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Enhancing *Agrobacterium*-mediated transformation in *Petunia hybrida* and *Nicotiana benthamiana*

MSc Thesis

By

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Abstract

Agrobacterium-mediated transformation plays a crucial role in microbiology, plant pathology and genetics. It forms the basis of the development of genetically modified crops with improved agronomic traits and an application of genomic sciences to plant biology. However, it is impossible to achieve a full biotechnological exploitation using *Agrobacterium*-mediated transformation in some economically important plant species due to low transformation efficiency. Here I report the usefulness of acetosyringone, lipoic acid, 2-aminoindan-2-phosphonic acid (AIP), and paclobutrazol as transformation enhancers in *Petunia hybrida* and *Nicotiana benthamiana* when included in co-culture media during the transformation process. The usefulness of Green Fluorescent Protein (GFP) and red fluorescent protein (dsRed) as reporters of transformation in these species has been investigated as well. Explants from fresh leaves were inoculated with cultures of Agl0(pMF1-JS14) and Agl1(pBinGlyRed-Asc1) strains containing the plant binary Ti plasmid vectors with genes encoding for GFP plus β-glucuronidase (GUS) and dsRed respectively. The inoculated explants were subjected to co-culture media containing varying acetosyringone concentrations (0, 100 and 400 µM), lipoic acid and AIP (0, 10 and 50 µM), paclobutrazol (0.5 and 5 mg/l). Transformation efficiencies were calculated by scoring both transient GFP, GUS and dsRed expression and stable transformation by counting the number of explants surviving on selection media. Although both reporters can be used in *P. hybrida* and *N. benthamiana*, dsRed is more reliable than GFP by resolving the problem of chlorophyll autofluorescence. Inclusion of acetosyringone increased transformation efficiency in the two plant species. However, increasing this compound from 100 to 400 µM did not increase the overall transformation efficiency. In *N. benthamiana*, lipoic acid increased the transformation efficiency using Agl0(pMF1-JS14) strain with 50 µM attaining the highest transformation efficiency. This compound also reduced tissue browning and necrosis of explants. AIP increased transformation efficiency in *P. hybrida* and *N. benthamiana*. However the optimum concentration workable with these species needs to be validated. Due to time limitation, the usefulness of paclobutrazol was not elucidated.

Acknowledgements

I would like to thank my supervisors Dr. Frans A. Krens and Ing. Iris Tinnenbroek for guidance, support, patience and encouragement. I also thank all the members of the Ornamental plants, Tissue culture and Gene transfer group in the Laboratory of Plant Breeding for the assistance during the laboratory experiments.

1. INTRODUCTION

1.1 The use of *Agrobacterium* in transformation

Plant transformation forms the basis of plant biotechnology. It involves the transfer of exogenous DNA into host plant tissues. The DNA donor species can be a related or unrelated plant species, or even a nonplant organism (Barampuram and Zhang, 2011). This is the fundamental basis of the development of genetically modified crops with improved agronomic traits. In some countries, transgenic varieties of economically important crops such as potatoes, maize, tomatoes, cotton and soybean are contributing a considerable percentage to the total crop productivity (Gelvin, 2003). *Agrobacterium*-mediated transformation plays a crucial role in microbiology, plant pathology and genetics. This method has been preferred as it has several advantages over direct DNA delivery systems. These include the ability to transfer large DNA segments with subsequent stable integration and consistent expression of the transferred DNA for many generations (Barampuram and Zhang, 2011; Ziemienowicz *et al*., 2001). However, in some plant species *Agrobacterium* achieves a lower transformation efficiency or frequency. Therefore, direct DNA delivery methods, such as electroporation and microparticle bombardment, are often explored in addition to *Agrobacterium*-mediated transformation.

1.2 The Tranfer DNA (T-DNA)

Agrobacterium tumefaciens is the most widely used bacterial species in plant transformation. It induces tumorigenesis in dicotyledonous plants, a phenotype known as crown gall disease (Zupan and Zambryski, 1995). When *A. tumefaciens* attaches to plant cells, it transfers part of its plasmid DNA (T-DNA) into the cells and subsequently the T-DNA is integrated into the plant genome. The T-DNA is found on the tumour inducing plasmid (Ti plasmid) (Figure 1) and is comprised of oncogenic genes that encode for enzymes involved in auxin and cytokinin biosynthesis (Lee *et al.*, 1992; Ziemienowicz *et al*., 2001). The virulence region of the Ti plasmid contains *Vir* genes that play a critical role in excision, transfer and integration of the T-DNA into host cells (Lee *et al*., 1992). Host-derived phenolic compounds are known to trigger the expression of *Vir* genes. Hence, plant exudates activate the virulence of *Agrobacterium*. Flanking the T-region are 25-bp borders which provide cleavage sites recognised by the *Vir* endonuclease (Gelvin, 2003). For use in plant genetic engineering, the Ti plasmid has been modified by removing the oncogenes to produce a disarmed Ti-plasmid. In addition, the T-DNA part is separated from the *Vir* functions by generating a binary vector system, one vector carrying the *Vir* genes and a separate vector carrying the genes-of-interest to be transferred (T-DNA). Genes of interests are incorporated in the T-DNA together with other constituents. These include selectable markers (e.g. antibiotic resistance) and reporter genes (e.g. β-glucuronidase (GUS), green fluorescent protein (GFP) and red fluorescent protein (dsRed), that enable selection of transformants and monitoring

of the transfer process (Lee and Gelvin, 2008). This artificial plasmid is known as a binary vector (Figure 2). There are different *Agrobacterium* strains used for transformation and nowadays researchers prefer the so called super-virulent strains which achieve a higher infectivity. These strains incite larger, fast appearing tumours and have a wider host range than other *A. tumefaciens* strains (Komari *et al.*, 1986). Examples of these strains are EHA105 or AGL0 (Wydro *et al.* 2006).

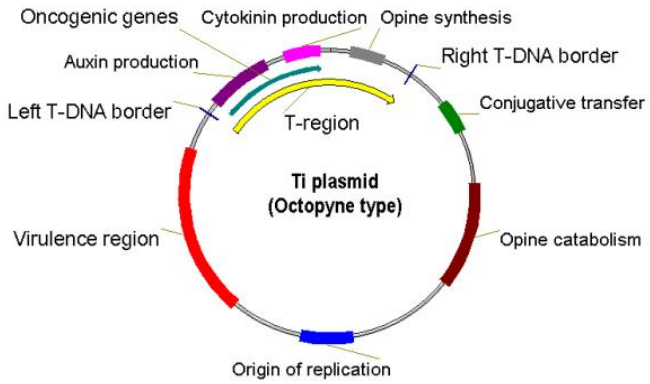

Figure 1: Tumour inducing (Ti) plasmid

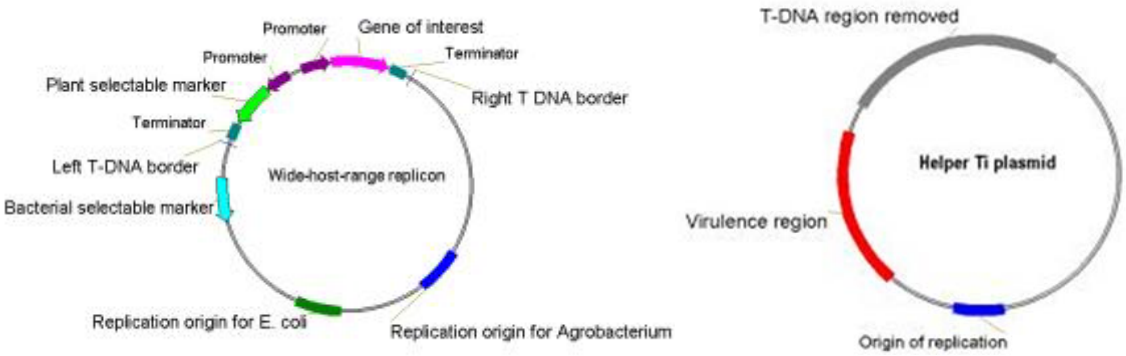

Figure 2: Binary vector

1.3 Reporter genes

Several reporter genes are widely used in plant transformation to monitor developmental and spatial gene expression patterns (Jach *et al*., 2001). β-glucuronidase (GUS), luciferase (LUC) and chloramphenicol acetyltransferase (CAT) have been used for several decades and perhaps are part of the pioneer reporter genes (Jach *et al*., 2001; Töpfer *et al*., 1988). More recently, green fluorescent protein (GFP) isolated from *Aequorea victoria* and dsRed (from *Discosoma* sp) became attractive due to some advantages. For instance, GFP allows non-

destructive detection and does not require specific substrates as compared to former reporters (Jach *et al*., 2001; Naylor, 1999). However, chlorophyll autofluorescence can be problematic when using GFP. The excitation peak wavelength of dsRed (553nm) is above that of chlorophyll and thereby resolving the problem of chlorophyll autofluorescence (Jach *et al*., 2001; Hakkila *et al*., 2002). Table 1 shows a brief description of some of the reporter genes mentioned above. In this study, I will check the usefulness of GFP and DsRed in monitoring transformation efficiency in *Nicotiana benthamiana* and *Petunia hybrida.*

Table 1: Advantages and disadvantages of GUS, GFP and dsRed

Reporter gene	Source	Advantages	Disadvantages
GUS	*Escherichia coli*	▪ Stable, well-characterized	▪ Destructive assay
GFP	*Aequorea victoria*	▪ No substrate addition ▪ Non-destructive	▪ Chlorophyll autofluorescence ▪ Can interfere with regeneration
dsRed	*Discosoma* sp	▪ No substrate addition ▪ Non-destructive ▪ No chlorophyll autofluorescence	▪ Not well-characterized ▪ Interference with plant growth unknown

1.4 Optimising transformation efficiency

Efficient transformation systems are becoming more important with the increasing availability of plant genome sequences. Transformation is used in functional genomics to enable interpretation of the role of genomic sequences to metabolic and physiological functions (Cervera *et al*., 1998; Dan *et al*., 2009). Thus, optimizing *Agrobacterium*-mediated transformation efficiency (TE) is required since it enables more of the targeted cells to take up exogenous DNA. This implies more susceptibility of explant cells to *Agrobacterium* infection. As a consequence, transforming the former recalcitrant species may become more feasible (Cervera *et al*., 1998).

There are several factors that have been reported to influence transformation efficiency. Some of these factors affect *Agrobacterium tumefaciens* virulence and some affect explant cell competence for transformation. More so, some factors affect the regeneration process to produce a transformed callus and subsequently transformed plant. Hence, the success rate of *Agrobacterium*-mediated transformation highly depends on the plant species, bacterial strain, transformation and regeneration conditions (Godwin *et al*., 1991; Krens *et al*., 1988; Sheikholeslam and Weeks, 1987).

In this study I am going to assess the suitability of four compounds as transformation enhancers. These are acetosyringone (Jin *et al*., 2005; Sheikholeslam and Weeks, 1987; Turk *et al*., 1994), 2-aminoindan-2-phosphonic acid (AIP) which is a salicylic acid inhibitor (Mauch-Mani and Slusarenko, 1996), lipoic acid (Dan *et al*., 2009) and paclobutrazol (Xiang *et al*., 2011).

1.4.1 Acetosyringone

Acetosyringone is one of the phenolic compounds exuded from plant wounds that has been reported to induce the expression of *Vir* genes in Ti-plasmid (Jin *et al*.,2005; Turk *et al*., 1994). The induction of *Vir* genes subsequently results in optimization of T-DNA transfer to host cells. Acetosyringone has been reported to increase transformation efficiency when a concentration ranging from 20 - 200 µM is applied to *Agrobacterium* cultures prior to inoculation (Sheikholeslam and Weeks, 1987) or during co-cultivation (Godwin *et al*., 1991). Since the concentration range is quite large, the question that remains is whether higher concentrations still increase or decrease the transformation efficiency. Thus, the effects of concentrations higher than 200 µM are not yet elucidated. In this regard, I hypothesize that an increase of acetosyringone concentration to 400 µM still increases transformation efficiency.

1.4.2 Salicylic acid inhibitor (AIP)

In some crops, endogenous salicylic acid has been proposed to signal the development of systemic acquired resistance to pathogen attacks (Gaffney *et al*., 1993, Malamy *et al*., 1990). In plants, salicylic acid is produced from two pathways via the Shikimate pathway (Figure 3). These are isochorismate and phenylalanine pathways. Isochorismate synthase (ICS) and phenylalanine ammonia-lyase (PAL) enzymes play critical roles in salicylic acid production via these pathways. Therefore, inhibition of these enzymes should reduce salicylic acid accumulation and renders plants with an increased susceptibility to pathogen infections. Mauch-Mani and Slusarenko (1996) reported that pre-treatment of *Arabidopsis* seedlings with 2-aminoindan-2-phosphonic acid (AIP), a highly specific PAL inhibitor made plants completely susceptible to Downy mildew. In this regard, I will check the effect of using AIP as a transformation enhancer whereby explant cells become more susceptible to *Agrobacterium* infection.

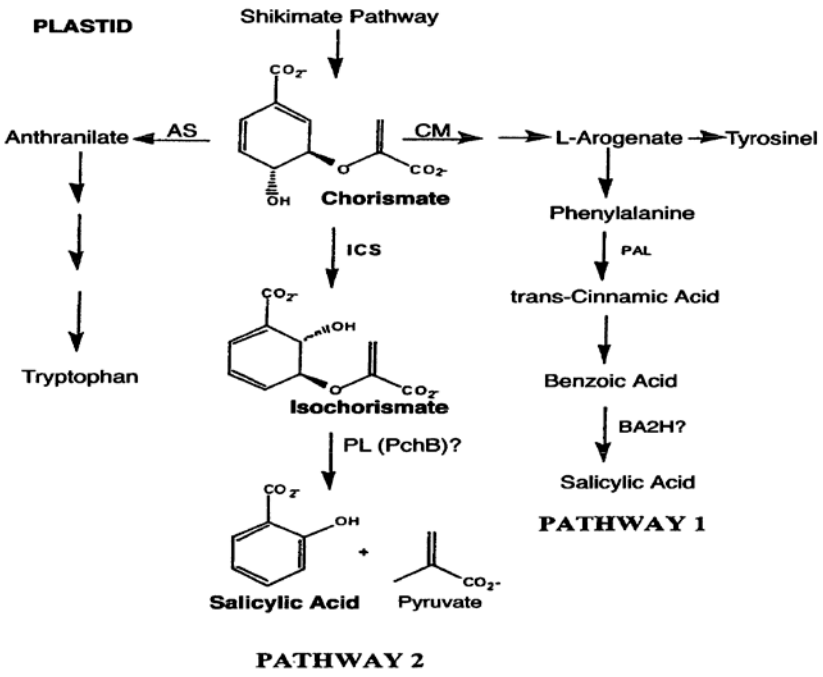

Figure 3: Salicylic acid biosynthetic pathway: Enzymes shown in abbreviation are: anthranilate synthase (AS), benzoic acid 2-hydroxylase (BA2H), , chorismate mutase (CM), isochorismate synthase (ICS), isochorismate pyruvate lyase (PL), and phenylalanine ammonia-lyase (PAL). Figure derived from Wildermuth *et al*. (2001).

1.4.3 Lipoic acid

Lipoic acid is a sulphur-containing compound that has an antioxidative activity (Dan *et al*., 2009). Transformed explants under tissue culture conditions often suffer from tissue browning or necrosis. One of the reasons is that when tissues are excised and inoculated by *Agrobacterium*, they suffer oxidative stress. With its antioxidative capacity, lipoic acid has been reported to reduce tissue necrosis while explants are in regeneration media. This is achieved by scavenging free radicals or reactive oxygen species (ROS). In this study, I would like to analyse the effect of lipoic acid on *Agrobacterium*-mediated transformation, thus checking whether lipoic acid can improve transformation efficiency.

1.4.4 Paclobutrazol

Paclobutrazol is a plant hormone known to retard plant growth. It has also been reported to increase the antioxidant enzyme activity (Jaleel *et al*., 2007; Kraus and Fletcher, 1993). This is achieved by increasing the activity of superoxide dismutases and ascorbate peroxidases that reduce the ROS. Xiang *et al.* (2011) have also reported paclobutrazol as an inhibitor for salicylic acid biosynthesis. This is achieved by inhibiting benzoic acid 2-hydroxylase (BA2H) that converts benzoic acid to salicylic acid. For this study, paclobutrazol assumes a function similar to AIP.

1.5 Study objectives

- To check the usefulness of GFP and dsRed in monitoring transformation efficiency.
- To test the effect on gene transfer of increasing the acetosyringone concentration, of using lipoic acid, salicylic acid inhibitor (AIP) and paclobutrazol in inoculation and/ or co-cultivation medium.
- To monitor the effect of the above mentioned objectives in two plant species: *Nicotiana benthamiana* and *Petunia hybrida.*

2. Materials and Methods

2.1 Plant material

To analyse the effects of different compounds mentioned in the study objectives, two plant species were used. These *are Nicotiana benthamiana* and *Petunia hybrida*. These species are generally regarded as not recalcitrant to *Agrobacterium*-mediated transformation. *P. hybrida* was sown in the greenhouse in pots whereas *N. benthamiana* was grown *in vitro*. Transformation was done using fresh unblemished leaves.

2.2 Culture of *Agrobacterium tumefaciens*

In this study, two *Agrobacterium* strains were used for transformation, Agl0(pMF1-JS14) and Agl1(pBinGlyRed-Asc1) containing the plant binary Ti plasmid vectors with genes encoding for GFP and dsRed respectively (Figure 4). In addition to GFP gene, plasmid pMF1-JS14 also contained the GUS reporter gene. The strains were grown in Luria Broth (LB) medium with filter sterilized kanamycin and rifampicin antibiotics at a concentration of 50 mg/l. This was grown overnight at 28^{o}C in a shaker (150 rpm) in a total volume of 10 ml (LB + antibiotics) in 50 ml tubes. Glycerol stock solutions were prepared for long term storage by adding 150 µl sterile 98% glycerol to 850 µl bacterial suspension in 2 ml eppendorf tubes and vortexing well to mix the solution. The tubes were stored at -80^{o}C. For transformation, overnight cultures were prepared from these glycerol stock as described above.

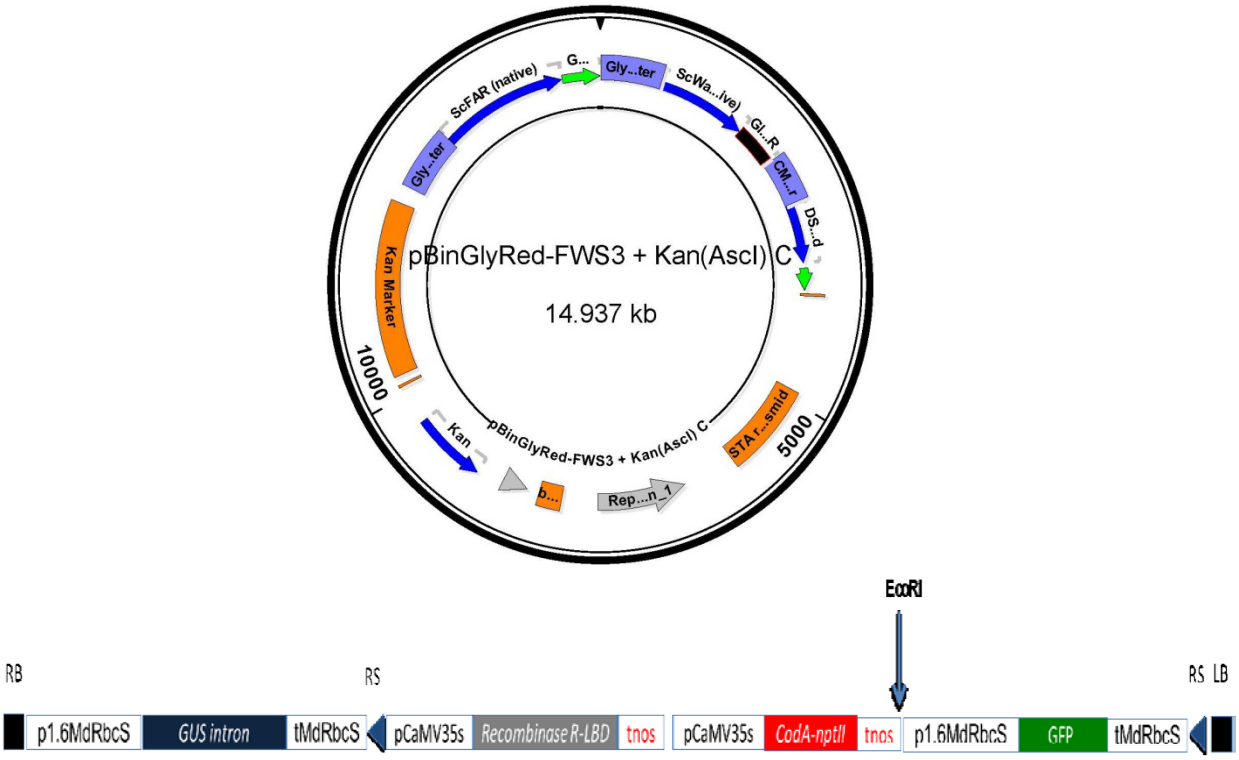

Figure 4: Binary vectors, pBinGlyRed-Asc1 (top) and pMF1-JS14 (bottom). pBinGlyRed-Asc1 contains DsRed gene, 35S CaMV promoter, two jojoba genes (*ScFAR* and *ScWS*), a selectable marker: the *nptII* (*neomycin phosphotransferase II*) gene. pMF1-JS14 contains several genes (*Recombinase R-LBD, CodA-nptII*, GFP and GUS) and a 35s promoter and NOS terminator.

2.3 *A. tumefaciens*-mediated transformation

2.3.1 *Petunia hybrida*

The procedure for transforming *Petunia* was derived from Horsch and Klee (1986). Fresh and unblemished leaves were harvested after about five weeks after sowing. Following sterilization with 1% (w/v) NaClO plus 0.1% (v/v) Tween 20 for 20 minutes, the leaves were rinsed three times with sterile distilled water. Leaf discs (6 mm diameter) were cut excluding the midrib and placed upside down on medium containing Murashige and Skoog (MS) basal salts, Vitamin B5 (pantothenic acid), 3% sucrose, 4.44 µM benzylaminopurine (BAP), 0.54 µM naphthalene acetic acid (NAA), 0.8% micro agar. The pH was adjusted to 5.8 in all media throughout the experiments. After two days at 24^{o}C, only viable expanding discs were incubated with one of the two *Agrobacterium* strains. An overnight culture was poured over discs and shaken for few seconds and then the leaf discs were blotted dry on sterile filter paper. Three Petri dish replicates each containing 20 leaf discs were used for each *Agrobacterium* strain. Controls were included by inoculating discs with LB medium instead of the *Agrobacterium*. The Petri dishes were incubated at 24^{o}C, wrapped in aluminium foil to keep them in darkness. After three days of co-cultivation with *Agrobacterium*, the discs were transferred to selection media, prepared the same as above but with the addition of 200 mg/l cefotaxime, 100 mg/l timentin and 200 mg/l kanamycin. The first two antibiotics control bacterial overgrowth and kanamycin was included for selection of stable transformants. Transient and stable GFP and dsRed expression was checked with a fluorescence microscope at 48 hours and between 20 - 30 days after transformation. The number of explants showing GFP and dsRed fluorescence as well as survival rate on selection media was determined to deduce the reliability of each reporter gene in monitoring transformation of *P. hybrida.*

2.3.2 *Nicotiana benthamiana*

Fresh *in vitro* grown leaves were collected and put in a Petri dish containing about 5 to 10 ml callus inducing medium (CIM). The CIM contained 4.4 g/l MS medium, 30 g/l sucrose, 5.37 µM NAA and 0.89 µM BAP. The leaves were placed upside down in CIM. The midrib was removed before cutting the leaf discs of about 1 cm^2. 100 µl *Agrobacterium* culture was diluted in 5 ml CIM to an OD_{600} of approximately 0.1 and this was poured over the leaf discs allowing them to float. The Petri dishes were wrapped in parafilm and aluminium foil to keep them in darkness and incubated at 24^{o}C for 3 days. After 3 days of co-cultivation with *Agrobacterium*, the discs were blotted with sterile Whatmann filter paper before placing them upside up to Petri dishes containing shoot inducing medium (SIM). The SIM was prepared by adding 4.4 g/l MS medium, 30 g/l sucrose, 0.54 µM NAA, 4.44 µM BAP, 8 g/l micro agar and pH adjusted to 5.8. In addition, 500 mg/l cefotaxime and 50 mg/l kanamycin were added after autoclaving. Control discs were divided into two treatments: grown on SIM

with and without kanamycin. Expression of GFP and dsRed was checked using the same method as for *P. hybrida*.

2.4 The effects of acetosyringone, lipoic acid, salicylic acid inhibitor (AIP) and paclobutrazol on transformation efficiency

Similar transformation procedures for both plant species were used in this analysis as described above. Separate experiments were done for analysing the effects of these compounds during transformation. For acetosyringone, three concentrations were analysed: 0, 100 and 400 µM. All these compounds were added in co-culture media. Lipoic acid and AIP were added at concentrations of 0, 10 and 50 µM (Dan *et al*., 2009; Mauch-Mani and Slusarenko, 1996). Paclobutrazol was applied at concentrations of 0.5 and 5 mg/l. Transient GFP and dsRed expression was scored only for the acetosyringone experiment. This is because the microscope UV-light unit broke down during the experiments. Since Agl0(pMF1-JS14) strain also contained GUS gene in addition to GFP, transient transformation assays were done using GUS staining in downstream experiments. However, transformation efficiency was determined from the number of explants surviving on selection media about 20 days post-transformation. Analysis of Variance (ANOVA) was performed using SPSS statistical package.

3. RESULTS

3.1 The usefulness of GFP and DsRed in monitoring transformation efficiency

Table 2 shows transient expression of dsRed after 48 hours of co-culture with *Agrobacterium* in *Petunia hybrida* and *Nicotiana benthamiana*. The larger the number of spots the higher the transformation efficiency. The majority of explants had 1 – 10 dsRed spots in both species. Due to technical problems with the GFP filter settings on the microscope, transient GFP expression was not recorded in this first experiment. However, in later experiments it was possible to visualize GFP fluorescence spots after 48 hours. In comparison, dsRed fluorescence spots were more clearly visible than GFP spots (Figure 5). This figure also shows stable transformation after 25 days of inoculation with *Agrobacterium*.

Table 2: Transient expression of GFP and dsRed reporter genes in *P. hybrida* and *N. benthamiana* 48 hours post inoculation with *A. tumefaciens*

Agrobacterium strain	Reporter gene	Plant species	No of explants tested	Explants without spots	Explants with 1-10 spots	Explants with 10-20 spots	Explants with >20 spots
Agl0(pMF1-JS14)	GFP	Nicotiana	60	-	-	-	-
		Petunia	60	-	-	-	-
Agl1(pBinGlyRed-Asc1)	dsRed	Nicotiana	60	14	23	13	10
		Petunia	60	21	27	8	4

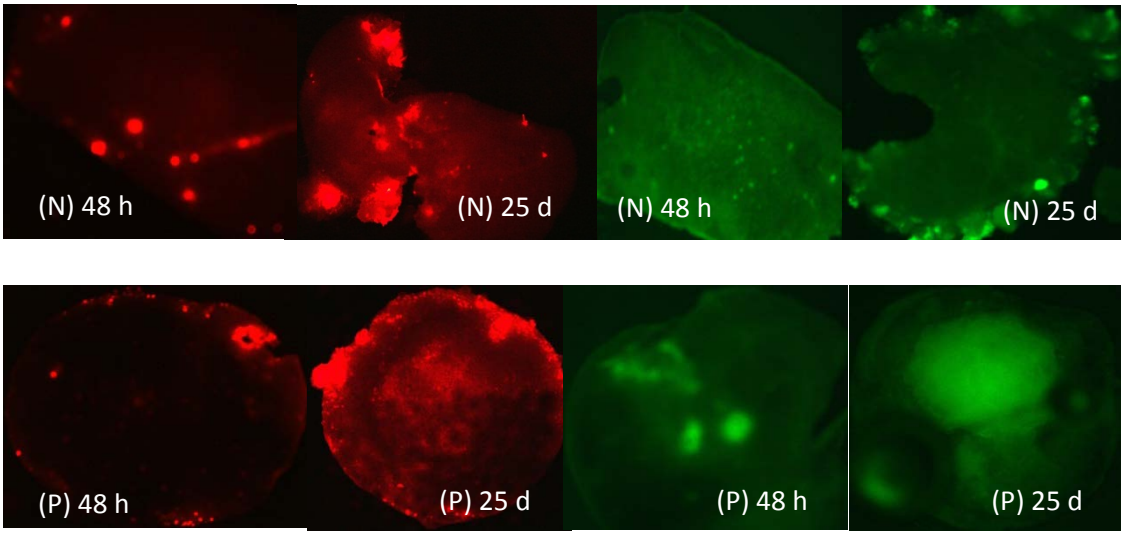

Figure 5: GFP and dsRed expression in *Petunia* (P) and *Nicotiana* (N) explants at 48 hours and 25 days after inoculation with *Agrobacterium tumefaciens*

3.2 The effect of acetosyringone on transient GFP and dsRed expression

To measure the effects of acetosyringone on transient GFP and dsRed expression, the number of fluorescence spots on each explant was determined. Low transient expression had two categories: 0 spots and 1 -10 spots whereas high transient expression ranged from 10 to over 20 spots. Figure 6 is an illustration of how the fluorescence spots were counted. In both species, the mean number of GFP spots ranged from 0 – 10 across all acetosyringone concentrations (Figure 7 and 8). This is contrary to dsRed as over 20 fluorescence spots could be counted. The average number of explants without fluorescence spots was significantly higher ($P<0.05$) for 0 µM acetosyringone than for 100 and 400 µM in all species. In *Petunia*, there was no significant difference between 100 and 400 µM on both *Agrobacterium* strains (Figure 7). However, the average number of explants with > 20 spots was higher with 400 µM than 100 µM although this was not significantly different.

In *N. benthamiana* (Figure 8), the average number of explants with and without spots was not significantly different between 100 and 400 µM with Agl0(pMF1-JS14). However, this was not the case with Agl1(pBinGlyRed-Asc1) where a significantly higher transient expression (10 to >20 spots) was observed with 400 µM. More so, all the explants treated with 400 µM acetosyringone concentration were transiently transformed.

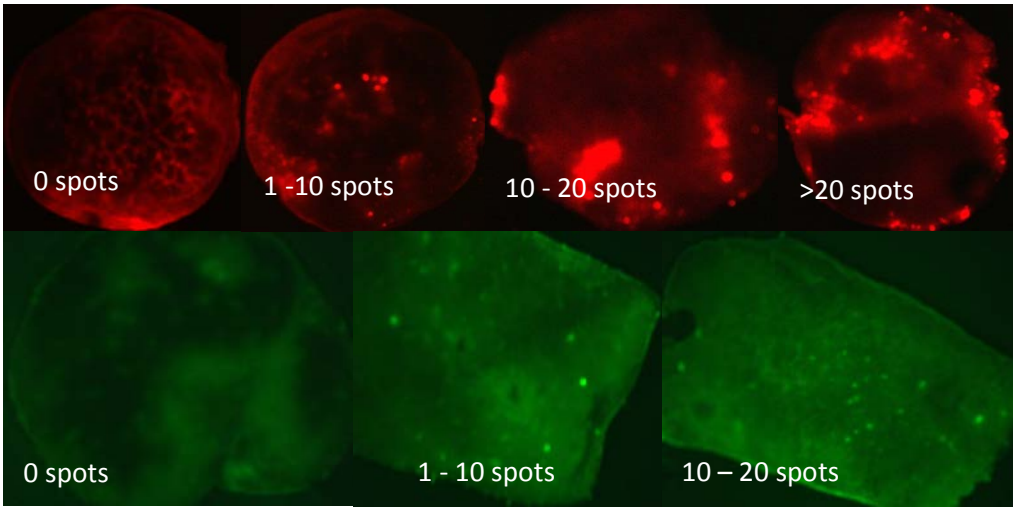

Figure 6: Illustration of how the number of GFP and dsRed fluorescence spots was determined.

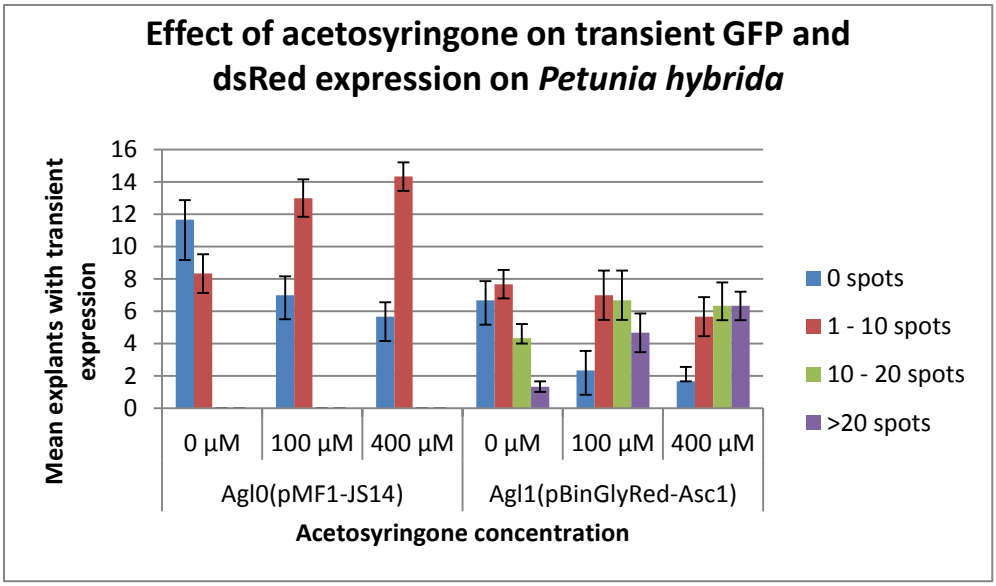

Figure 7: The effects of acetosyringone on transient GFP and dsRed expression on *Petunia hybrida* observed as mean±SE number of explants with fluorescence spots.

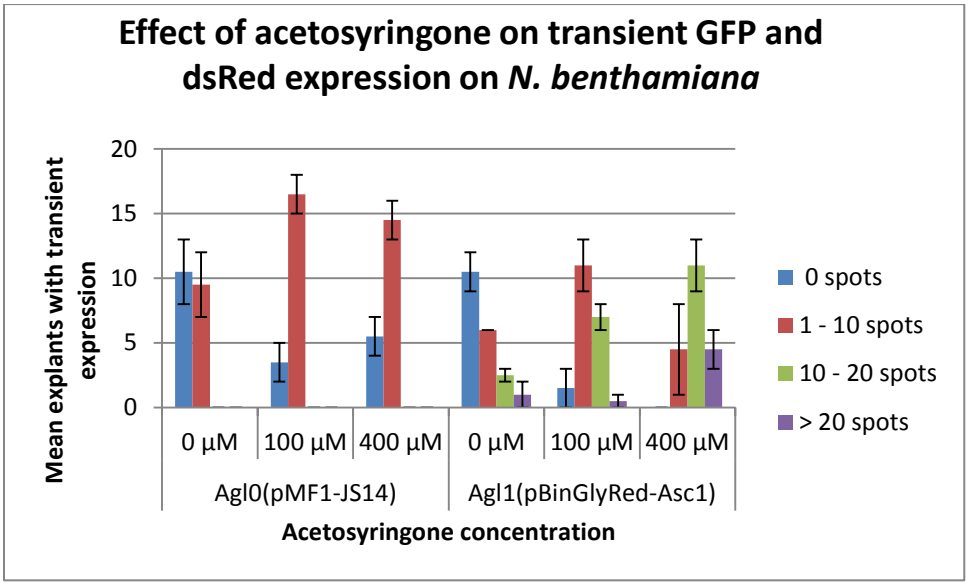

Figure 8: The effects of acetosyringone on transient GFP and dsRed expression on *N. benthamiana* observed as mean±SE number of explants with fluorescence spots.

3.3 The effect of acetosyringone on stable transformation efficiency

The average number of explants surviving on selection media was significantly lower ($P<0.05$) on explants without any acetosyringone treatment (0 µM) than with AS treatment (100 and 400 µM) (Table 3 and Figure 9). There was no significant difference between 100 and 400 µM in the means of explants surviving on kanamycin. This was observed between both species and *Agrobacterium* strains.

Table 3: The effect of acetosyringone on stable transformation efficiency. The means with different letters represent significantly (P<0.05) different.

Plant species	Agrobacterium strain	AS concentration	Explants tested on Kanamycin	Replicates	Mean No. of Explants surviving on Kanamycin.	% Transformation efficiency
Petunia	Agl0(pMF1-JS14)	0 µM	20	3	5.3±0.88 [a]	26.5
		100 µM	20	3	8.7±1.20 [b]	43.5
		400 µM	20	3	7.7±0.88 [b]	38.5
	Agl1(pBinGlyRed-Asc1)	0 µM	20	3	4.3±1.20 [a]	21.5
		100 µM	20	3	8.0±1.00 [b]	40.0
		400 µM	20	3	8.7±1.76 [b]	43.5
Nicotiana	Agl0(pMF1-JS14)	0 µM	20	3	5.7±1.20 [a]	28.5
		100 µM	20	3	11.3±1.45 [b]	56.5
		400 µM	20	3	9.3±2.03 [b]	46.5
	Agl1(pBinGlyRed-Asc1)	0 µM	20	3	5.3±1.20 [a]	26.5
		100 µM	20	3	9.0±1.00 [b]	45
		400 µM	20	3	8.0±1.73 [b]	40

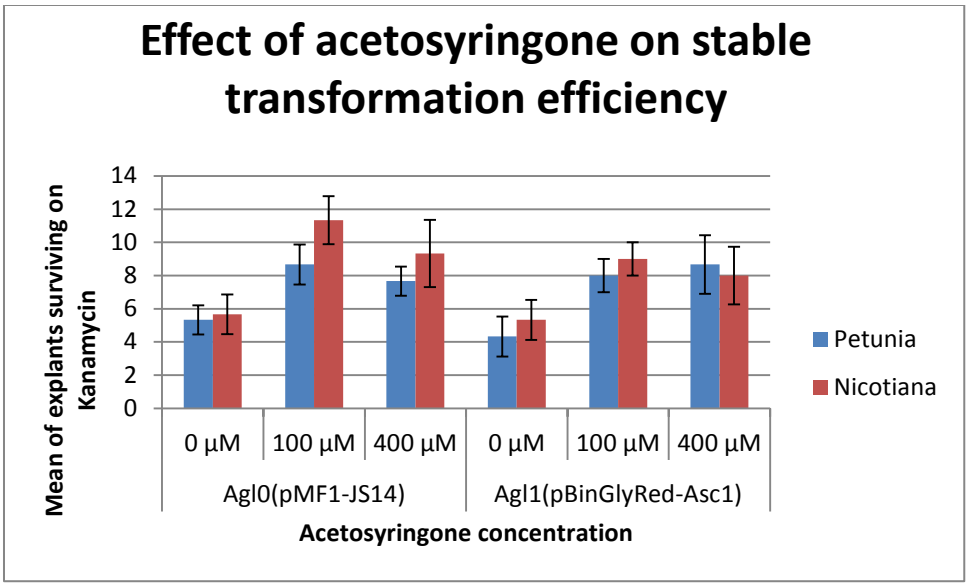

Figure 9: The effect of varying acetosyringone concentrations on stable transformation efficiency in *P. hybrida* and *N. benthamiana* species.

3.4 The effects of lipoic acid on transformation efficiency

Transient GFP and dsRed expression was impossible in this experiment due to technical problems with the microscope. Only stable transformation efficiency was determined using *N. benthamiana* because *Petunia* explants unexpectedly died in due course. Table 4 and Figure 10 show the average number of explants surviving on selection media and hence transformation efficiency. The highest average number of explants surviving on kanamycin was observed with 50 µM and 10 µM using Agl0(pMF1-JS14) and Agl1(pBinGlyRed-Asc1) respectively. However, only 50 µM achieved a significantly different effect ($P<0.05$) from 0 and 10 µM using Agl0(pMF1-JS14). The effect of 10 µM using Agl1(pBinGlyRed-Asc1) was not significantly differently from 0 and 50 µM. The explants treated with 10 and 50 µM were greener and more vigorous, and produced healthier calluses than explants on media without lipoic acid. This was observed on explants treated with Agl0(pMF1-JS14) (Figure 11).

Table 4: The effect of lipoic acid on transformation efficiency of *N. benthamiana*, 20 days after inoculation with *Agrobacterium*

Plant Species	*Agrobacterium* strain	Lipoic acid concentration (µM)	No. of explants tested	Reps[y]	Explants surviving on kanamycin (mean ± SE)	% TE[z]
Nicotiana	Agl0(pMF1-JS14)	0	20	3	6.5±1.5 [a]	32.5
		10	20	3	9±1 [a]	45
		50	20	3	12.5±0.5 [b]	62.6
	Agl1(pBinGlyRed-Asc1)	0	20	3	7±1 [a]	35
		10	20	3	10.5±2.5 [a]	52.5
		50	20	3	7.5±0.5 [a]	37.5

[y] is Replicates, [z] is transformation efficiency. Means with different letters denote significant difference ($P<0.05$).

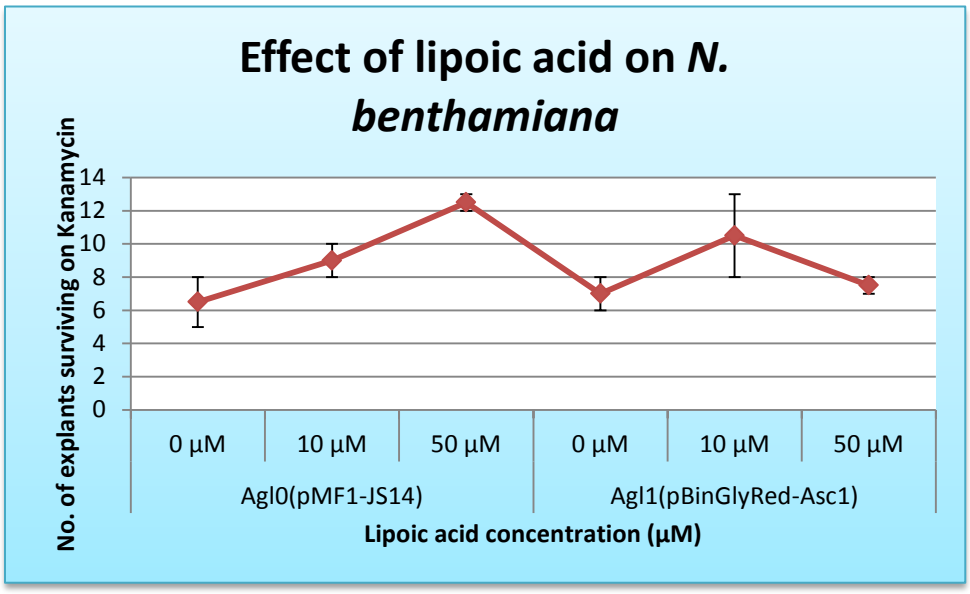

Figure 10: Graphical illustration of the effect of lipoic acid on stable transformation efficiency in *N. benthamiana*, 20 days after inoculation with *Agrobacterium*

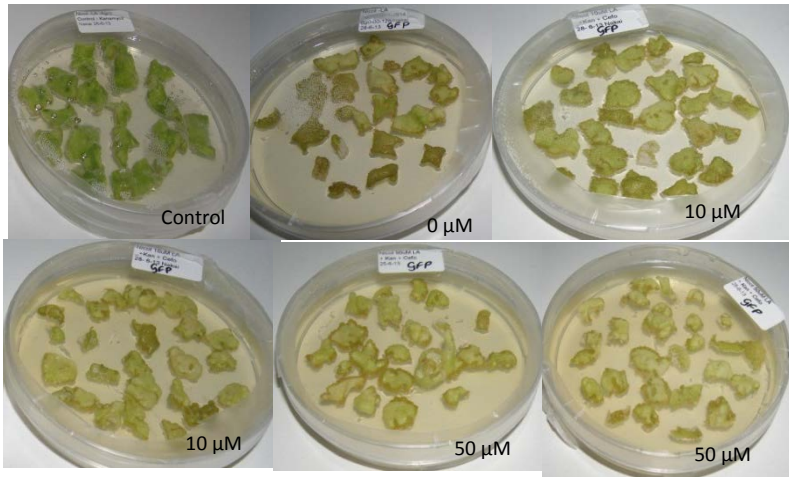

Figure 11: Explants in Petri dishes treated with different lipoic acid concentrations. Control was not treated with *Agrobacterium*, lipoic acid or kanamycin.

3.5 The effects of salicylic acid inhibitor (AIP) on transformation efficiency

3.5.1 Effect of AIP on transient GUS expression 48 hours after inoculation with *Agrobacterium*

Figure 12 below shows the intensity of GUS staining on *Nicotiana* (N) and *Petunia* (P) explants treated with different AIP concentrations. Staining on *Petunia* was not that intense as compared to *Nicotiana* and hence less visible. I observed that the stain intensity for *Nicotiana* explants that were treated with 50 µM AIP was higher than for 0 and 10 µM. More so, this pattern is also observed in Figure 13 where the average number of explants showing any GUS staining is highest with 50 µM AIP and significantly different from 0 and 10 µM. Thus, GUS expression increased with AIP concentration. In *Petunia,* 10 µM resulted in highest GUS expression and this was significantly different from 0 and 50 µM .

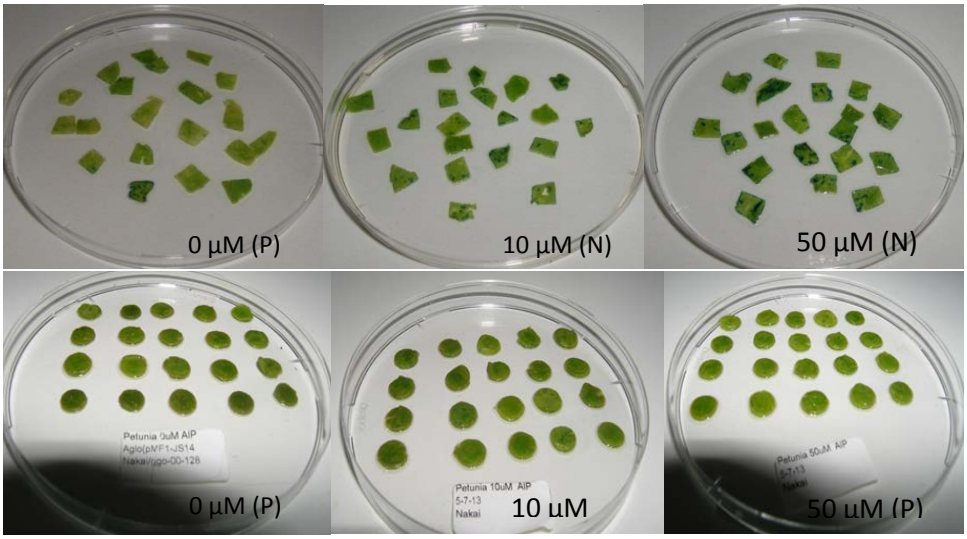

Figure 12: Plates showing transient GUS expression on *Nicotiana* (N) and *Petunia* (P) explants treated with different AIP concentrations during co-cultivation with *Agrobacterium* strain Agl0(pMF1-JS14).

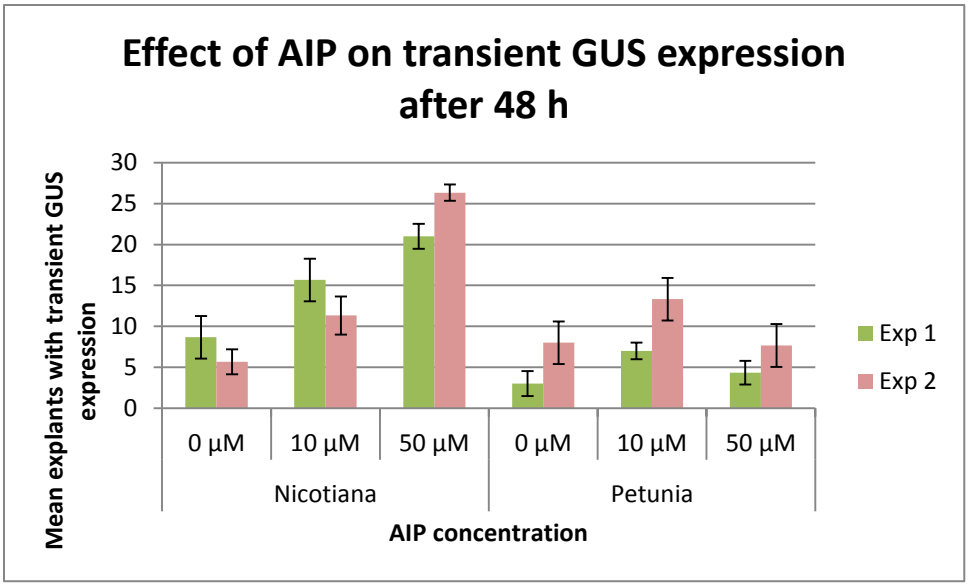

Figure 13: Graph showing the effect of AIP on transient GUS expression after 48 h of co-cultivation with *Agrobacterium* strain Agl0(pMF1-JS14) done in two experiments (Exp 1 and 2).

3.5.2 Effect of AIP on stable transformation efficiency

Due to time limitation, the results of this experiment were recorded 13 days after transformation. Only the number of explants with viable growing calluses on media containing kanamycin was recorded, instead of the number of explants surviving on kanamycin. Figure 14 illustrates how this was determined, showing explants with and without growing calluses on media containing kanamycin. Table 5 shows the percentage transformation efficiency achieved by different AIP concentrations. In both species, the explants treated without AIP had the lowest transformation efficiency of 13.3% (*Petunia*) and 30.0 % (*Nicotiana*). In contrast to transient GUS expression assay, there were almost no differences between 10 and 50 µM AIP in both species.

Table 5: The effect of AIP on stable transformation efficiency

Plant species	*Agrobacterium* strain	AIP concentration	Explants tested on Kanamycin	No. of Explants with surviving calli	% Transformation efficiency
Petunia	Agl0(pMF1-JS14)	0 µM	60	8	13.3
		10 µM	100	29	29.0
		50 µM	72	21	29.2
Nicotiana	Agl0(pMF1-JS14)	0 µM	80	24	30.0
		10 µM	60	41	68.33
		50 µM	60	42	70.0

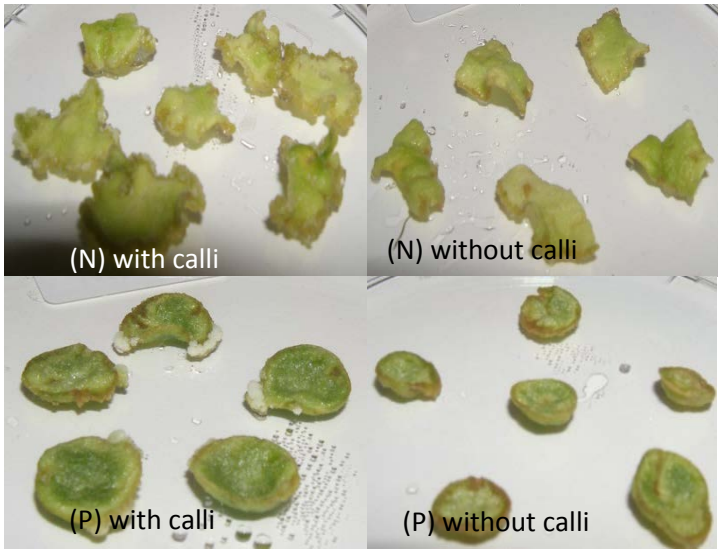

Figure 14: *Nicotiana* (N) and *Petunia* (P) explants with and without growing calli on kanamycin media

3.6 The effects of paclobutrazol on transformation efficiency

The effects of paclobutrazol in this project were not elucidated because the compound that was in stock had expired and had no time left to order new compound. I tried to use the one in stock for transient GUS expression with *Nicotiana* explants but all the explants became greasy and died during inoculation. This may have been caused by the expiry of this compound.

4. DISCUSSION

The expression of GFP and dsRed in *Petunia* and *N. benthamiana* explants after 48 h and 25 days after transformation with *Agrobacterium* confirms the usefulness of these reporter genes in both transient and stable *Agrobacterium*-mediated transformation assays. At 48 h, fluorescence spots indicate transformed cells by *Agrobacterium* and after 25 days, the spots become larger and clearly visible (Figure 5) because of cell proliferation from stably transformed cells. Although both reporter genes can be useful in these species, dsRed is more reliable than GFP for transient expression. This is because dsRed mitigates the chlorophyll autofluorescence drawback often encountered when using GFP. However, as the transformed cells keep on dividing producing calli, GFP expression can be confirmed with much confidence and distinguished from other fluorescing organelles. In comparison, chlorophyll autofluorescence was more pronounced in *Petunia* than *N. benthamiana* explants. This is because *Petunia* leaves are darker in colour compared to the latter, probably because of a higher chlorophyll content. More chlorophyll results in a higher level of autofluorescence.

It is hypothesized in this study that acetosyringone increases transformation efficiency when included in co-culture media during transformation by *Agrobacterium*. From the transient expression results, fluorescence spots ranged from 0 to 10 for GFP and from 0 to >20 for dsRed. In first instance, this may indicate a lower transformation when explants are transformed with Agl0(pMF1-JS14) strain than with Agl1(pBinGlyRed-Asc1). This is not true since this pattern was not observed on the stable transformation efficiency. This may have been a result of differences in reliability of these two reporter genes as discussed in the above paragraph. Interestingly, the average number of explants without any fluorescence spots was significantly higher for 0 µM acetosyringone than for 100 and 400 µM for all strains and species. This confirms the results from earlier reports (Godwin *et al*., 1991; Sheikholeslam and Weeks, 1987) that this wound response molecule increases transformation efficiency by activating *Agrobacterium* virulence genes. In *Petunia hybrida*, there was no significant difference in transient GFP and dsRed expression between 100 and 400 µM. This was also the case in *N. benthamiana* with GFP but a significantly higher dsRed transient expression was observed with 400 µM. Acetosyringone treatment (100 and 400 µM) increased the number of explants that were stably transformed. Since there were no significant differences in the effect of these two concentrations on stable transformation efficiency of both species, and also on transient expression in *Petunia*, the use of 400 µM seems not to increase the overall transformation efficiency.

Lipoic acid was reported by Dan *et al.* (2009) as a transformation enhancer in soybean, tomato, wheat and cotton. In this report, its usefulness in enhancing transformation efficiency was examined in *N. benthamiana*. The average number of explants surviving on kanamycin was directly proportional to lipoic acid concentrations using Agl0(pMF1-JS14) strain, with 50 µM attaining the highest transformation efficiency (Figure 10). This

observation suggests an increase in transformation efficiency when this compound is included in co-culture media. Since lipoic acid is known as a metabolic antioxidant (Dan *et al*. 2009), it may reduce oxidative stress imposed to cells or tissues by *Agrobacterium* during the transformation process. Hence reduction in oxidative stress consequently reduces cell death, and promotes proliferation of transformed cells and tissues. The addition of lipoic acid (both 10 and 50 µM) achieved healthier explants with viable calluses (Figure 11). With Agl1(pBinGlyRed-Asc1) strain, 10 µM lipoic acid treatment had the highest number of explants survival but this was not significantly differently from 0 and 50 µM. This may suggests differences in optimum lipoic acid concentrations between these two strains.

Transient GUS expression assay was done using explants of both species treated with different AIP concentrations. In *Nicotiana*, explants treated with 50 µM AIP had the highest number of GUS stained explants as well as stain intensity (Figure 12 and 13). AIP inhibits the production of salicylic acid (Mauch-Mani and Slusarenko, 1996). Consequently, the plant cells become more susceptible to pathogen attack because of reduced systemic acquired resistance. Since the pMF1-JS14 plasmid contains a GUS gene intron, no GUS expression is expected in *Agrobacterium* cells. This is because *Agrobacterium*, as a prokaryote lacks the eukaryotic RNA splicing apparatus (Dan *et al.*, 2009). Therefore, the increase in transient GUS expression with AIP concentration shows an increase in transformation efficiency when AIP is used in co-culture media during *Nicotiana* transformation. In *Petunia*, the GUS expression was less than in *Nicotiana* probably because of the differences in these species in susceptibility to *Agrobacterium*. In Petunia, 10 µM AIP concentration showed a higher GUS expression and this may be the optimum concentration for this species.

The number of explants with growing calluses on kanamycin was determined 13 days after transformation (Table 5). Although there were significant differences between 10 and 50 µM AIP concentrations in transient GUS expression, there were almost no differences in stable transformation efficiency between 10 and 50 µM AIP in both species. However, cultures without AIP in both species showed a significantly lower transformation efficiency which is congruous with GUS expression assay results. Thus as described above, the inclusion of AIP in the co-culture media seems to increase the susceptibility of cells to *Agrobacterium* attack, and hence an increased transformation efficiency. Nevertheless, differences between species may be a result of differences in AIP optimum concentrations. Since paclobutrazol also inhibits one of the enzymes in salicylic acid biosynthesis, its usefulness in enhancing transformation efficiency has to be elucidated as well in order to confirm how AIP resulted in a higher transformation efficiency.

5. CONCLUSIONS AND RECOMMENDATIONS

GFP and dsRed are useful reporter genes in *P. hybrida* and *N. benthamiana* for both transient and stable *Agrobacterium*-mediated transformation assays. However, dsRed is more reliable than GFP. This is because its excitation peak wavelength is above that of chlorophyll and thus resolves the problem of chlorophyll autofluorescence often encountered with GFP.

Acetosyringone increases transformation efficiency in *P. hybrida* and *N. benthamiana*. However, increasing this compound from 100 to 400 µM does not increase transformation efficiency in both species.

In *N. benthamiana*, lipoic acid increases transformation efficiency using Agl0(pMF1-JS14) strain with 50 µM attaining the highest transformation efficiency. This compound also reduces tissue browning and necrosis when included in co-culture media. This is not yet elucidated in *Petunia hybrida*. Repeating this experiment is necessary to affirm the validity of these results.

The inclusion of AIP in co-culture media during transformation enhances transformation efficiency in *P. hybrida* and *N. benthamiana*. However, the optimum concentration workable with these species still needs to be validated. Moreover, the usefulness of paclobutrazol has to be investigated as well in order to confirm the influence of the salicylic acid pathway on transformation efficiency.

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