culture volumes.

† The maximum recommended culture volume applies to the capacity of the QIAfilter Maxi Cartridge. If higher yields of low-copy plasmids are desired, the lysates from two QIAfilter Maxi Cartridges can be loaded onto one QIAGEN-tip 500.

Important points before starting

- New users are advised to familiarize themselves with the detailed protocol provided in this handbook. In addition, extensive background information is provided on our plasmid resource page www.qiagen.com/goto/plasmidinfo.
- If working with low-copy vectors, it may be beneficial to increase the lysis buffer volumes to increase the efficiency of alkaline lysis, and thereby the DNA yield. In case additional Buffers P1, P2, and P3 are needed, their compositions are provided in Appendix B: Composition of Buffers, on page 36. Alternatively, the buffers and additional QIAfilter Cartridges may be purchased separately
- **Optional**: Remove samples at the indicated steps to monitor the procedure on an analytical gel (see page 33).
- In contrast to the standard protocol, the lysate is not incubated on ice after addition of Buffer P3.
- Blue (marked with a ▲) denotes values for the QIAfilter Plasmid Midi Kit; red (marked with a ●) denotes values for the QIAfilter Plasmid Maxi Kit.

Things to do before starting

- Add the provided RNase A solution to Buffer P1 before use. Use 1 vial RNase A (centrifuge briefly before use) per bottle Buffer P1 for a final concentration of 100 µg/ml.
- Check Buffer P2 for SDS precipitation due to low storage temperatures. If necessary, dissolve the SDS by warming to 37°C.
- Prechill Buffer P3 at 4°C.
- **Optional**: Add the provided LyseBlue reagent to Buffer P1 and mix before use. Use 1 vial LyseBlue reagent per bottle Buffer P1 for a final dilution of 1:1000 (e.g., 10 µl LyseBlue into 10 ml Buffer P1). LyseBlue provides visual identification of optimum buffer mixing, thereby preventing the common handling errors that lead to inefficient cell lysis and incomplete precipitation of SDS, genomic DNA, and cell debris. For more details see "Using LyseBlue reagent" on page 14.

Procedure

1. **Pick a single colony from a freshly streaked selective plate and inoculate a starter culture of 2–5 ml LB medium containing the appropriate selective antibiotic. Incubate for approx. 8 h at 37°C with vigorous shaking (approx. 300 rpm).**

   Use a tube or flask with a volume of at least 4 times the volume of the culture.

2. **Dilute the starter culture 1/500 to 1/1000 into selective LB medium. For high-copy plasmids, inoculate ▲ 25 ml or ● 100 ml medium with ▲ 25–50 µl or ● 100–200 µl of starter culture. For low-copy plasmids, inoculate ▲ 50–100 ml or ● 250 ml medium with ▲ 100–200 µl or ● 250–500 µl of starter culture. Grow at 37°C for 12–16 h with vigorous shaking (approx. 300 rpm).**

   Use a flask or vessel with a volume of at least 4 times the volume of the culture. The culture should reach a cell density of approximately 3–4 x 10^9 cells per milliliter, which typically corresponds to a pellet wet weight of approximately 3 g/liter medium (see page 11).

3. **Harvest the bacterial cells by centrifugation at 6000 x *g* for 15 min at 4°C.**

   ⊗ If you wish to stop the protocol and continue later, freeze the cell pellets at –20°C.

4. **Resuspend the bacterial pellet in ▲ 4 ml or ● 10 ml Buffer P1.**

   For efficient lysis it is important to use a vessel that is large enough to allow complete mixing of the lysis buffers. Ensure that RNase A has been added to Buffer P1.

   If LyseBlue reagent has been added to Buffer P1, vigorously shake the buffer bottle before use to ensure LyseBlue particles are completely resuspended. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.

5. **Add ▲ 4 ml or ● 10 ml Buffer P2, mix thoroughly by vigorously inverting the sealed tube 4–6 times, and incubate at room temperature (15–25°C) for 5 min.**

   Do not vortex, as this will result in shearing of genomic DNA. The lysate should appear viscous. Do not allow the lysis reaction to proceed for more than 5 min. After use, the bottle containing Buffer P2 should be closed immediately to avoid acidification from CO_2 in the air.

   If LyseBlue has been added to Buffer P1 the cell suspension will turn blue after addition of Buffer P2. Mixing should result in a homogeneously colored suspension. If the suspension contains localized colorless regions or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colored suspension is achieved.

   During the incubation prepare the QIAfilter Cartridge:

   Screw the cap onto the outlet nozzle of the QIAfilter Midi or QIAfilter Maxi Cartridge.

   Place the QIAfilter Cartridge in a convenient tube.

6. **Add ▲ 4 ml or ● 10 ml chilled Buffer P3 to the lysate, and mix immediately and thoroughly by vigorously inverting 4–6 times. Proceed directly to step 7. Do not incubate the lysate on ice.**

   Precipitation is enhanced by using chilled Buffer P3. After addition of Buffer P3, a fluffy white material forms and the lysate becomes less viscous. The precipitated material contains genomic DNA, proteins, cell debris, and KDS. The lysate should be mixed thoroughly to ensure even potassium dodecyl sulfate precipitation. If the mixture still appears viscous, more mixing is required to completely neutralize the solution. It is important to transfer the lysate into the QIAfilter Cartridge immediately to prevent later disruption of the precipitate layer.

   If LyseBlue reagent has been used, the suspension should be mixed until all trace of blue has gone and the suspension is colorless. A homogeneous colorless suspension indicates that the SDS has been effectively precipitated.

7. **Pour the lysate into the barrel of the QIAfilter Cartridge. Incubate at room temperature for 10 min. Do not insert the plunger!**

   Important: This 10 min incubation at room temperature is essential for optimal performance of the QIAfilter Midi or QIAfilter Maxi Cartridge. Do not agitate the QIAfilter Cartridge during this time. A precipitate containing proteins, genomic DNA, and detergent will float and form a layer on top of the solution. This ensures convenient filtration without clogging. If, after the 10 min incubation, the precipitate has not floated to the top of the solution, carefully run a sterile pipet tip around the walls of the cartridge to dislodge it.

8. **Equilibrate a ▲ QIAGEN-tip 100 or ● QIAGEN-tip 500 by applying ▲ 4 ml or ● 10 ml Buffer QBT, and allow the column to empty by gravity flow.**

   Flow of buffer will begin automatically by reduction in surface tension due to the presence of detergent in the equilibration buffer. Allow the QIAGEN-tip to drain completely. QIAGEN-tips can be left unattended, since the flow of buffer will stop when the meniscus reaches the upper frit in the column.

9. **Remove the cap from the QIAfilter Cartridge outlet nozzle. Gently insert the plunger into the ▲ QIAfilter Midi or ● QIAfilter Maxi Cartridge and filter the cell lysate into the previously equilibrated QIAGEN-tip.**

   Filter until all of the lysate has passed through the QIAfilter Cartridge, but do not apply extreme force. Approximately ▲ **10 ml** and ● **25 ml** of the lysate are generally recovered after filtration.

   Optional: Remove a ▲ 240 µl or ● 120 µl sample of the filtered lysate and save for an analytical gel (sample 1) to determine whether growth and lysis conditions were optimal.

10. **Allow the cleared lysate to enter the resin by gravity flow.**

    Optional: Remove a ▲ 240 µl or ● 120 µl sample from the flow-through and save for an analytical gel (sample 2) to determine the efficiency of DNA binding to the QIAGEN resin.

11. **Wash the QIAGEN-tip with ▲ 2 x 10 ml or ● 2 x 30 ml Buffer QC.**

    Allow Buffer QC to move through the QIAGEN-tip by gravity flow. The first wash is sufficient to remove all contaminants in the majority of plasmid DNA preparations. The second wash is especially necessary when large culture volumes or bacterial strains producing large amounts of carbohydrates are used.

    Optional: Remove a ▲ 400 µl or ● 240 µl sample from the combined wash fractions and save for an analytical gel (sample 3).

12. **Elute DNA with ▲ 5 ml or ● 15 ml Buffer QF.**

    Collect the eluate in a 15 ml or 50 ml tube (not supplied). Use of polycarbonate centrifuge tubes is not recommended as polycarbonate is not resistant to the alcohol used in subsequent steps.

    Note: For constructs larger than 45–50 kb, prewarming the elution buffer to 65°C may help to increase yield.

    Optional: Remove a ▲ 100 µl or ● 60 µl sample of the eluate and save for an analytical gel (sample 4).

    ⊗ If you wish to stop the protocol and continue later, store the eluate at 4°C. Storage periods longer than overnight are not recommended.

13. **Precipitate DNA by adding ▲ 3.5 ml or ● 10.5 ml (0.7 volumes) room-temperature isopropanol to the eluted DNA. Mix and centrifuge immediately at ≥15,000 x *g* for 30 min at 4°C. Carefully decant the supernatant.**

    All solutions should be at room temperature to minimize salt precipitation, although centrifugation is carried out at 4°C to prevent overheating of the sample. Alternatively, disposable conical bottom centrifuge tubes can be used for centrifugation at 5000 x *g* for 60 min at 4°C. Isopropanol pellets have a glassy appearance and may be more difficult to see than the fluffy, salt-containing pellets that result from ethanol precipitation. Marking the outside of the tube before centrifugation allows the pellet to be more easily located. Isopropanol pellets are also more loosely attached to the side of the tube, and care should be taken when removing the supernatant.

14. **Wash DNA pellet with ▲ 2 ml or ● 5 ml of room-temperature 70% ethanol, and centrifuge at ≥15,000 x *g* for 10 min. Carefully decant the supernatant without disturbing the pellet.**

    Alternatively, disposable conical-bottom centrifuge tubes can be used for centrifugation at 5000 x *g* for 60 min at 4°C. The 70% ethanol removes precipitated salt and replaces isopropanol with the more volatile ethanol, making the DNA easier to redissolve.

15. **Air-dry the pellet for 5–10 min, and redissolve the DNA in a suitable volume of buffer (e.g., TE buffer, pH 8.0, or 10 mM Tris-Cl, pH 8.5).**

    Redissolve the DNA pellet by rinsing the walls to recover the DNA, especially if glass tubes have been used. Pipetting the DNA up and down to promote resuspension may cause shearing and should be avoided. Overdrying the pellet will make the DNA difficult to redissolve. DNA dissolves best under slightly alkaline conditions; it does not easily dissolve in acidic buffers.

Determination of yield

To determine the yield, DNA concentration should be determined by both UV spectrophotometry at 260 nm and quantitative analysis on an agarose gel. For reliable spectrophotometric DNA quantification, A_{260} readings should lie between 0.1 and 1.0.

Agarose gel analysis

We recommend removing and saving aliquots during the purification procedure (samples 1–4). If the plasmid DNA is of low yield or quality, the samples can be analyzed by agarose gel electrophoresis to determine the stage of the purification procedure where the problem occurred (see page 33).

Appendix Id – DNeasy plant mini kit

Plant Tissue (Mini Protocol)

Protocol: Purification of Total DNA from Plant Tissue (Mini Protocol)

Important points before starting

- If using the DNeasy Plant Mini Kit for the first time, read "Important Notes" (page 14).
- Ensure that you are familiar with operating the TissueRuptor or the TissueLyser. See "Disruption and homogenization using the TissueRuptor", page 15, or "Disruption and homogenization using the TissueLyser System", page 15. Refer to the *TissueRuptor User Manual* or the *TissueLyser Handbook* for operating instructions.
- Buffer AP1 may develop a yellow color upon storage. This does not affect the procedure.
- All centrifugation steps are carried out at room temperature (15–25°C) in a microcentrifuge.

Things to do before starting

- Buffer AP1 and Buffer AW1 concentrate may form precipitates upon storage. If necessary, warm to 65°C to redissolve (before adding ethanol to Buffer AW1). Do not heat Buffer AW1 after ethanol has been added.
- Buffer AW2 and Buffer AW1 are supplied as concentrates. Before using for the first time, add the appropriate amount of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
- Preheat a water bath or heating block to 65°C.

Procedure

1. **For disruption using the TissueRuptor, follow step 2; for disruption using the TissueLyser, follow steps 3–6.**

   Alternatively, plant or fungal tissue can be ground to a fine powder under liquid nitrogen using a mortar and pestle. Transfer the tissue powder and liquid nitrogen to an appropriately sized tube and allow the liquid nitrogen to evaporate. Do not allow the sample to thaw. Proceed immediately to step 7.

2. **TissueRuptor procedure: Place the sample material (≤100 mg wet weight or ≤20 mg lyophilized tissue) into a 2 ml microcentrifuge tube. Add liquid nitrogen to the tube, and freeze the sample for 30 s. Keep the sample submerged in liquid nitrogen, and disrupt for approximately 30 s at full speed. Allow the liquid nitrogen to evaporate, and proceed immediately to step 7.**

   Alternatively, fresh or lyophilized material can be directly disrupted in lysis buffer (after step 7) without using liquid nitrogen, but this may cause shearing of high-molecular-weight DNA. We do not recommend disrupting frozen material in lysis buffer as this can result in low yields and degraded DNA.

3. **TissueLyser procedure: Place the sample material (≤100 mg wet weight or ≤20 mg lyophilized tissue) into a 2 ml safe-lock microcentrifuge tube, together with a 3 mm tungsten carbide bead. Freeze the tubes in liquid nitrogen for 30 s.**

   When using lyophilized tissue, the tubes do not need to be frozen in liquid nitrogen.

4. **Place the tubes into the TissueLyser Adapter Set 2 x 24, and fix into the clamps of the TissueLyser. Immediately grind the samples for 1 min at 30 Hz.**

5. **Disassemble the adaptor set, remove the tubes, and refreeze in liquid nitrogen for 30 s.**

   When using lyophilized tissue, the tubes do not need to be frozen in liquid nitrogen.

6. **Repeat step 4, reversing the position of the tubes within the adaptor set. Proceed immediately to step 7.**

   To prevent variation in sample homogenization, the adaptor sets should be removed from the TissueLyser and disassembled after the first disruption step. For the second disruption step, the adaptor sets should be reassembled so that the tube order is reversed. Rotating the racks of tubes in this way ensures that all samples are thoroughly and equally disrupted.

   Note: The majority of plant tissue is ground to a fine powder after 2 disruption steps, however, for some materials one disruption step may be sufficient. Other tissues, such as seeds and roots, may require disruption steps. Optimization of the disruption procedure may be required for some plant material.

7. **Add 400 µl Buffer AP1 and 4 µl RNase A stock solution (100 mg/ml) to a maximum of 100 mg (wet weight) or 20 mg (dried) disrupted plant or fungal tissue and vortex vigorously.**

   No tissue clumps should be visible. Vortex or pipet further to remove any clumps. Clumps of tissue will not lyse properly and will therefore result in a lower yield of DNA. In rare cases, where clumps cannot be removed by pipetting and vortexing, a disposable micropestle may be used.

   Note: Do not mix Buffer AP1 and RNase A before use.

8. **Incubate the mixture for 10 min at 65°C. Mix 2 or 3 times during incubation by inverting tube.**

   This step lyses the cells.

9. **Add 130 µl Buffer P3 to the lysate, mix, and incubate for 5 min on ice.**

   This step precipitates detergent, proteins, and polysaccharides.

10. **Recommended: Centrifuge the lysate for 5 min at 20,000 x *g* (14,000 rpm).**

    Some plant materials can generate very viscous lysates and large amounts of precipitates during this step. This can result in shearing of the DNA in the next step (see "Lysate filtration with QIAshredder", page 19). In this case, optimal results are obtained if the majority of these precipitates are removed by centrifugation for 5 min at 20,000 x *g* (14,000 rpm). After centrifugation, apply supernatant to QIAshredder Mini spin column and continue with step 11.

11. **Pipet the lysate into the QIAshredder Mini spin column (lilac) placed in a 2 ml collection tube, and centrifuge for 2 min at 20,000 x *g* (14,000 rpm).**

    It may be necessary to cut the end off the pipet tip to apply the lysate to the QIAshredder Mini spin column. The QIAshredder Mini spin column removes most precipitates and cell debris, but a small amount will pass through and form a pellet in the collection tube. Be careful not to disturb this pellet in step 12.

12. **Transfer the flow-through fraction from step 11 into a new tube (not supplied) without disturbing the cell-debris pellet.**

    Typically 450 µl of lysate is recovered. For some plant species less lysate is recovered. In this case, determine the volume for the next step.

13. **Add 1.5 volumes of Buffer AW1 to the cleared lysate, and mix by pipetting.**

    For example, to 450 µl lysate, add 675 µl Buffer AW1. Reduce the amount of Buffer AW1 accordingly if the volume of lysate is smaller. A precipitate may form after the addition of Buffer AW1, but this will not affect the DNeasy procedure.

    Note: Ensure that ethanol has been added to Buffer AW1. See "Things to do before starting", page 22.

    Note: It is important to pipet Buffer AW1 directly onto the cleared lysate and to mix immediately.

14. **Pipet 650 µl of the mixture from step 13, including any precipitate that may have formed, into the DNeasy Mini spin column placed in a 2 ml collection tube (supplied). Centrifuge for 1 min at ≥6000 x *g* (corresponds to ≥8000 rpm for most microcentrifuges), and discard the flow-through.* Reuse the collection tube in step 15.**

15. **Repeat step 14 with remaining sample. Discard flow-through* and collection tube.**

* Flow-through fractions contain Buffer AW1, and are therefore not compatible with bleach. See page 6 for safety information.

16. **Place the DNeasy Mini spin column into a new 2 ml collection tube (supplied), add 500 µl Buffer AW2, and centrifuge for 1 min at ≥6000 x *g* (≥8000 rpm). Discard the flow-through and reuse the collection tube in step 17.**

    Note: Ensure that ethanol is added to Buffer AW2. See "Things to do before starting", page 22.

17. **Add 500 µl Buffer AW2 to the DNeasy Mini spin column, and centrifuge for 2 min at 20,000 x *g* (14,000 rpm) to dry the membrane.**

    It is important to dry the membrane of the DNeasy Mini spin column since residual ethanol may interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during elution. Discard flow-through and collection tube.

    After washing with Buffer AW2, the DNeasy Mini spin column membrane is usually only slightly colored. In the rare case that the membrane remains significantly colored after washing with Buffer AW2, refer to "Darkly colored membrane or green/yellow eluate after washing with Buffer AW2" in the Troubleshooting Guide on page 43.

    Note: Following the centrifugation, remove the DNeasy Mini spin column from the collection tube carefully so the column does not come into contact with the flow-through, as this will result in carryover of ethanol.

18. **Transfer the DNeasy Mini spin column to a 1.5 ml or 2 ml microcentrifuge tube (not supplied), and pipet 100 µl Buffer AE directly onto the DNeasy membrane. Incubate for 5 min at room temperature (15–25°C), and then centrifuge for 1 min at ≥6000 x *g* (≥8000 rpm) to elute.**

    Elution with 50 µl (instead of 100 µl) increases the final DNA concentration in the eluate significantly, but also reduces overall DNA yield. If larger amounts of DNA (>20 µg) are loaded, eluting with 200 µl (instead of 100 µl) increases yield. See "Elution", page 19.

19. **Repeat step 18 once.**

    A new microcentrifuge tube can be used for the second elution step to prevent dilution of the first eluate. Alternatively, the microcentrifuge tube can be reused for the second elution step to combine the eluates. See "Elution", page 19.

    Note: More than 200 µl should not be eluted into a 1.5 ml microcentrifuge tube because the DNeasy Mini spin column will come into contact with the eluate.

Appendix II – Nutrient media and other solutions

Nutrient media used for explants following Agobacterium-mediated transformations. Based on Preil, Walter (1994), but the rooting induction medium was changed. The RI medium was based on "Hormon free MS medium" from Clarke et al. (2008) as they could not report any significant advantage over a rooting induction medium with hormones. Standard MS medium with hormones bought in 1x jars were used for basal formulation.

pH was adjusted to 5.7-5.8 for all media.

Medium name	Basal formulation	Sucrose	Hormones	Gelrite	Selection
Callus induction (CI)	Full strength MS	3 %	0.2 mg/l CPA 0.2 mg/l BAP	0.35g/l	500 mg/l Cefotaxim* 10 mg/l Kanamycin*
Somatic embryo induction (SEI)	Full strength MS	3 %	0.3 mg/l NAA 0.15 mg/l 2iP	0.35g/l	500 mg/l Cefotaxim* 10 mg/l Kanamycin*
Somatic embryo maturation (SEM)	Full strength MS	3 %	0.05 mg/l BAP No auxin	0.35g/l	500 mg/l Cefotaxim* 10 mg/l Kanamycin*
Rooting induction (RI)	Full strength MS	2 %	No hormones	0.35g/l	No selection

CPA = (4-Chlorophenoxy)acetic acid, BAP = 6-Benzylaminopurine, NAA = 1-Naphthaleneacetic acid, 2iP= 2-Isopentenyl adenine, MS = Murashige and Skoog (1962)

* Callus inducing medium without selection was used for co-cultivation, and control shoot discs were grown on medium without selection.

GG-buffer

The GG-buffer was used to store DNA after final elution of plasmid purification. It was also used (with 1% agarose) in pipette tips for transformation by electrophoresis.

Ingredient	Concentration
Glucine	50 mM
Glutamine	70 mM

LB medium

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

Ingredient	Concentration
Tryptone	10 g/l
NaCl	10 g/l
Yeast extract	5 g/l

* Agar was added in when the LB medium was used on petri dishes.

MS-II

MS-II was used as a washing medium prior to *Agrobacterium*-mediated transformations, to remove traces of LB medium from bacterial cultivation.

Ingredient	Concentration
MS	Full strength (1 jar/l)
Sucrose	20 g/l (2%)

TBE buffer and 1% agarose gel

5X Tris-Borate-EDTA (TBE) buffer was made as a stock, and used in 1X concentrations for gel electrophoresis.

Ingredient	Concentration
Tris	54g/l
Boric acid	27.5 g/l
EDTA 0.5M	20ml/l

For 1% agarose gels, 1% agarose and 2µl GelRed was added to 1X TBE buffer.

PCR master mix

A 10X PCR master mix was made prior to PCR. This consisted of:

Ingredient	Concentration
dNTP	2mM
Left primer	2mM
Right primer	2mM
TaqPolymerase	1 unit
Buffer 10X	1 x
RNAse free dH_2O	Fill to 90µl

1 µl template DNA was added to 1X PCR master mix prior to PCR.

Appendix III – Plasmid Isolation data

This section shows the raw data from plasmid isolation prior to transformation by electrophoresis.

Qiagen plasmid midi kit:

Sample ID	DNA (Ng/ul)
1	53,41
2	21,26
3	43,36
4	43,72
5	22,84
6	21,53
7	27,73
8	29,13
Average	34.12

Max kit yield: 100 µg Final elution volume: 100 µl

Average plasmid yield = Average consentration * elution volume = 34.12 ng/µl * 100 µl = 3412 ng = 3.412 µg

Yield percentage = average yield/max yield = 3.412 µg/ 100 µg = 0.03412 = 3.4%

Genomed Jetquick plasmid miniprep:

Sample ID	DNA (ng/ul)	Sample ID	DNA (ng/ul)
1	30,34	20	27,03
2	29,54	21	24,61
3	52,51	22	26,12
4	40,45	23	22,8
5	48,63	24	22,54
6	50,13	25	31,9
7	50,55	26	43,03
8	31,87	27	47,53
9	19,58	28	40,24
10	30,49	29	22,15
11	18,93	30	52,96
12	27,85	31	46,4
13	36,77	32	44,75
14	25,36	33	0,68
15	24,45	34	52,09
16	29,69	35	43,57
17	16,24	Average	32.3
18	-4,21		
19	22,98		

Max kit yield: 50 µg

Final elution volume: 50 µl

Average plasmid yield = 32.3

Average consentration * elution volume = 32.3 ng/µl * 50 µl = 1615 ng = 1.615 µg

Yield percentage = average yield/max yield = 1.615 µg/ 50 µg = 0.0323 = 3.2 %

Qiafilter Plasmid Maxi Kit:

Sample ID	DNA (Ng/ul)
1	21,64
2	30,73
3	30,34
4	29,54
Average	28,06

Max kit yield: 500 Final elution volume: 500 µl

Average plasmid yield = Average consentration * elution volume = 28.06 ng/µl * 500 µl = 14030 ng = 14.03 µg

Yield percentage = average yield/max yield = 14.03 µg/ 500 µg = 0.028 = 2.8%

Due to low plasmid yields using all kits, the samples were combined and precipitated to increase concentration. New concentrations are shown below.

Sample ID	DNA (ng/ul)	Used in pipette tip
1	220,93	Pipette A
2	241,13	Pipette B
3	256,23	Pipette C
4	245,3	Pipette D
5	280,02	Positive Control during PCR
7	245,45	Not used
8	287,86	Not used
9	321,3	Not used
10	205,98	Not used
11	233,55	Not used
12	326,22	Not used

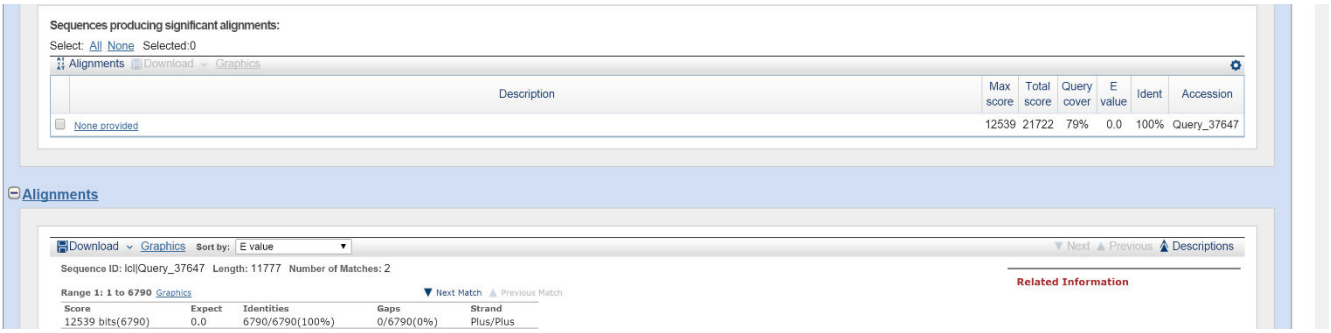
Appendix IV – Electrophoresis data

Complete overview of data from transformation by electrophoresis. The ampere (start) decreased rapidly, and were closer to the ampere stop for most of the time.

Date	Plant no.	Pipette tip	Shoot no.	Volt	Time (minutes)	Ampere (mA) start	Ampere (mA) stop
02.12.14	1	A	1.1	60	10	0.80	0.70
			1.2	60	8	0.78	0.68
			1.3	65	10	0.76	0.68
			1.4	65	8	0.79	0.68
	2	A	2.1	50	10	0.75	0.58
			2.2	50	9	0.66	0.58
			2.3	50	10	0.69	0.61
03.12.14	3	A	3.1	50	8	0.68	0.57
			3.2	50	8	0.58	0.54
	4	A	4.1	45	9	0.54	0.47
			4.2	45	10	0.50	0.46
04.12.14	5	B	5.1	60	10	0.42	0.33
			5.2	60	10	0.53	0.45
	6	B	6.1	50	10	0.55	0.50
			6.2	50	10	0.69	0.54
			6.3	40	10	0.61	0.34
	7	B	7.1	40	8	0.55	0.43
			7.2	40	10	0.54	0.45
09.01.15	8	C	8.1	40	10	0.50	0.46
			8.2	35	8	0.57	0.52
	9	C	9.1	40	10	0.53	0.42
			9.2	50	10	0.55	0.48
			9.3	50	10	0.59	0.52
			9.4	40	10	0.47	0.44
12.01.15	10	D	10.1	40	9	0.60	0.53
			10.2	40	8	0.59	0.47
			10.3	40	9	0.62	0.53
			10.4	40	10	0.63	0.57
	11	D	11.1	40	10	0.47	0.37
			11.2	50	10	0.57	0.48
			11.3	50	9	0.57	0.47
			11.4	50	10	0.46	0.36
			11.5	60	10	0.57	0.48
13.01.15	12	D	12.1	50	8	0.55	0.49
			12.2	40	10	0.49	0.41
			12.3	55	8	0.57	0.50
			12.4	45	10	0.58	0.46
	13	D	13.1	50	10	0.54	0.43
			13.2	45	10	0.57	0.50
			13.3	45	8	0.63	0.52
			13.4	45	10	0.59	0.48
			13.5	45	10	0.54	0.43
Total	**13**	**4**	**42**				

Appendix V – BLAST result

The plasmid pJAM1983 was compared to common binary vectors using BLAST (Version BLASTN 2.2.32+, run date 30/7.15) to determine the plasmid backbone. A 100% identity was achieved with pBIN19, indicating that this is the backbone used for pJAM1983. The query cover is 79%, where the remaining 21% corresponds to the gene insert (Figure 2)



Norwegian University
of Life Sciences

Postboks 5003
NO-1432 Ås, Norway
+47 67 23 00 00
www.nmbu.no