# The effect of salicylic acid and lipoic acid on *Agrobacterium*-mediated transformation in *Petunia hybrida* and *Nicotiana benthamiana*

MSc thesis

By

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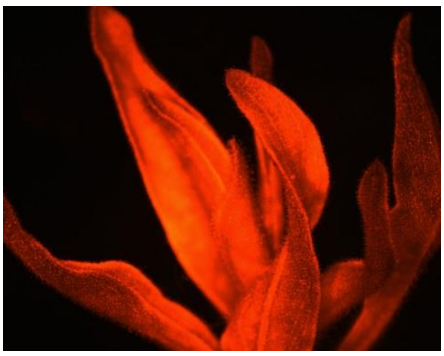
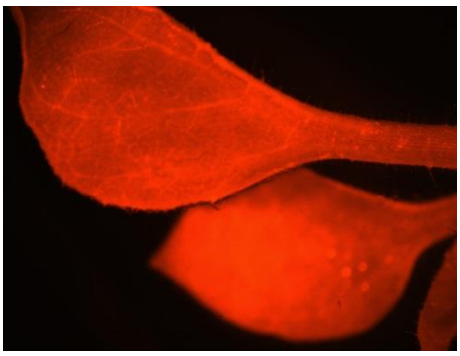


(*Petunia hybrida*)



(*Nicotiana benthamiana*)

Wageningen University and Research Center Plant breeding, The Netherlands

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## Abstract

*Agrobacterium*-mediated transformation plays a curial role in microbiology, biotechnology, 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. Nevertheless, some economically important plant species are suffered a low transformation efficiency by using *Agrobacterium*-mediated transformation. This paper reports the effect of paclobutrazol (PBZ) and 2-aminoindan-2-phosphonic acid (AIP) as salicylic acid (SA) inhibitor and lipoic acid (LA) on *Agrobacterium*-mediated transformation in *Petunia hybrida* and *Nicotiana benthamiana.* Explants from unblemished fresh leaves were inoculated with cultures of Agl1 (pBinGlyRed-Asc1) strains which contain the plant binary Ti plasmid vectors with gene encoding for dsRed. For each compound, two exposure time (long and short) and varying concentrations (0μM, 1μM, 10μM, 50μM and 100μM) were tested. Transformation efficiencies were calculated by counting dsRed spots via fluorescence microscope, stable transformation by counting the number of explants surviving on selection media and ultimate transformation by counting the number of regeneration shoots from callus on selection media. Leaves from transformant shoots were collected to run PCR to confirm genetic transformation. In *P.hybrida* and *N.benthamiana*, PBZ and AIP block SA biosynthesis and increase the *Agrobacterium*-mediated transformation efficiency, the optimum concentration and exposure time that worked were variable. And adding SA was lead to low Agrobacterium-mediated transformation efficiency. In both two species, explants were more vigorous and less browning and necrosis by adding LA. For *N. benthamiana*, LA increased the transformation efficiency at the concentration level of 100μM. But for *P.hybrida*, the LA has less efficiency. PCR result indicated that a transformant shoot with observed fluorescence could be transgenic, but a transformant shoot without fluorescence could also be transgenic plant due to gene silencing. Due to time limitation, the PCR for regeneration shoots from *N.benthamiana* was not included.

**Key words**: *Agrobacterium*-mediated transformation; *Petunia hybrida*; *Nicotiana benthamiana*; salicylic acid; 2-aminoindan-2-phosphonic acid; Paclobutrazol; lipoic acid; plant defence transformation efficiency.

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## Acknowledgements

I would like to thank my supervisors Dr. Frans Krens, Nur Fatihah Hasan Nudin, and Bernadette van Kronenburg for guidance, support, patience and encouragement. Thanks Linda Kodde for guidance me in DNA extraction and PCR. I also want to thank all the member of the Ornamentals, Tissue culture and Gene transfer research group of Wageningen UR Plant Breeding for the assistance during the laboratory experiments.

## List of abbreviation

SA = Salicylic acid

AIP = 2-aminoindan-2-phosphonic acid

PBZ = Paclobutrazol

LA = Lipoic acid

%TE = Stable transformation efficiency

%RE = Ultimate transformation efficiency

Ept = Exposure time

Cpd. = Compound

Conc. = Concentration

NOE= No. of explant tested

NOS= No. of dsRed spot

NOEcalli4w = No. of explants + calli grown in SM/SIM medium for four weeks

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# 1. Introduction

## 1.1 *Agrobacterium*-mediated transformation

Plant genetic engineering allowed introduction of genes into crop plants from related or unrelated plants or even a non-plant organism, and result in better agronomical traits. Introduction of genes of interests into the target cell by *Agrobacterium tumefaciens* is one of the major methods for delivering exogenous DNA to plant cells (Barampuram and Zhang, 2011). Up to now, the importance of *Agrobacterium*-mediated transformation is increasing constantly, with an ever increasing acreage in the world on which transgenic varieties of economically important crops such as potatoes, tomatoes, corns, cotton, canola and soybeans are cultivated in many countries (Gelvin, 2003). *Agrobacterium*-mediated transformation is important for microbiology, biotechnology, plant pathology and genetics (Dan et al., 2009). This method has many advantages over direct DNA delivery systems, for example, it has the ability to transfer large intact DNA segments, it yields insertions with defined ends and at low copy number, stable integration and inheritance and consistent expression of the transferred DNA for many generations (Barampuram and Zhang, 2011). Nevertheless, in some monocots and recalcitrant plant species, *Agrobacterium*-mediated transformation achieved lower rate of success. Therefore, direct DNA transfer methods like micro-particle bombardment, electroporation, microinjection have been developed (Barampuram and Zhang, 2011).

## 1.2 The transfer DNA (T-DNA) and reporter genes

*Agrobacterium* is widely used for DNA delivery system for plant (Ziemienowicz et al., 2001). Many plant biologists exploit *Agrobacterium tumefaciens* to introduce DNA into host plants for molecular and genetic studies (Zupan and Zambryski, 1995). It induced tumorigenesis in dicotyledonous plant and cause the crown gall disease (Horsch and Klee, 1986; Zupan and Zambryski, 1995). The plasmid of *A.tumefaciens* is called the tumor-inducing (Ti) plasmid (Figure 1) (Hooykaas, 1989) which influence its tumor-inducing capability (Barampuram and Zhang, 2011). Ti plasmid contains a form of transferred DNA (T-DNA) which includes two types of genes: oncogenic genes and opine synthesis genes and both are located inside the T-DNA of the Ti plasmid. In addition, the T-DNA is referred to as the T-region when it is located on the Ti plasmid (Gelvin, 2003). The oncogenic genes encode enzymes which are involved in auxin and cytokinin biosynthesis (Barampuram and Zhang, 2011). The virulence (*vir*) region of the Ti plasmid is responsible to rendering the plant cell susceptible to *A.tumefaciens* transformation (Stachel and Zambryski, 1986), and generation of the T-strand which is integrated as the T-DNA into host cells (Krens et al., 1982). Virulence gene is triggered by host-derived phenolic compounds by plant wound (Gelvin, 2003; Lee et al., 1992), therefore virulence of *Agrobacterium* is activated by the plant exudates. In plant genetic engineering, the Ti plasmid has been modified by deletion of either the complete set of oncogenes or the tumorigenic T-DNA to produce a disarmed Ti plasmid (Sheikholeslam and Weeks, 1987; Torisky et al., 1997). Furthermore, the T-region and *Vir* genes could be separated into two different replicons by generation a binary vector system: one vector carrying the *Vir* genes (the replicon contained the *Vir* gene became the *Vir* helper), and the other vector carrying the genes-of-interest to be transferred (T-DNA). Moreover, in the vector the binary vector the genes-of interest are often accompanied in the T-DNA with different other constituents (Gelvin, 2003), such as selectable markers and reporter genes that capacitate to select transformants and monitor the transfer process (Lee and Gelvin, 2008).

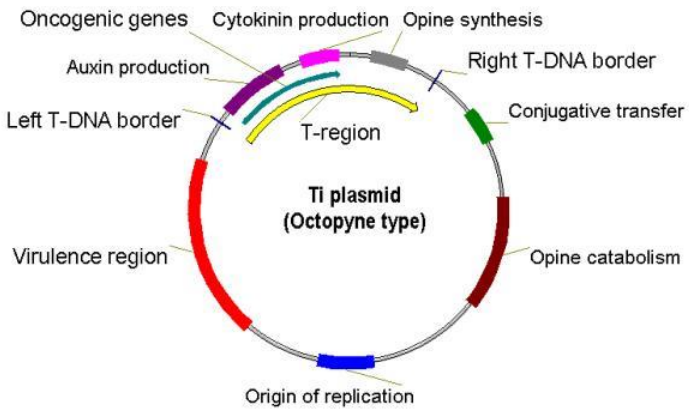



Figure 1 - Tumor inducing (Ti) plasmid of *A.tumefaciens* [1]

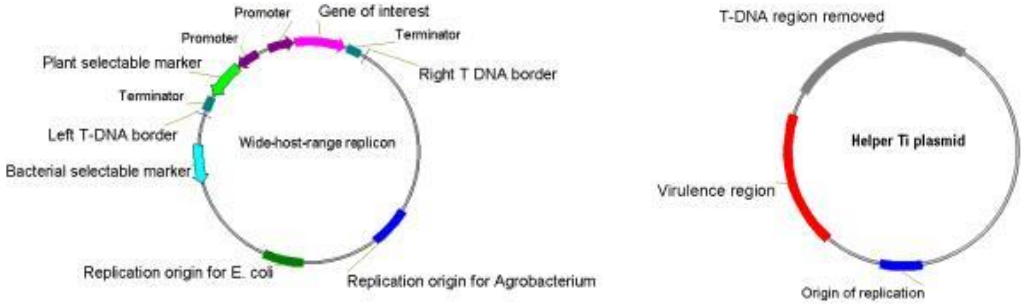



Figure 2 - Binary vector[2]

In order to monitor developmental and spatial gene expression patterns, there are several reporter genes that are widely used in plant transformation (Jach et al., 2001). These include β-glucuronidase (GUS), luciferase (LUC), chloramphenicol acetyltransferase (CAT), green fluorescent protein (GFP) and neomycin phosphotransferase- (NPT)-II, which have been used for several decades (Jach et al., 2001; Töpfer et al., 1988). Recently, red fluorescent protein (dsRed) isolate from reef corals (*Discosoma* sp.) became attractive due to some advantages. For example, it has no need for substrate addition and is non-destructive, the excitation peak wavelength is above the excitation peak of chlorophyll and thereby avoiding the problem of chlorophyll auto-fluorescence (Hakkila et al., 2002; Jach et al., 2001). However, the dsRed has been mainly used in transient expression assays and nothing is known yet about the interference of dsRed expression with plant growth, development and metabolism of the target plant (Jach et al., 2001).

## 1.3 Optimizing transformation efficiency

An efficient plant transformation system is crucially important to understand physiological, biochemical and molecular mechanisms of metabolic pathways. It also can be used in biotechnology and improve the application of genomic technologies (Dan et al., 2009). However, there are several factors that have been reported to influence the transformation efficiency. For example, the acetosyringone, a natural wound response molecule, was proven to increase the transformation rate

[1] Picture derive from http://www.cambia.org/daisy/AgroTran/g1/843.html
[2] Picture derive from http://www.cambia.org/daisy/AgroTran/g1/848.html

(Sheikholeslam and Weeks, 1987); different plant species and bacterial strains show different transformation rates (Krens et al., 1988; Pena et al., 1995; Sheikholeslam and Weeks, 1987). In summary, the bacterial strain, the presence of inducers, the type of plant material used for co-cultivation are of influence to *Agrobacterium*-mediated transformation efficiency (Wenck et al., 1999).

In our research group we want to genetically modify ornamental plants and modify flower color. Preferably in lily, but lily is difficult to transform with low efficiencies. As model we chose *Petunia hybrida* and *Nicotiana benthamiana* to be able to quickly monitor the effect of tested parameters. Transformation knows two phases and consists of gene transfer (transient) and stable gene integration (stable transformation). Gene transfer indicated the plasmid contain target gene uptake by explant after co-cultivation. And stable gene integration means that the target gene integrated into explant cell after co-cultivation. Those two phases depend on the interaction between the bacterium and the plant. In this research we chose two approaches, helping the plant feel better by adding an antioxidant, lipoic acid, and helping bacterium by lowering plant defense by adding salicylic acid inhibitors to optimizing *Agrobacterium*-mediated transformation efficiency.

### 1.3.1 Salicylic acid

A physiological immunity of plants, which is also known as systemic acquired resistance (SAR) is induced by pathogen attack. In plants, the accumulation of salicylic acid (SA) is associated with SAR responses (Gaffney et al., 1993). SA induces pathogensis-related gene expression and enhances the resistance of plants to pathogense (D'Maris Amick Dempsey et al., 2011). Therefore SA has been proposed as an endogenous signal for induction of SAR in many plant species (Gaffney et al., 1993; Malamy et al., 1990). For instance, silencing of SA biosynthesis in *Nicotiana benthamiana* plants increased the susceptibility to *Tobacco Mosaic Virus* (Zhu et al., 2014). The SA biosynthesis in plant has two distinct pathways (Figure 3), one is isochorismate (IC) pathway (route 1) and the other one is Phenylalanine ammonia-lyase (PAL) pathway (route 2) (D'Maris Amick Dempsey et al., 2011). Thus, inhibition of these enzymes should reduce the accumulation of SA.

During the *Agrobacterium*-plant interactions, SA is implicated in the signaling process (Yuan et al., 2008). Yuan et al., (2007) reported that SA can shut down the expression of the *Vir* regulon directly. Plants were more susceptible to *Agrobacterium* infection when there was a defect of SA accumulation and more resistance when overproducing SA. Anand et al., (2008) also reported that *Nicotiana benthamiana* plant treated with SA has low susceptibility to *Agrobacterium* infection. In this study, I will check whether blocking SA synthesis by using SA inhibitors will raise transformation efficiency. As a control, we add SA to see whether it has lower transformation efficiency.

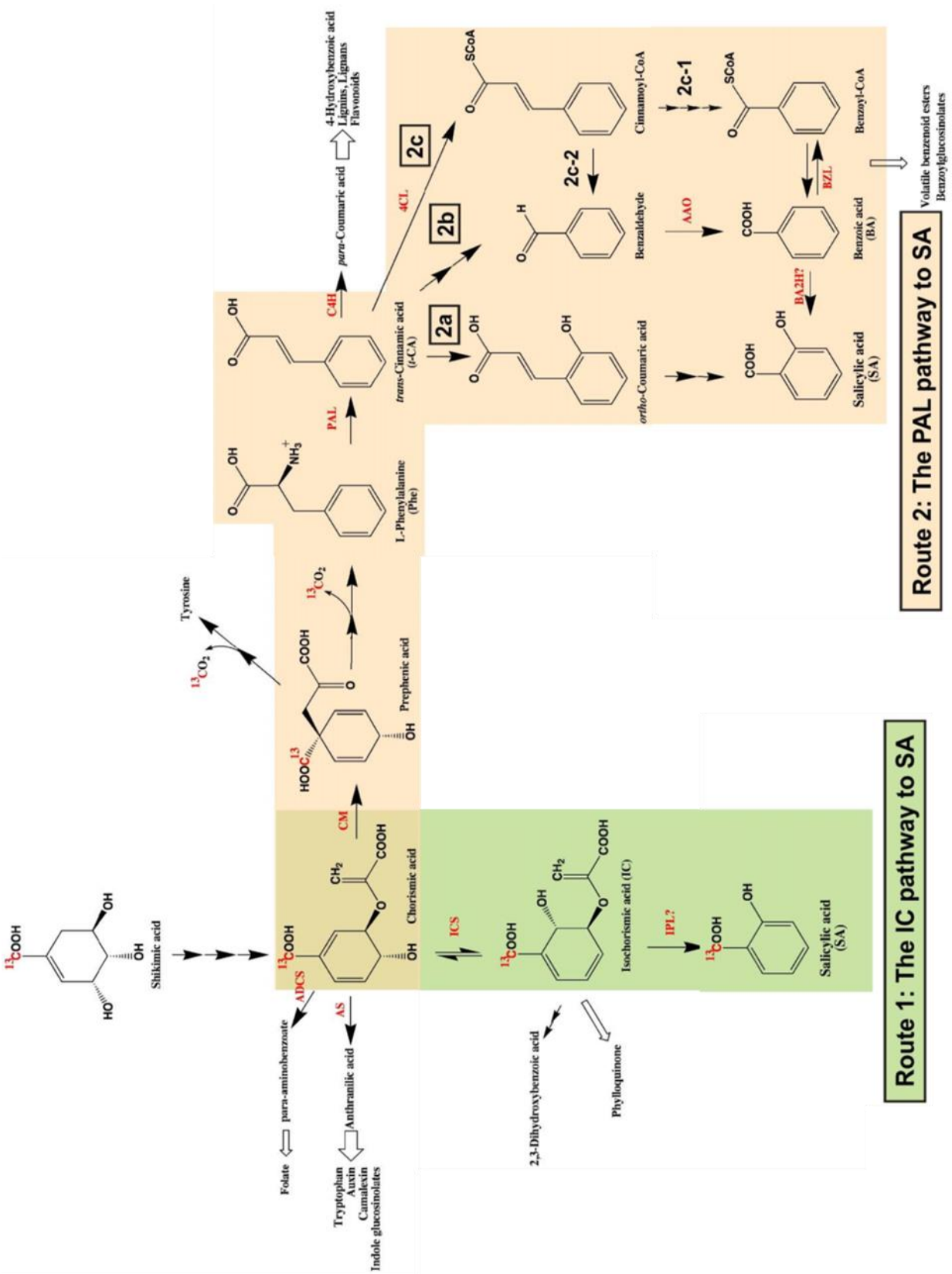



Figure 3 - Salicylic acid biosynthetic pathway. Two distinct pathways, route one is isochorismate (IC) pathway, and route two is phenylalanine ammonia-lyase (PAL) pathway. Figure derived from D'Maris Amick Dempsey et al., 2011.

### 1.3.2 2-aminoindan-2-phosphonic acid

2-aminoindan-2-phosphonic acid (AIP) can inhibit PAL activity leading to reduced pathogen-induced SA accumulation in tobacco, cucumber and *Arabidopsis* (D'Maris Amick Dempsey et al., 2011; Mauch-Mani and Slusarenko, 1996). This compound also has a negative effect on plant growth, fresh weight and root development (Janas et al., 1998). In this study, I will check the effect of AIP as a transformation enhancer by checking the susceptibility to *Agrobacterium* infection.

### 1.3.3 Paclobutrazol

Paclobutrazol (PBZ) [(2RS-3RS)-1-4(chlorophenhyl)-4,4-dimethyl-2-1,2,4-triazollyl-penten-3-ol] is a member from the triazole family (Sankar et al., 2007). Triazole compounds are involved in a variety of morphological and biochemical responses in plants. Those responses include inhibition of shoot elongation, stimulation of root growth, increase of cytokinin synthesis and provide protection from various environmental stresses (Bayat and Sepehri, 2012). Paclobutrazol has been reported as an inhibitor of benzoic acid 2-hydroxylase (BA2H) that converts benzoic acid to salicylic acid (Xiang et al., 2011). In this study, paclobutrazol is assumed to have a similar function as AIP.

### 1.3.4 Lipoic acid

Lipoic acid is a sulfur-containing compound that is involved in several multi-enzyme complexes and has anti-oxidative activity (Dan et al., 2009). Under tissue culture condition, there are three common problems for transformed explant, which are browning, necrosis and recalcitrance. One possible explanation is that during excision and inoculation with A. *tumefaciens* the plant tissues or cells are suffering from oxidative stress. The outcome of plant cell culture and transformation is influenced by oxidative stress, free radicals or reactive oxygen species (ROS). Based on Dan et al., (2009), the three common problems in plant transformation were resolved when applying LA by scavenging free radicals or ROS in plant transformation. In this study, I will analyze the effect of LA on *Agrobacterium*-mediated transformation therefore to check whether LA can improve transformation efficiency.

## 1.4 Study objective

The hypothesis of this study is that blocking SA biosynthesis by SA inhibitors like AIP and PBZ could increase the plant's susceptibility and gene transfer events. LA should improve the condition for the plant after inoculation with *Agrobacterium*.

- To check the effect of increasing concentrations of salicylic acid, of SA inhibitors (AIP and Paclobutrazol) and of lipoic acid on *Agrobacterium*-mediated transformation efficiency in *Petunia hybrida* and *Nicotiana benthamiana.*
- To compare two exposure times (long and short) of salicylic acid, of SA inhibitors (AIP and paclobutrazol) and of lipoic acid on *Agrobacterium*-mediated transformation efficiency in *Petunia hybrida* and *Nicotiana benthamiana.*

# 2 Materials and Methods

## 2.1 Plant Materials

As mention in the study objectives, two model plant species were used for the experiments which are *Petunia hybrida* and *Nicotiana benthamiana.* It is because these two species are not recalcitrant to *Agrobacterium*-mediated transformation, changes in transformation efficiency can be easily measured. *P. hybrida* were sown in pots in the greenhouse whereas *N. benthamiana* were sown in petri dish *in vitro*. Fresh unblemished leaves were used for transformation. In this experiment, leaf disc transformation system was used because it can score expression of various T-DNA markers within a short period and also it can provide long-term selection for growth of transformed callus and shoots (Horsch et al., 1986). In addition, in this study, one *Agrobacterium* strain was used for transformation which is AGL1(pBinGlyRed-Asc1) (Figure 4). It contains the plant binary Ti plasmid vectors with genes encoding of dsRed. Except of salicylic acid, AIP, paclobutrazol and lipoic acid, four additional compounds were used in these experiments. They were acetosyringone which can increase transformation efficiency with concentration level at 1ml/l; cefotaxin and timentin are two antibiotics, they can control bacterial overgrowth; and the last one was kanamycin that is used for selection of stable transformations.

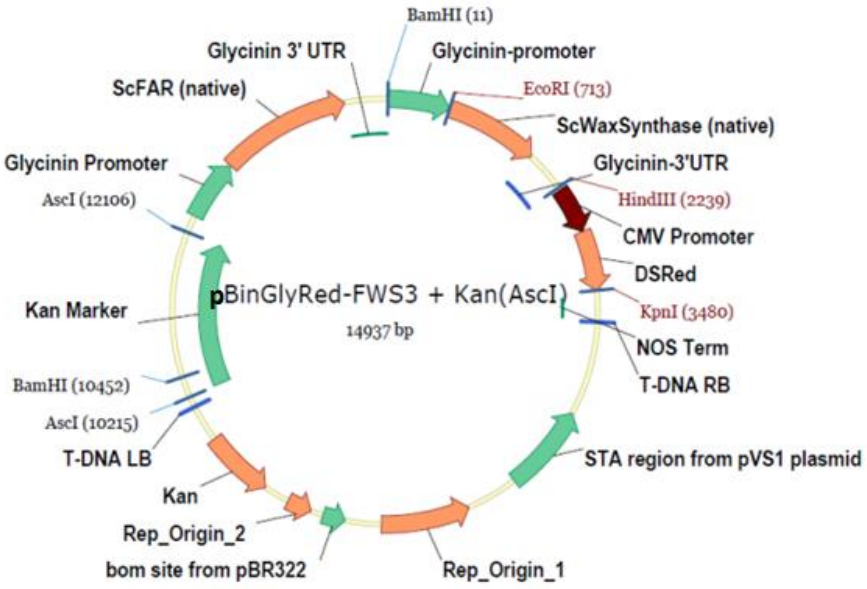



Figure 4 - Binary vector: pBinGlyRed-Asc1

## 2.2 Culture of *Agrobacterium tumefaciens*

The *Agrobacterium* strain was grown overnight (about 12 hours) in liquid luria broth (LB) medium (Table 1) with 50mg/l filter sterilized kanamycin and 50mg/l filter sterilized rifampicin antibiotics. The liquid LB medium was then divided into twenty 50 ml tubes, each tube contains 10 ml LB and *Agrobacterium* strain. For a control of measuring the concentration of *Agrobacterium,* only add kanamycin and rifampicin antibiotics into the liquid LB medium and put 10 ml into a 50 ml tube.

These tubes were put at 28℃ in a shaker (150 rpm) for overnight culture. The concentration of *Agrobacterium* overnight culture was checked using Spectrophotometer with $OD_{600}$, and then centrifuged at 3000rpm, 15mins and 8℃. For *Petunia hybrida*, when $OD_{600}$ was larger than 1, re-suspended the pallet with liquid MS-30 (Table 1) plus acetosyringone. And checked OD again, adjust it to about 1. For *Nicotiana benthamiana*, when $OD_{600}$ was larger than 1, re-suspended the pallet with callus induction medium (CIM, Table 3). And check OD again; adjust it to 0.1 to 0.2.

Table 1 - Mediums used for *Agrobacterium* culture.

| Type of medium | Contents (for one liter) |
|---|---|
| Liquid Luria Broth (LB) medium for bacteria culture | 20g of "LB Broth low salt" in 1L distilled water |
| Liquid MS-30 medium for bacteria suspension | Murashige and Skoog (MS) basal salts, Vitamin B5 (Pantothenic acid), 3% sucrose. Adjust pH to 5.8. |

## 2.3 *Agrobacterium*-mediated transformation

### 2.3.1 *Petunia hybrida*

Unblemished fresh leaves were collected in greenhouse after four to six weeks after sowing. Leaves were sterilized in glass jars (about 10 leaves per glass jar) with 1% (w/v) Na-hypochlorite (24.9ml Na-hypochlorite in 275.1ml demi-water), 0.1% (v/v) Tween 20 (1 drop in 300ml 1% w/v Na-hypochlorite) and shaken for 10 minutes, then sterile distilled water was used to wash the leaves for three times. After leaf sterilization, leaf discs were cut (about 6 mm in diameters) excluding the midrib and the edge and randomly placed up-side-down on the regeneration medium (RM, Table 2) with the four different compounds to be tested separately, each compound was tested at five concentrations (0μM, 1μM, 10μM, 50μM, 100μM) for two days with 24-26℃ in the light (16hL/8hD). Three petri dish replicates and each contain 15 leaf discs were used for *Agrobacterium* strain. After two days pre-culture, good growing leaf discs were collected and placed in 50ml tubes which contained the *Agrobacterium* in liquid MS-30. Gently shake the tubes to make sure all explants were good contact with *A. tumefaciens* suspension. Blot the explants dry on sterile filter paper and then placed them back (up-side-down) on the regeneration medium from which they derived. For further co-cultivation, petri dishes were incubated at 24-26 ℃ in the dark. For controls, these were inoculating the leave discs with liquid MS-30 medium instead of the *Agrobacterium*. After three days co-cultivation with *Agrobacterium*, all the leave discs (explants) were transferred to the kanamycin selection medium (Km SM, Table 2) with the same orientation and placed them in the light (16hL/8hD) at 24-26℃. For controls, explants were transferred to the selection medium (SM, Table 2). At this stage, I have two schemes, long exposure time and short exposure time. Long exposure time was explants to compound in three days of co-cultivation and 14 days on Km SM labelled as Set A., and short exposure time was explant to compound only in three days of co-cultivation labelled as Set B. So for the long exposure time the Km SM contained four different compounds separately; for the short exposure time the Km SM do not contain any of those compounds. I used the fluorescence microscope to check the transient dsRed expression at two time points, one is 3 days after co-cultivation (represent as t=3 days) and the other is 14 days after co-cultivation (represent as t=14 days) on Km SM. Refreshed the Km SM every two weeks, at this stage, so after 14 days in Km SM, the Km SM did not contain the four compounds anymore. Then 30 days (about 4 weeks) after

growth on Km SM, I checked how many explant survived and showed callus development, and the % transformation efficiency (TE) was calculated as follows:

$\%TE = \left(\frac{explant\ \ with\ \ calli\ \ (t{=}30\ \ days\ \ in\ \ Km\ \ SM)}{explant\ \ tested}\right) \times 100\%$ . Meanwhile shoot regeneration from callus, transferred the regenerate shoots into shoot medium (STM, Table 2) and keep the rest of the calli in Km SM for those explants that have no shoot appearing yet. If the transgenic *P.hybrida* shoots were not rooting in two weeks, transferred the shoots to rooting medium (RTM, Table 2). Care is taken that each shoot, represents an individual transformation event by harvesting only one shoot per explant. After 90 days (about 12 weeks) growth on Km SM, check the regenerated shoot on Km SM and calculated the % Ultimate transformation efficiency (RE) as follows:

$\%RE = \left(\frac{regenerated\ \ shoots\ \ (t{=}12\ \ weeks)}{explant\ \ tested}\right) \times 100\%$ . Two to three leaves were collected from transformant shoots to run polymerase chain reaction (PCR) to confirm genetic transformation. Transformation protocol of *P.hybrida* was summarized in Figure 5 below.

Table 2 - Mediums used in *P. hybrida Agrobacterium*-mediated transformation.

| **Type of medium** | **Contents (for one liter)** |
|---|---|
| Regeneration medium | 4.4g/l Murashige and Skoog (MS) basal salts, Vitamin B5 (Pantothenic acid), 3% sucrose, 4.44µM benzylaminopurine (BAP), 0.54 µM naphthalene acetic acid (NAA), 0.8% micro agar. Adjust pH to 5.8. After autoclave, add 20 mg/l acetosyringone. |
| SM | 4.4g/l MS basal salts, Vitamin B5 (Pantothenic acid), 3% sucrose, 4.44µM BAP, 0.54 µM NAA, 0.8% micro agar. Adjust pH to 5.8. After autoclave add 20 mg/l acetosyringone, 200mg/l cefotaxime and 100mg/l timentin. |
| Km SM | 4.4g/l MS basal salts, Vitamin B5 (Pantothenic acid), 3% sucrose, 4.44µM BAP, 0.54 µM NAA, 0.8% micro agar. Adjust pH to 5.8. After autoclave add 20 mg/l acetosyringone, 200mg/l cefotaxime 100mg/l, timentin and 200mg/l kanamycin. |
| shoot medium (STM) | 4.4g/l MS basal salts, Vitamin B5 (Pantothenic acid), 3% sucrose, 1% glucose, 4.44µM BAP, 0.54 µM NAA, 0.8% micro agar. Adjust pH to 5.8. After autoclave add 1mg/ml zeatin, 200mg/l cefotaxime and 200mg/l kanamycin |
| rooting medium (RTM) | 4.4g/l MS basal salts, Vitamin B5 (Pantothenic acid), 3% sucrose, 1% glucose, 0.8% micro agar. Adjust pH to 5.8. After autoclave adds 250 mg/l cefotaxime and 200mg/l kanamycin (omitted). |

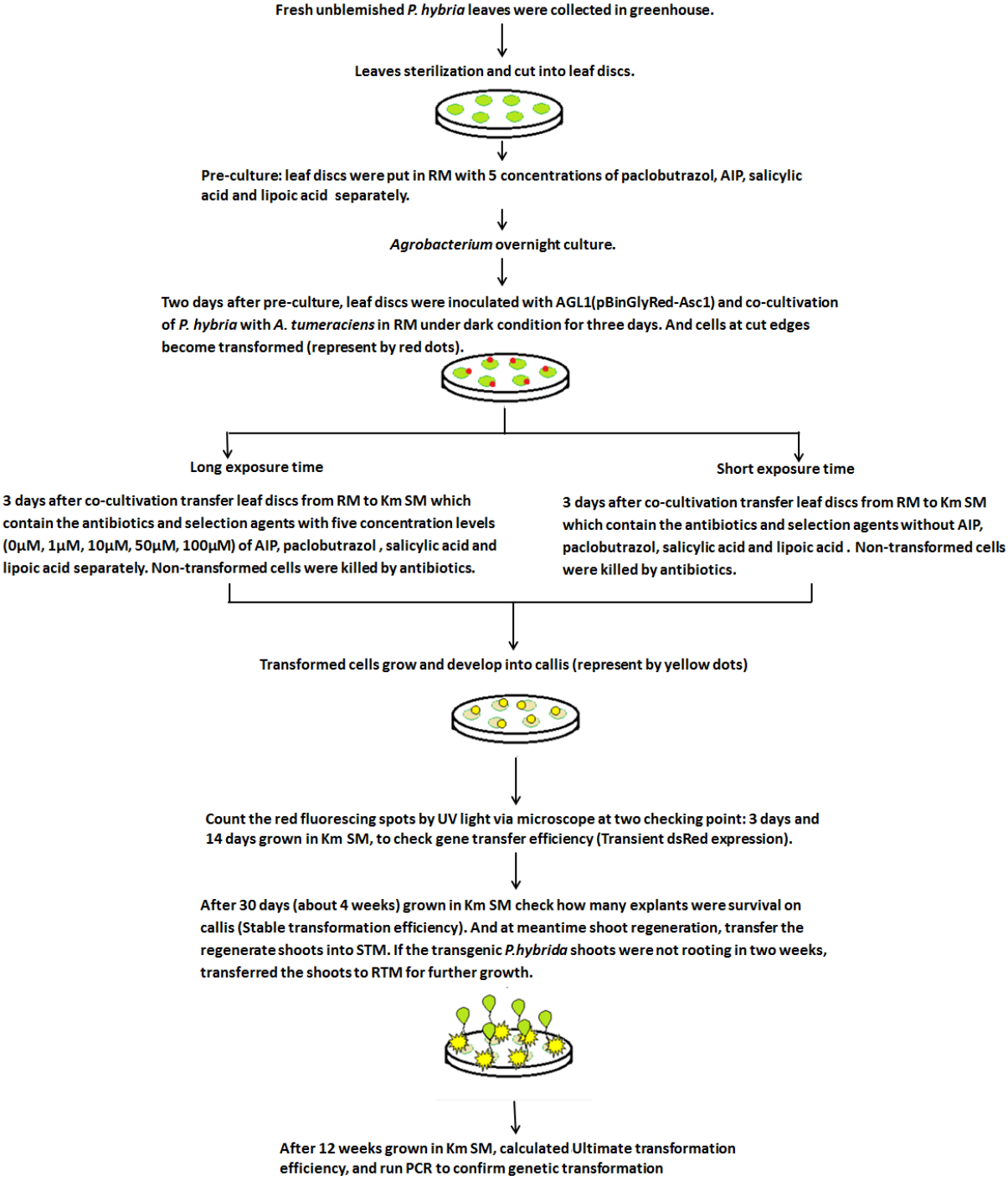



Figure 5 - Protocol of *P. hybrida Agrobacterium*-mediated transformation.

### *2.3.2 Nicotiana benthamiana*

*Nicotiana benthamiana* seeds were sterilized and put in petri dishes with germination medium (Table 3). Petri dishes were stored for four days in the refrigerator in the dark. On the fourth day, petri dishes with the seeds were transferred to 24℃ and 16hL/8hD for further germination. when seeds were germinated, seedlings was transferred to a container with MS-30 medium (Table 3). After four to six weeks, unblemished fresh leaves were collected from MS-30 container. leaf discs were cut (about 6 mm in diameters) excluding the midrib and the edge and randomly placed up-side-down in a petri dish containing 5-10ml callus induction medium (CIM, Table 3) with *Agrobacterium* and four different compounds to be tested separately. For each compound it was tested at five concentrations (0µM, 1µM, 10µM, 50µM, 100µM). Because the CIM is liquid so it is important to keep leaf discs floating by adding few CIM liquid on the leaf discs surface due to the water surface tension, otherwise the leaves will drown in the medium. The leaves discs were put at 24-26℃ in the dark for three days for co-cultivation. For each concentration, three petri dish replicates and each contain 15 leaf discs were used for *Agrobacterium* strain. After three days co-cultivation, leaf discs were transferred to the petri dishes with Shoot induction medium (SIM, Table 3), turned upside down (so the leaves will be upside up again) with the same orientation and placed them in the light (16hL/8hD) at 24-26℃. For controls, explants were transferred to the shoot induction medium minus (SIM-, Table 3). At this stage, I have two schemes same as *P. hybrida*, long exposure time and short exposure time. For the long exposure time the SIM contained four different compounds separately labelled as Set A; for the short exposure time the SIM do not contain any of those compounds labelled as Set B. I used the fluorescence microscope to check the transient dsRed expression at same checking points as *P.hybrida*, one is 3 days after co-cultivation (represent as t=3 days) and the other is 14 days after co-cultivation (represent as t=14 days) on SIM. Refreshed the SIM every two weeks, at this stage, so after 14 days in SIM, the SIM did not contain the four compounds anymore. Then 30 days (about 4 weeks) after growth on SIM, I checked how many explant survived and showed callus development, and the %TE was calculated as followed:

$\%TE = \left(\frac{explant\ \ with\ \ calli\ \ (t{=}30\ \ days\ \ in\ \ SIM)}{explant\ \ tested}\right) \times 100\%$. Meanwhile shoot regeneration from callus, transferred the regenerate shoots into root induction medium (RIM, Table 3) and keep the rest of the calli in SIM for those explants that have no shoot appearing yet. Care is taken that each shoot, represents an individual transformation event by harvesting only one shoot per explant. After 90 days (about 12 weeks) growth on SIM, check the new regenerated shoots on SIM and calculated the %RE. Two to three leaves were collected from transformant shoots to run polymerase chain reaction (PCR) to confirm genetic transformation. Transformation protocol of *N.benthamiana* was summarized in Figure 6 below.

Table 3 - Mediums used in *N. benthamiana Agrobacterium*-mediated transformation

| Type of medium | Contents |
|---|---|
| Germination medium | 4.4g/l Murashige and Skoog (MS) medium (including vitamins), 3% sucrose, 0.8% micro agar. Adjust pH to 5.8. |
| MS-30 | 4.4g/l MS medium (including vitamins), 3% sucrose, 0.8% micro agar. Adjust pH to 5.8. |
| Callus induction medium (CIM) | 4.4g/l MS medium (including vitamins), 0.88 µM BAP, 5.4µM NAA, 3% sucrose. Adjust pH to 5.8. After autoclave add 20 mg/l acetosyringone. |
| Shoot induction medium (SIM) | 4.4g/l MS medium (including vitamins), 4.44µM BAP, 0.54µM NAA, 3% sucrose. Adjust pH to 5.8. After autoclave add 20 mg/l acetosyringone, 500mg/l cefotaxime and 50ml/l kanamycin. Reduced the concentration of cefotaxime from 500mg/l to 300mg/l after three weeks in SIM. Then decrease the concentration of cefotaxime from 300 mg/l to 150mg/l after three weeks in SIM. |
| Shoot induction medium minus (SIM -) | 4.4g/l MS medium (including vitamins), 4.44µM BAP, 0.54µM NAA, 3% sucrose. Adjust pH to 5.8. After autoclave add 20 mg/l acetosyringone and 500mg/l cefotaxime. |
| Root induction medium (RIM) | 4.4g/l MS medium (including vitamins), 0.27 µM indole acetic acid (IAA),3% sucrose, 50ml/l kanamycin. Adjust pH to 5.8. |

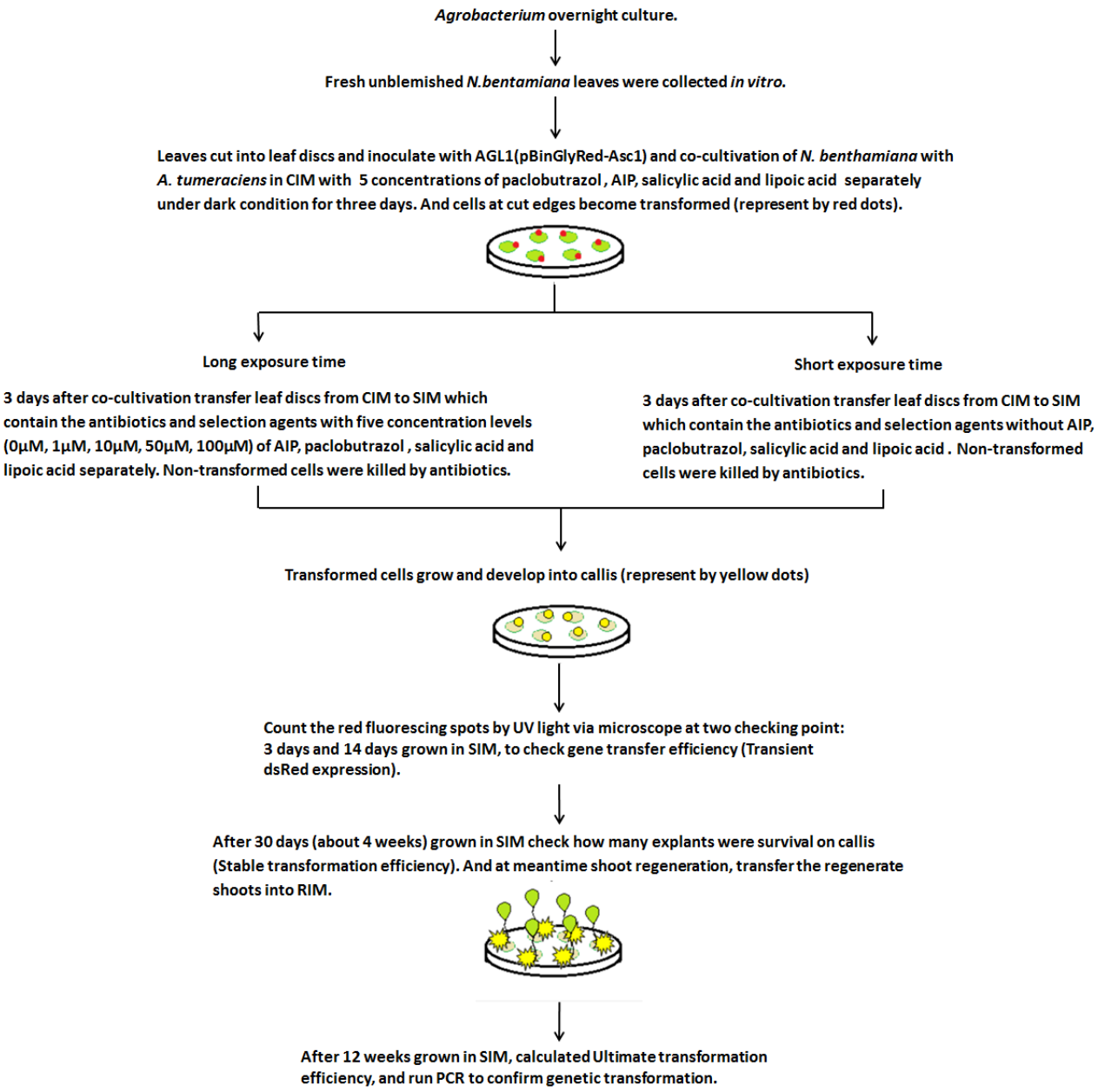



Figure 6 - Protocol of *N. benthamiana Agrobacterium*-mediated transformation.

## 2.4 Data collection and analysis

The raw data was collected by counting the dsRed spots from pictures taken by a fluorescence microscope and were put into Excel. The standard of counting dsRed is shown in Figure 7 and after 14 days grown in SM or SIM, the standard of counting dsRed is shown in Figure 8. Statistical analysis was performed by using one-way analysis of variance (ANOVA) test and univariate general linear model (GLM) test from SPSS and two-way analysis of variance from GenStat follow with Tukey's 95% confidence intervals and Duncan's multiple range test (DMRT). The P values <0.05 were considered as significant, which means that concentrations and/or exposure times may have effect on transformation efficiency.

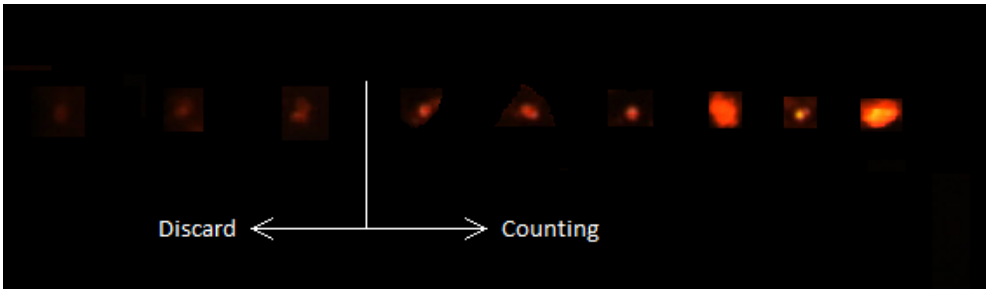



Figure 7 – General standard of dsRed counting

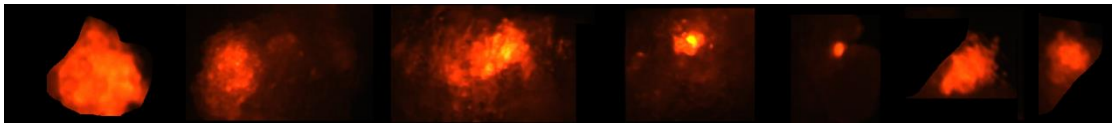


Figure 8 - Standard of dsRed counting for checking point t=14 days in SM/SIM, calli in the figure were counted as one dsRed spot (irrespective of the size)

# 3 Results

## 3.1 The effect of AIP, PBZ, SA and LA on transient expression

To measure the effects of AIP, PBZ, SA and LA on dsRed expression on *P.hybrida* and *N.benthamiana*, the number of fluorescence spots on each explant was determined with different concentrations and at different time intervals. i.e. long days and short days. The standard of counting the fluorescence spots was described in section 2.3. The larger the number of spots the higher transient dsRed expression, means the higher gene transfer efficiency. Due to limited time, the experiment with *P. hybrida* was only repeated once with AIP and PBZ and the result is discussed in the following paragraph. For each compound, the results of different exposure times and concentrations of *P.hybrida* and *N. benthamiana* have been determined. A short summary of the effects of AIP, PBZ, SA and LA can be found in the end of this section.

### 3.1.1 The effect of AIP on transient dsRed expression

The transient dsRed expression of *P.hybrida* after three days in SM medium has significant different on exposure time and concentration ($P < 0.05$). As Figure 9 and Table 4 shows that at checking point t=3 days, 1μM AIP with long exposure time has the largest number of dsRed spots. And the number of dsRed sport with 0μM AIP with long exposure time is higher than short exposure time. With long exposure time, concentrations higher than 10μM has less dsRed spots. With short exposure time, except for 100μM, the higher concentrations the lower number of dsRed spots. At checking point t=14 days, no significant different on exposure time and concentration were found as Table 5 shows below. 0μM AIP with long exposure time has the largest number of dsRed spots, but according to Table 4, 100μM AIP with short exposure time has very close value to it. Because there was no significant different was found on concentrations with two exposure time, so I check the different between concentrations with one exposure time separately (appendix 3 and 4), but also no significant different was found. The repeat experiment of AIP has the similar result as the first experiment (result shown in appendix 1 and 2).

For *N. benthamiana*, as shown in Figure 9 and Table 4, at checking point t=3 days, result shows that there was a significant different on concentration but no significant different on exposure time. The largest number of dsRed spots was 10μM AIP with long exposure time. At checking point t=14 days, a significant different on exposure time was appear. 100μM AIP with long exposure time has the highest number of dsRed spots. As Table 6 shows, the dsRed spots with long exposure time have more spots than short exposure time.

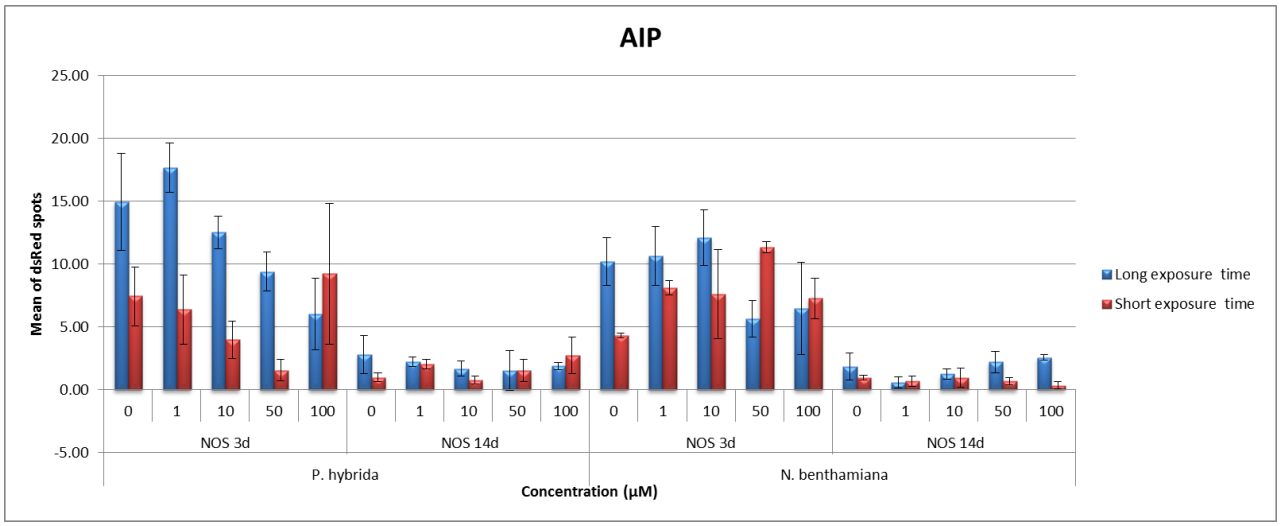
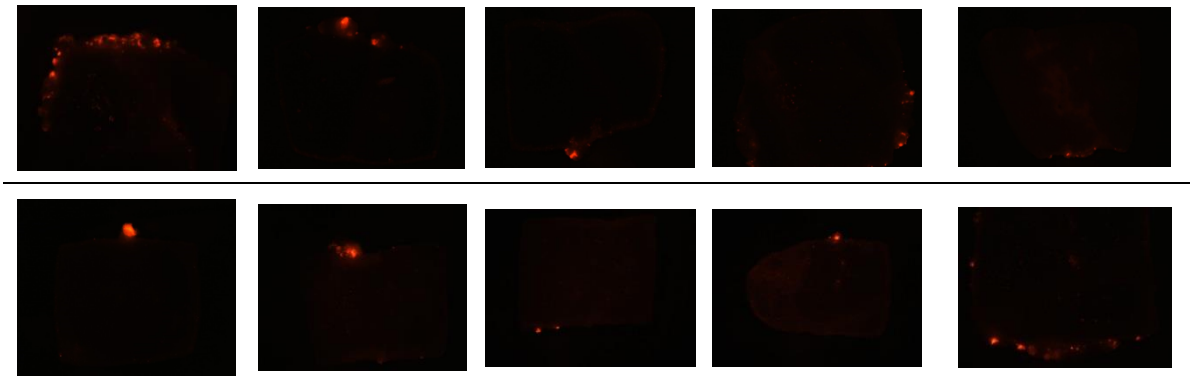



Figure 9 - The effect of AIP on transient dsRed expression. Error bar indicate standard deviation.

Table 4 - The effect of AIP on transient dsRed expression. The means with different letters represent significantly (P<0.05) different

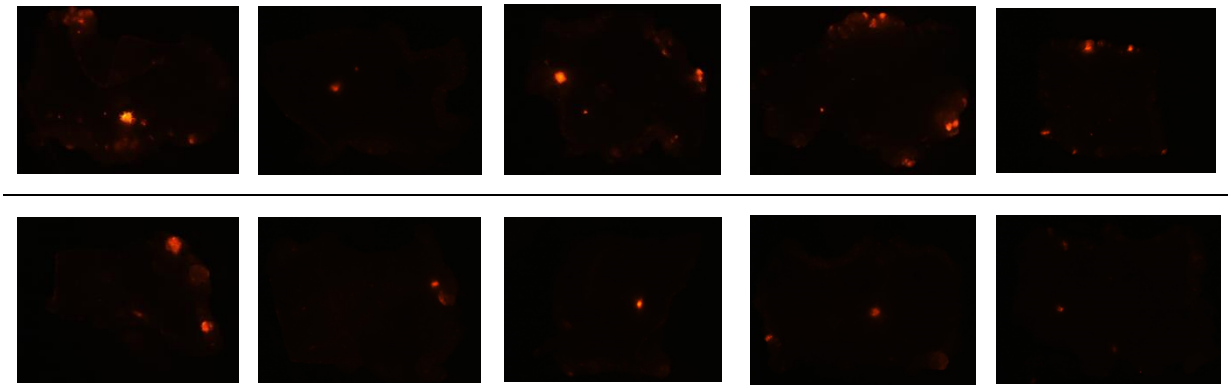
| Plant | Exposure time | Conc. (µM) | Rep. | dsRed spots (t=3days) | | dsRed spots (t=14days) | |
|---|---|---|---|---|---|---|---|
| *P.hybrida* | long | 0 | 3 | 14.93±3.86 | cd | 2.78±1.52 | a |
| | long | 1 | 3 | 17.67±1.97 | d | 2.20±0.37 | a |
| | long | 10 | 3 | 12.51±1.30 | bcd | 1.67±0.61 | a |
| | long | 50 | 3 | 9.40±1.57 | abc | 1.51±1.58 | a |
| | long | 100 | 3 | 6.00±2.84 | ab | 1.87±0.29 | a |
| | short | 0 | 3 | 7.45±2.35 | abc | 0.98±0.37 | a |
| | short | 1 | 3 | 3.68±2.74 | ab | 2.02±0.39 | a |
| | short | 10 | 3 | 3.96±1.50 | a | 0.77±0.31 | a |
| | short | 50 | 3 | 1.53±0.85 | a | 1.53±0.87 | a |
| | short | 100 | 3 | 9.22±5.61 | abc | 2.71±1.48 | a |
| *N.benthamiana* | long | 0 | 3 | 10.20±1.91 | bcde | 1.82±1.08 | bcd |
| | long | 1 | 3 | 10.62±2.35 | cde | 0.58±0.44 | a |
| | long | 10 | 3 | 12.09±2.24 | e | 1.24±0.43 | abc |
| | long | 50 | 3 | 5.65±1.46 | a | 2.20±0.87 | cd |
| | long | 100 | 3 | 6.47±3.65 | ab | 2.56±0.21 | d |
| | short | 0 | 3 | 4.29±0.19 | a | 0.96±0.20 | ab |
| | short | 1 | 3 | 8.11±0.55 | abcde | 0.67±0.40 | a |
| | short | 10 | 3 | 7.60±3.54 | abcd | 0.93±0.76 | ab |
| | short | 50 | 3 | 11.36±0.46 | de | 0.66±0.31 | a |
| | short | 100 | 3 | 7.27±1.60 | abc | 0.33±0.28 | a |

Table 5 – Transient dsRed expression in *P.hybrida* of long and short exposure time (EPT) treated with AIP at checking point t=14 days.



*Upper line: long exposre time; lower line: short exposre time;
*From left to right: AIP concentration 0µM, 1µM, 10µM, 50µM, 100µM.

Table 6 - Transient dsRed expression in *N.benthamiana* of long and short exposure time (EPT) treated with AIP at checking point t=14 days.



*Upper line: long exposre time; lower line: short exposre time;
*From left to right AIP concentration 0µM, 1µM, 10µM, 50µM, 100µM.

### 3.1.2 The effect of PBZ on transient dsRed expression

At checking point t=3 days, the transient dsRed expression of *P.hybrida* treated with PBZ has a significant different at concentration but not at exposure time (Figure 10 and Table 7). The highest number of dsRed spots is obtained at 0µM PBZ with long exposure time but with high standard deviation. 1µM PBZ with long exposure time has the second highest number of dsRed spots but with low standard deviation. At checking point t=14 days, the significant different was found on concentrations and exposure time. The highest number of spots is obtained at 10µM PBZ with short exposure time. The number of dsRed sport with short exposure time is higher than long exposure time and too high concentration of PBZ like 100µM has very low number of dsRed spots (Table 8). The repeat experiment of AIP has the similar result as the first experiment (appendix 1 and 2).

For *N.benthamiana*, at checking point t=3 days, there was a significant different at concentrations as well as exposure time. The number of dsRed spots with long exposure time was lower than short exposure time (Table 7). The highest number of dsRed spots is obtained at 100µM PBZ with short

exposure time but should be aware that the stand deviation was large. 10µM PBZ with short exposure time was the second highest number of dsRed spot with small stand deviation. At checking point t=14 days, there was no significant different at concentrations and exposure time. As can be seen from Table 9, the number of dsRed at different concentration with two exposure times were no big different. The highest number of dsRed spots is obtained at 100µM PBZ with short exposure time.

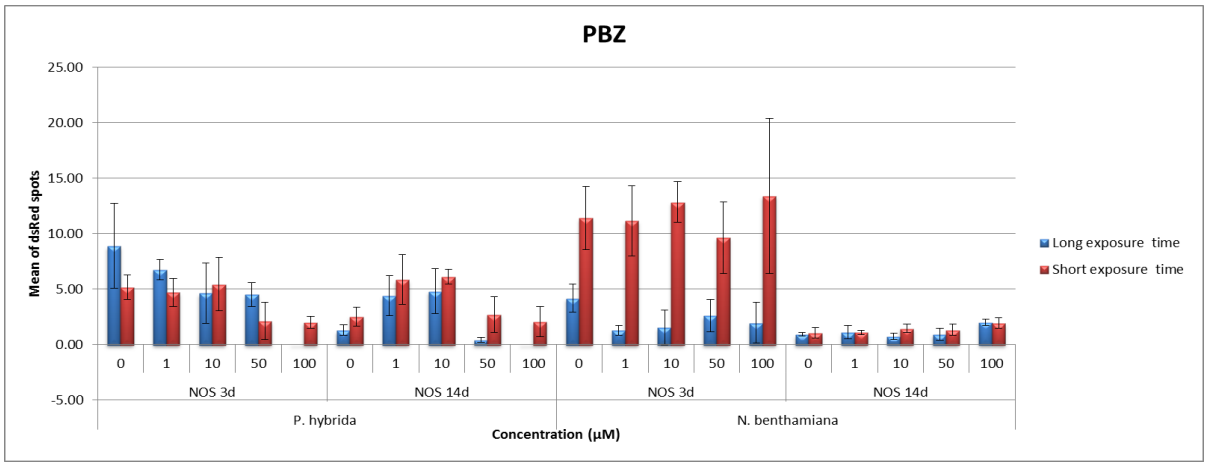
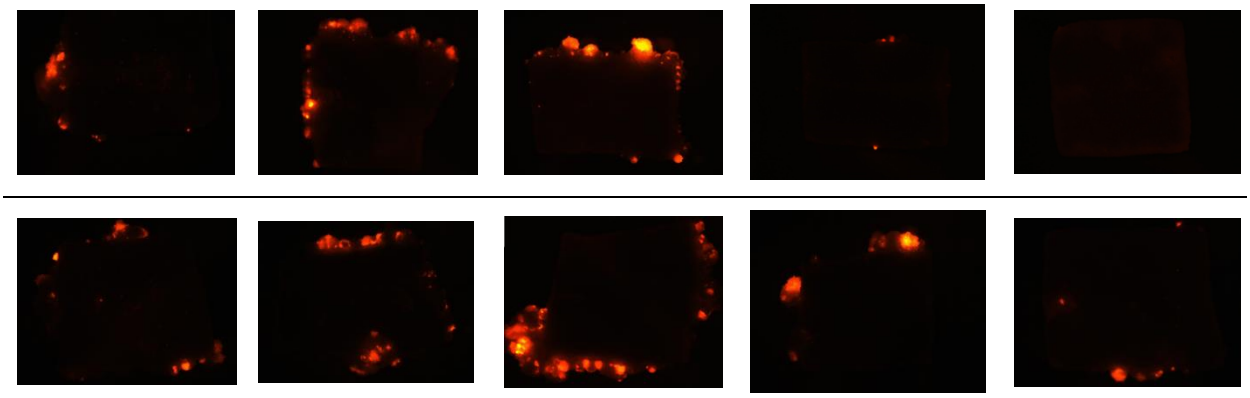



Figure 10 - The effect of PBZ on transient dsRed expression. Error bar indicate standard deviation.

Table 7 - The effect of PBZ on transient dsRed expression. The means with different letters represent significantly (P<0.05) different

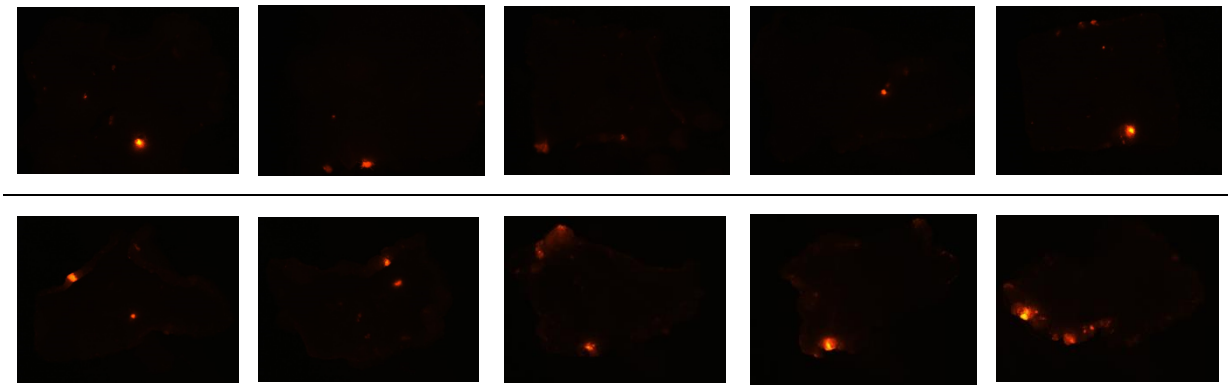
| Plant | Exposure time | Conc. (µM) | Rep. | dsRed spots (t=3days) | | dsRed spots (t=14days) | |
|---|---|---|---|---|---|---|---|
| *P.hybrida* | long | 0 | 3 | 8.89±3.82 | c | 1.29±0.47 | ab |
| | long | 1 | 3 | 6.75±0.91 | bc | 4.40±1.81 | bcd |
| | long | 10 | 3 | 4.65±2.73 | abc | 4.80±2.03 | bcd |
| | long | 50 | 3 | 4.51±1.07 | abc | 0.42±0.22 | a |
| | long | 100 | 3 | 0.00±0.00 | a | 0.00±0.00 | a |
| | short | 0 | 3 | 5.15±1.10 | abc | 2.51±0.83 | abcd |
| | short | 1 | 3 | 4.69±1.27 | abc | 5.84±2.24 | cd |
| | short | 10 | 3 | 6.11±1.80 | abc | 6.11±0.66 | d |
| | short | 50 | 3 | 2.11±1.67 | ab | 2.71±1.60 | abcd |
| | short | 100 | 3 | 2.00±0.53 | ab | 2.07±1.38 | abc |
| *N.benthamiana* | long | 0 | 3 | 4.16±1.27 | abc | 0.91±0.16 | ab |
| | long | 1 | 3 | 1.20±0.50 | a | 1.11±0.63 | ab |
| | long | 10 | 3 | 1.53±1.57 | a | 0.74±0.31 | a |
| | long | 50 | 3 | 2.62±1.45 | ab | 0.93±0.54 | ab |
| | long | 100 | 3 | 1.96±1.84 | a | 1.20±0.31 | b |
| | short | 0 | 3 | 11.42±2.86 | cd | 1.05±0.49 | ab |
| | short | 1 | 3 | 11.15±3.18 | bcd | 1.09±0.21 | ab |
| | short | 10 | 3 | 12.84±1.82 | cd | 1.45±0.37 | ab |
| | short | 50 | 3 | 9.64±3.21 | abcd | 1.33±0.48 | ab |
| | short | 100 | 3 | 13.42±6.99 | d | 1.93±0.47 | ab |

Table 8 - Transient dsRed expression in *P.hybrida* of long and short exposure time (EPT) treated with PBZ at checking point t=14 days.



*Upper line: long exposre time; lower line: short exposre time;
*From left to right: PBZ concentration 0µM, 1µM, 10µM, 50µM, 100µM.

Table 9 - Transient dsRed expression *in N.benthamiana* of long and short exposure time (EPT) treated with PBZ at checking point t=14 days.



*Upper line: long exposre time; lower line: short exposre time;
*From left to right: PBZ concentration 0µM, 1µM, 10µM, 50µM, 100µM.

### 3.1.3 The effect of SA on transient dsRed expression

At checking point t=3 days, the transient dsRed expression of *P.hybrida* treated with SA has a significant different at exposure time but no different at concentrations. The number of dsRed spot with long exposure time was lower than short exposure time (Figure 11 and Table 10). The highest number of dsRed spots is obtained at 1µM SA with short exposure time. At checking point t=14 days, a significant different was found at exposure time as well as concentration (Table 11). The number of dsRed spot with long exposure time was lower than short exposure time. The highest number of spots is obtained at 1µM SA with short exposure time.

For *N.benthamiana*, at checking point t=3 days, significant different at concentrations and exposure times were found. The dsRed spots with long exposure time has lower number than short exposure, and as the concentrations increase, the number of dsRed spots were decrease (Figure 11 and Table 10). The highest number of dsRed spots is obtained at 0µM SA with short exposure time. At checking

point t=14 days, there was a significant different at concentrations but not at exposure times (Table 12). The highest number of dsRed spots is obtained at 50µM SA with long or short exposure time.

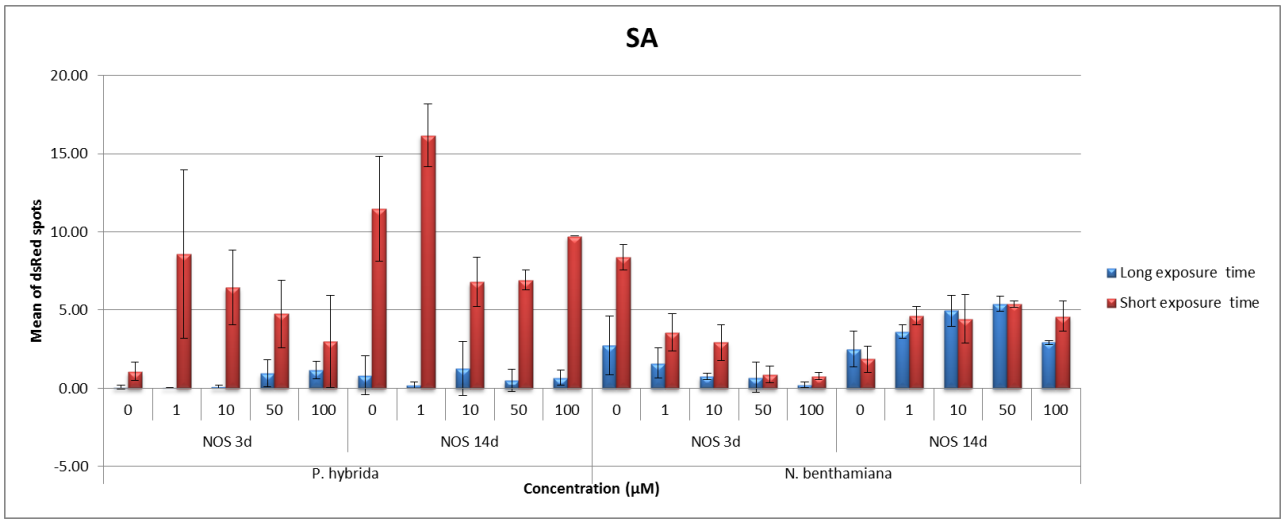
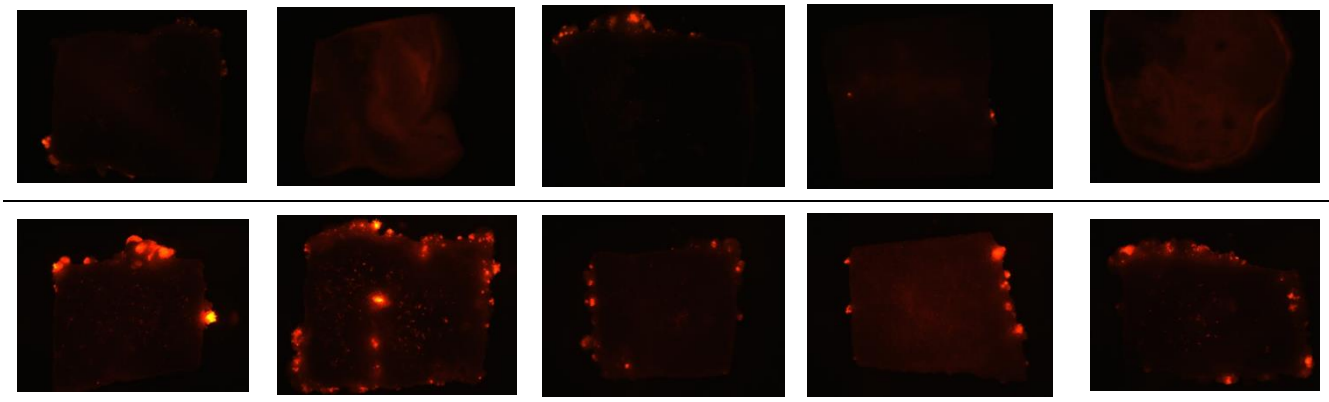



Figure 11 - The effect of SA on transient dsRed expression. Error bar indicate standard deviation.

Table 10 - The effect of SA on transient dsRed expression. The means with different letters represent significantly (P<0.05) different

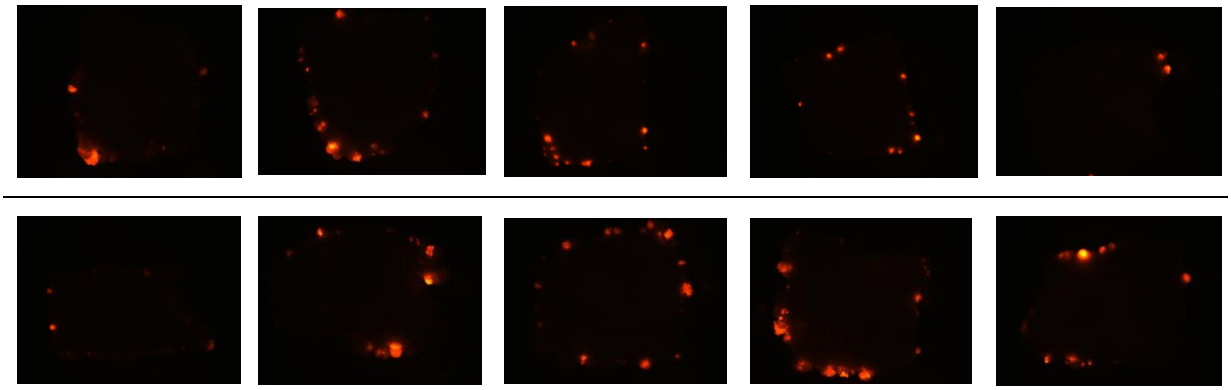
| Plant | Exposure time | Conc. (µM) | Rep. | dsRed spots (t=3days) | | dsRed spots (t=14days) | |
|---|---|---|---|---|---|---|---|
| *P.hybrida* | long | 0 | 3 | 0.07±0.12 | a | 0.82±1.25 | a |
| | long | 1 | 3 | 0.02±0.04 | a | 0.19±0.22 | a |
| | long | 10 | 3 | 0.09±0.10 | a | 1.27±1.74 | a |
| | long | 50 | 3 | 0.96±0.86 | ab | 0.50±0.71 | a |
| | long | 100 | 3 | 1.15±0.57 | ab | 0.67±0.47 | a |
| | short | 0 | 3 | 1.07±0.59 | ab | 11.47±3.37 | c |
| | short | 1 | 3 | 8.58±5.38 | d | 16.18±2.01 | d |
| | short | 10 | 3 | 6.44±2.40 | cd | 6.80±1.58 | b |
| | short | 50 | 3 | 4.76±2.15 | bcd | 6.93±0.64 | b |
| | short | 100 | 3 | 3.00±2.95 | abc | 9.73 | bc |
| *N.benthamiana* | long | 0 | 3 | 2.73±1.87 | bc | 2.51±1.15 | ab |
| | long | 1 | 3 | 1.60±0.96 | ab | 3.62±0.44 | abcd |
| | long | 10 | 3 | 0.76±0.21 | a | 4.96±0.99 | cd |
| | long | 50 | 3 | 0.69±0.96 | a | 5.40±0.48 | d |
| | long | 100 | 3 | 0.22±0.17 | a | 2.93±0.12 | abc |
| | short | 0 | 3 | 8.38±0.84 | d | 1.87±0.83 | a |
| | short | 1 | 3 | 3.58±1.21 | c | 4.64±0.60 | bcd |
| | short | 10 | 3 | 2.93±1.14 | bc | 4.44±1.54 | bcd |
| | short | 50 | 3 | 0.89±0.52 | a | 5.38±0.22 | d |
| | short | 100 | 3 | 0.75±0.24 | a | 4.60±0.93 | bcd |

Table 11 - Transient dsRed expression in *P.hybrida* of long and short exposure time (EPT) treated with SA at checking point t=14 days.



*Upper line: long exposre time; lower line: short exposre time;
*From left to right: SA concentration 0µM, 1µM, 10µM, 50µM, 100µM.

Table 12 - Transient dsRed expression in *N.benthamiana* of long and short exposure time (EPT) treated with SA at checking point t=14 days.



*Upper line: exposure time 3 days; lower line: exposure time 14 days;
*From left to right: SA concentration 0µM, 1µM, 10µM, 50µM, 100µM.

### 3.1.4 The effect of LA on transient dsRed expression

At checking point t=3 days, there was a significant different at concentration and exposure time. The number of dsRed spots with long exposure time was lower than short exposure time. The highest number of dsRed spots is obtained at 10µM LA with short exposure time (Figure 12 and Table 13). At checking point t=14 days, significant different was found in exposure time but no significant different at concentration (Table 14). The number of dsRed spots with long exposure time was less than short exposure time. The highest number of dsRed spots is obtained at 50µM SA with short exposure time.

For *N.benthamiana*, at checking point t=3 days, there was a significant different at concentration but no significant different at exposure time (Figure 12 and Table 13). The highest number of dsRed spots is obtained at 1µM LA with short exposure time. At checking point t=14 days, there was a significant different at exposure times as well as concentrations. The highest number of spots is obtained at 10µM LA with short exposure time. Except for 0µM LA, the number of dsRed spots of 1µM, 10µM,

50µM and 100µM LA with short exposure time were higher than long exposure time (Table 15).

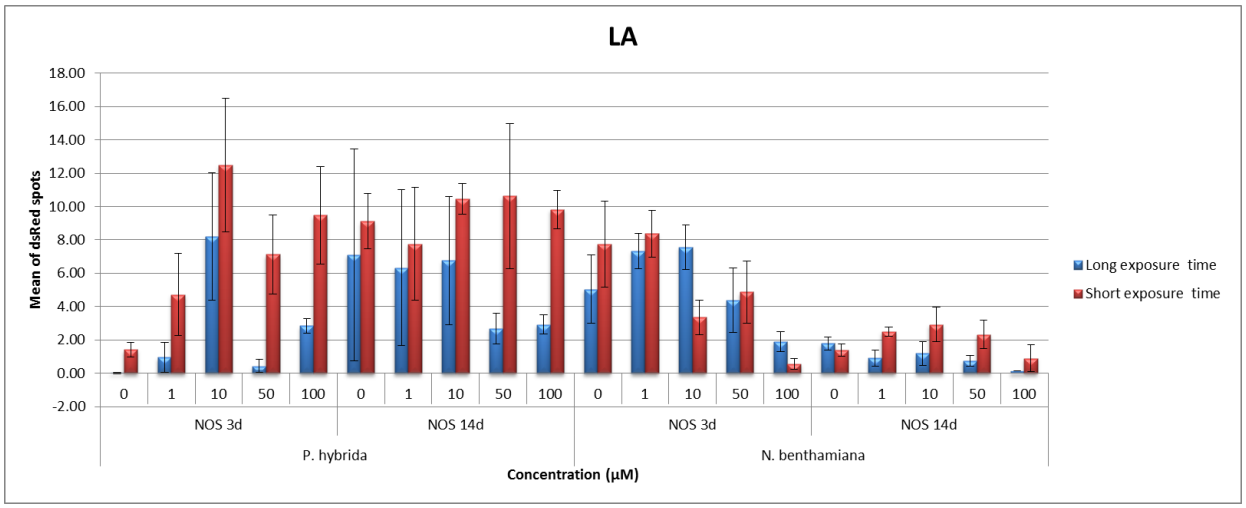
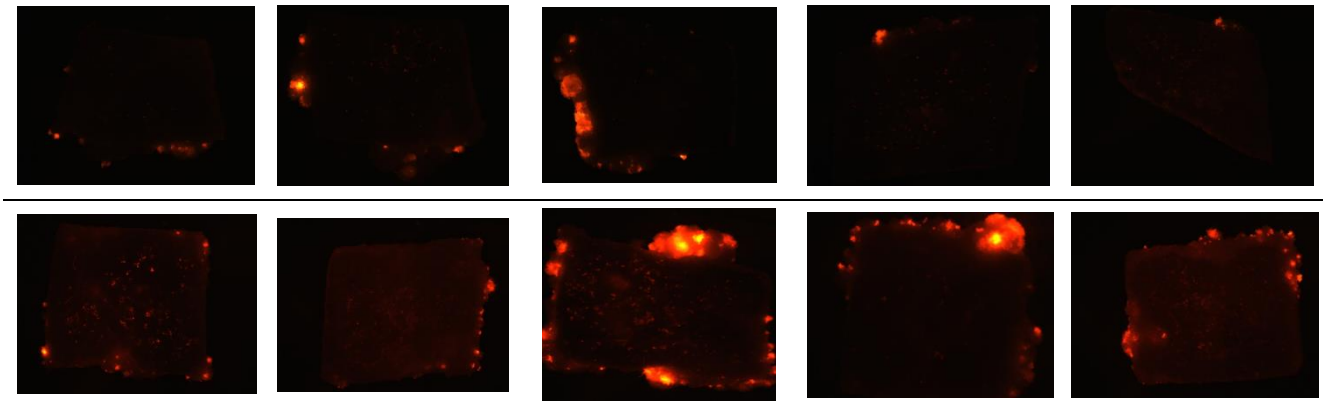



Figure 12 - The effect of LA on transient dsRed expression. Error bar indicate standard deviation.

Table 13 - The effect of LA on transient dsRed expression. The means with different letters represent significantly (P<0.05) different.

| Plant | Exposure time | Conc. (µM) | Rep. | dsRed spots (t=3days) | | dsRed spots (t=14days) | |
|---|---|---|---|---|---|---|---|
| *P.hybrida* | long | 0 | 3 | 0.02±0.04 | a | 7.11±6.35 | a |
| | long | 1 | 3 | 0.96±0.91 | ab | 6.33±4.69 | a |
| | long | 10 | 3 | 8.20±3.82 | cde | 6.76±3.85 | a |
| | long | 50 | 3 | 0.44±0.39 | a | 2.69±0.93 | a |
| | long | 100 | 3 | 2.84±0.43 | abc | 2.93±0.57 | a |
| | short | 0 | 3 | 1.42±0.43 | ab | 9.11±1.65 | a |
| | short | 1 | 3 | 4.71±2.46 | abcd | 7.76±3.39 | a |
| | short | 10 | 3 | 12.49±3.99 | e | 10.46±0.92 | a |
| | short | 50 | 3 | 7.13±2.37 | bcde | 10.62±4.33 | a |
| | short | 100 | 3 | 9.47±2.91 | de | 9.82±1.15 | a |
| *N.benthamiana* | long | 0 | 3 | 5.05±2.05 | cde | 1.78±0.40 | bc |
| | long | 1 | 3 | 7.33±1.05 | def | 0.91±0.50 | ab |
| | long | 10 | 3 | 7.56±1.35 | ef | 1.20±0.72 | ab |
| | long | 50 | 3 | 4.38±1.94 | bcd | 0.75±0.31 | ab |
| | long | 100 | 3 | 1.90±0.61 | ab | 0.13±0.00 | a |
| | short | 0 | 3 | 7.75±2.58 | ef | 1.40±0.37 | bc |
| | short | 1 | 3 | 8.38±1.42 | f | 2.51±0.27 | cd |
| | short | 10 | 3 | 3.36±1.04 | abc | 2.93±1.04 | d |
| | short | 50 | 3 | 4.87±1.88 | bcde | 2.33±0.85 | cd |
| | short | 100 | 3 | 0.57±0.33 | a | 0.90±0.81 | ab |

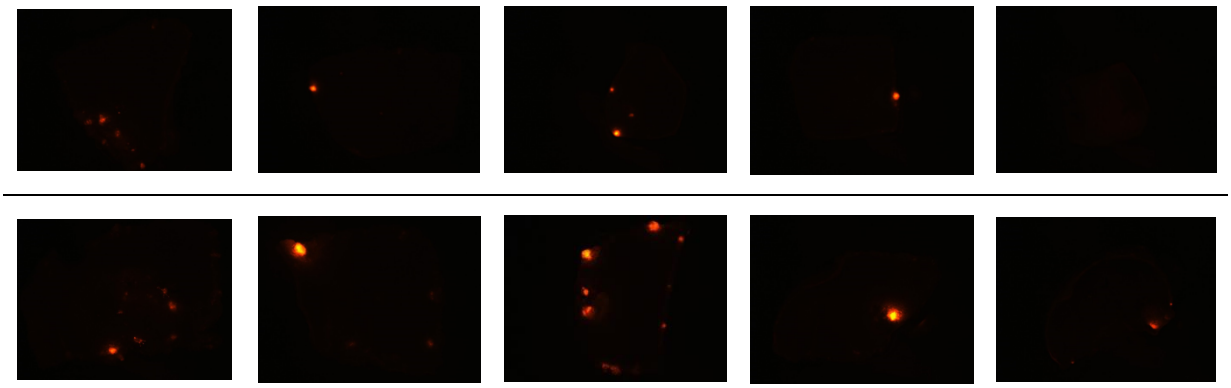
Table 14 - Transient dsRed expression in *P.hybrida* of long and short exposure time (EPT) treated with LA at checking point t=14 days.



*Upper line: long exposre time; lower line: short exposre time;

*From left to right: LA concentration 0μM, 1μM, 10μM, 50μM, 100μM.

Table 15 - Transient dsRed expression in *N.benthamiana* of long and short exposure time (EPT) treated LA at checking point t=14 days.



*Upper line: long exposre time; lower line: short exposre time;

*From left to right: LA concentration 0μM, 1μM, 10μM, 50μM, 100μM.

### 3.1.5 Summary

The highest transient dsRed expression with suitable concentration and exposure time of each compound were summarized in Table 16. *For P.hybrida*, at the checking point t= 3 days, 1μM AIP with long exposure time has the highest number of dsRed spots, and 10μM LA with short exposure time also has large number of dsRed spots. At the checking point t=14 days, unexpected, with short exposure time 1μM SA has the highest number of dsRed spots. Meanwhile, 50μM LA with short exposure time has the second highest number. For *N.benthamiana*, at the checking point t= 3 days, 10μM PBZ with short exposure time has the highest number of dsRed spots, and 10μM AIP with long exposure time also has large number of dsRed spots. At the checking point t=14 days, same as *P.hybrida*, unexpected, with 50μM SA has the highest number of dsRed spots.

Table 16 - The highest transient dsRed expression for each compound in two plant species.

| Plant species | Cpd. | dsRed spots (t=3 days) | dsRed spots (t=14 days) |
|---|---|---|---|
| *P.hybrida* | AIP | 1µM, long exposure time | 0µM, long exposure time |
| | PBZ | 0µM, long exposure time | 10µM, short exposure time |
| | SA | 1µM, short exposure time | 1µM, short exposure time |
| | LA | 10µM, short exposure time | 50µM, short exposure time |
| *N.benthamiana* | AIP | 10µM, long exposure time | 100µM, long exposure time |
| | PBZ | 10µM, short exposure time | 100µM, short exposure time |
| | SA | 0µM, short exposure time | 50µM, long or short exposure time |
| | LA | 1µM, short exposure time | 10µM, short exposure time |

## 3.2 The effect of AIP, PBZ, SA and LA on stable transformation efficiency

The effect of different compounds on stable transformation efficiency (%TE) was determined by calculating the number of explants with callus growth on Km SM or SIM after 30 days (4 weeks), the larger the number of explants with callus, the higher %TE. The following paragraph shows the effects of AIP, PBZ, SA and LA on %TE separately. For each compound, the result of the effect of exposure time and concentration were illustrated in the following paragraphs.

### 3.2.1 The effect of AIP on stable transformation efficiency

Figure 13 below illustrates the effect of AIP on stable transformation efficiency, and its can be seen that for *P.hybrida*, the highest %TE is obtained at 1µM AIP with long exposure time. The P value of exposure time and concentration were both smaller than 0.05 which indicated there was a significant different of these two factors (Table 17). However, for *N. benthamiana*, there was no significant difference for concentration as well as for exposure time. The %TE with different exposure times and different concentrations were similar to each other (Table 17).

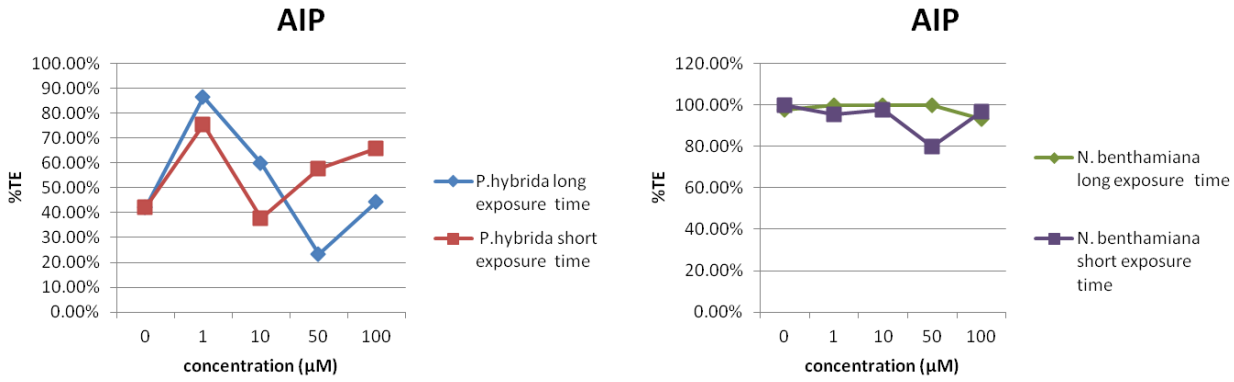



Figure 13 - The effect of AIP on stable transformation efficiency.

Table 17 - The effect of AIP on stable transformation efficiency. The means with different letters represent significantly (P<0.05) different.

| Compound | Plant | Exposure time | Conc. (µM) | Explant + calli (t=30days) | | %TE |
|---|---|---|---|---|---|---|
| AIP | *P.hybrida* | long | 0 | 6.33±2.51 | ab | 42.22 |
| | | long | 1 | 13.00±1.73 | d | 86.67 |
| | | long | 10 | 9.00±1.00 | bc | 60.00 |
| | | long | 50 | 3.33±2.30 | a | 23.17 |
| | | long | 100 | 6.67±2.08 | ab | 44.44 |
| | | short | 0 | 6.33±0.58 | ab | 42.22 |
| | | short | 1 | 11.33±1.53 | cd | 75.56 |
| | | short | 10 | 5.00±1.73 | a | 37.68 |
| | | short | 50 | 8.67±2.31 | bc | 57.78 |
| | | short | 100 | 9.67±1.53 | bc | 66.03 |
| | *N.benthamiana* | long | 0 | 14.67±0.58 | a | 97.78 |
| | | long | 1 | 15.00±0.00 | a | 100.00 |
| | | long | 10 | 15.00±0.00 | a | 100.00 |
| | | long | 50 | 15.00±0.00 | a | 100.00 |
| | | long | 100 | 14.00±0.00 | a | 93.33 |
| | | short | 0 | 15.00±0.00 | a | 100.00 |
| | | short | 1 | 14.33±0.58 | a | 95.56 |
| | | short | 10 | 14.67±0.58 | a | 97.78 |
| | | short | 50 | 12.00±4.36 | a | 80.00 |
| | | short | 100 | 14.50±0.71 | a | 96.67 |

### 3.2.2 The effect of PBZ on stable transformation efficiency

Figure 14 below illustrates the effect of PBZ on stable transformation efficiency, and its can be seen that for *P.hybrida*, the highest %TE is obtained at 10µM PBZ with short exposure time. There were significant different at different concentrations and exposure times (Table 18). However, for *N.benthamiana*, the same result with AIP, no significant difference for concentration as well as for exposure time. The %TE with different exposure times and different concentrations were very similar to each other (Table 18).

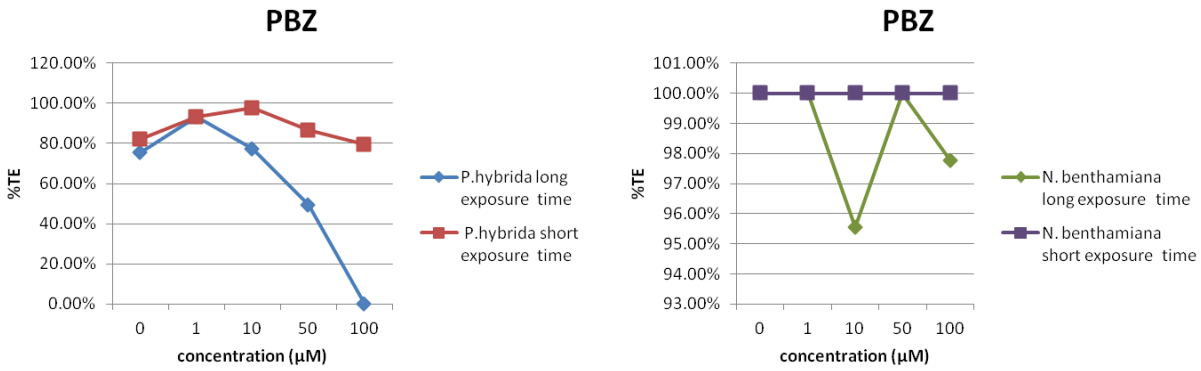



Figure 14 - The effect of PBZ on stable transformation efficiency

Table 18 - The effect of PBZ on stable transformation efficiency. The means with different letters represent significantly (P<0.05) different.

| Compound | Plant | Exposure time | Conc. (µM) | Explant + calli (t=30days) | | %TE |
|---|---|---|---|---|---|---|
| PBZ | *P.hybrida* | long | 0 | 11.33±2.31 | bc | 76.00 |
| | | long | 1 | 14.00±1.00 | c | 93.00 |
| | | long | 10 | 11.33±1.53 | bc | 77.00 |
| | | long | 50 | 6.67±3.06 | b | 49.00 |
| | | long | 100 | 0.00±0.00 | a | 0.00 |
| | | short | 0 | 13.33±1.12 | c | 82.22 |
| | | short | 1 | 13.67±0.58 | c | 93.17 |
| | | short | 10 | 14.67±0.58 | c | 97.78 |
| | | short | 50 | 13.00±1.73 | c | 86.67 |
| | | short | 100 | 11.67±3.51 | bc | 79.68 |
| | *N.benthamiana* | long | 0 | 15.00±0.00 | a | 100.00 |
| | | long | 1 | 15.00±0.00 | a | 100.00 |
| | | long | 10 | 14.33±1.16 | a | 95.56 |
| | | long | 50 | 15.00±0.00 | a | 100.00 |
| | | long | 100 | 14.67±0.58 | a | 97.78 |
| | | short | 0 | 15.00±0.00 | a | 100.00 |
| | | short | 1 | 15.00±0.00 | a | 100.00 |
| | | short | 10 | 15.00±0.00 | a | 100.00 |
| | | short | 50 | 15.00±0.00 | a | 100.00 |
| | | short | 100 | 15.00±0.00 | a | 100.00 |

### 3.2.3 The effect of SA on stable transformation efficiency

Figure 15 below illustrates the effect of SA on stable transformation efficiency, and its can be seen that for *P.hybrida*, the highest %TE is obtained at 0µM SA with short exposure time. There was a significant different at different exposure times, which was with short exposure time, the %TE was higher than with long exposure time. But there was no significant different at varying concentrations (Table 19). However, for *N. benthamiana*, the same result with AIP and PBZ, no significant difference for concentration as well as for exposure times. The %TE with different exposure times and different concentrations were very similar to each other (Table 19).

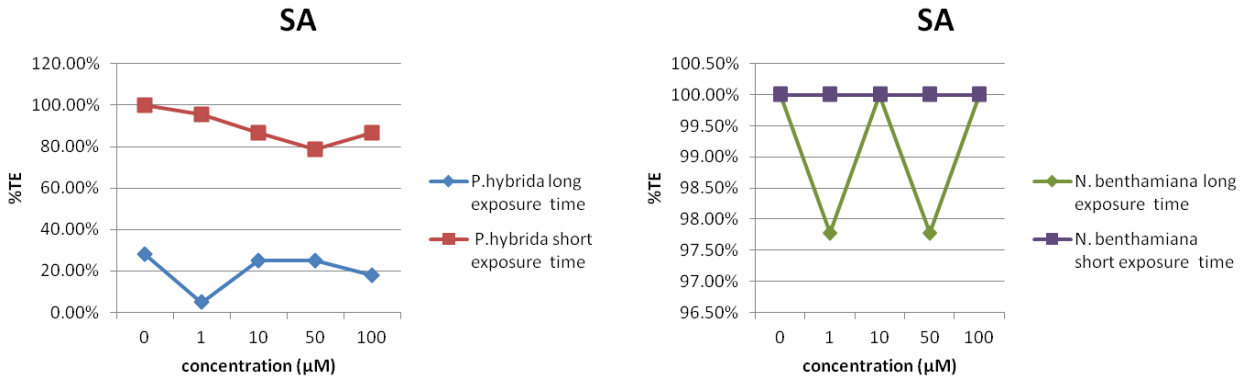



Figure 15 - The effect of SA on stable transformation efficiency

Table 19 - The effect of SA on stable transformation efficiency. The means with different letters represent significantly (P<0.05) different.

| Compound | Plant | Exposure time | Conc. (µM) | Explant + calli (t=30days) | | %TE |
|---|---|---|---|---|---|---|
| SA | *P.hybrida* | long | 0 | 3.67±6.35 | abc | 28.21 |
| | | long | 1 | 0.67±0.58 | a | 4.76 |
| | | long | 10 | 3.00±5.20 | ab | 25.00 |
| | | long | 50 | 1.50±2.12 | a | 25.00 |
| | | long | 100 | 2.67±3.01 | ab | 17.78 |
| | | short | 0 | 15.00±0.00 | d | 100.00 |
| | | short | 1 | 14.00±1.00 | d | 95.56 |
| | | short | 10 | 13.00±0.00 | cd | 86.67 |
| | | short | 50 | 11.33±3.22 | bcd | 78.63 |
| | | short | 100 | 13 | cd | 86.67 |
| | *N.benthamiana* | long | 0 | 15.00±0.00 | a | 100.00 |
| | | long | 1 | 14.67±0.58 | a | 97.78 |
| | | long | 10 | 15.00±0.00 | a | 100.00 |
| | | long | 50 | 14.67±0.58 | a | 97.78 |
| | | long | 100 | 15.00±0.00 | a | 100.00 |
| | | short | 0 | 15.00±0.00 | a | 100.00 |
| | | short | 1 | 15.00±0.00 | a | 100.00 |
| | | short | 10 | 15.00±0.00 | a | 100.00 |
| | | short | 50 | 15.00±0.00 | a | 100.00 |
| | | short | 100 | 15.00±0.00 | a | 100.00 |

### 3.2.4 The effect of LA on stable transformation efficiency

Figure 16 below illustrates the effect of LA on stable transformation efficiency, and its can be seen that for *P.hybrida*, the highest %TE is obtained at 1µM LA with long and short exposure times. There was no significant different at different exposure times and concentrations (Table 17). For *N.benthamiana*, it has similar result with AIP and PBZ and SA, no significant different at different

exposure times. For 100µM LA although it has a different between the other concentrations, but this result may not precise. Because 100µM LA only has two replications, due to contamination, the stable transformation efficiency of these two replications was almost the same. So assume the 100µM LA has three replications, there should be no significant difference for concentrations, same as other three compounds mentation in the earlier paragraph. In addition, for both two plant species, explants treated 1µM, 10µM and 50µM LA were greener and more vigorous compare with 0µM and 100µM LA. Also calluses were healthier like Figure 17 shows below.

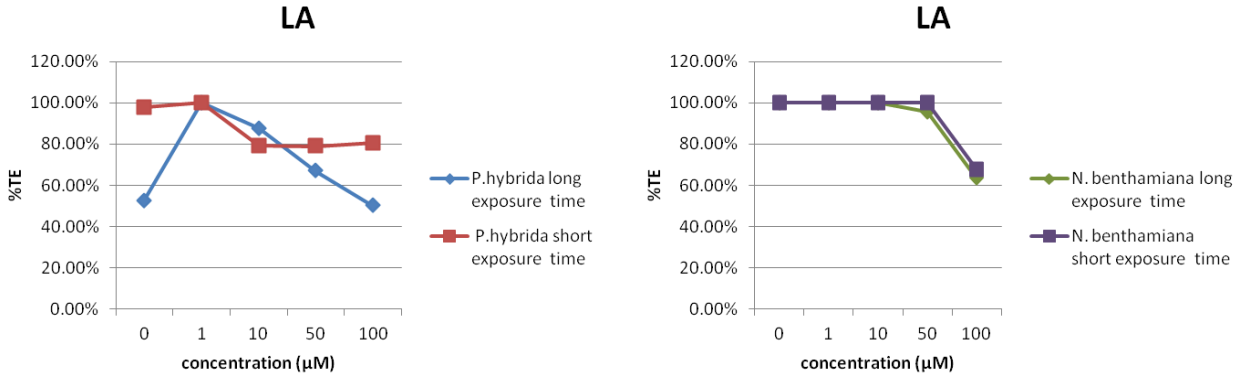



Figure 16 - The effect of LA on stable transformation efficiency

Table 20 - The effect of LA on stable transformation efficiency. The means with different letters represent significantly (P<0.05) different.

| Compound | Plant | Exposure time | Conc. (µM) | Explant + calli (t=30days) | | %TE |
|---|---|---|---|---|---|---|
| LA | *P.hybrida* | long | 0 | 7.67±7.10 | ab | 52.54 |
| | | long | 1 | 15.00 | b | 100.00 |
| | | long | 10 | 12.67±3.22 | ab | 87.52 |
| | | long | 50 | 8.33±3.06 | ab | 66.87 |
| | | long | 100 | 5.33±0.58 | a | 50.11 |
| | | short | 0 | 14.67±0.58 | b | 97.78 |
| | | short | 1 | 12.33±2.08 | ab | 100.00 |
| | | short | 10 | 11.67±2.52 | ab | 79.21 |
| | | short | 50 | 11.33±1.16 | ab | 78.97 |
| | | short | 100 | 11.33±2.08 | ab | 80.56 |
| | *N.benthamiana* | long | 0 | 15.00±0.00 | b | 100.00 |
| | | long | 1 | 15.00±0.00 | b | 100.00 |
| | | long | 10 | 15.00±0.00 | b | 100.00 |
| | | long | 50 | 14.33±0.58 | b | 95.56 |
| | | long | 100 | 9.50±0.71 | a | 63.33 |
| | | short | 0 | 15.00±0.00 | b | 100.00 |
| | | short | 1 | 15.00±0.00 | b | 100.00 |
| | | short | 10 | 15.00±0.00 | b | 100.00 |
| | | short | 50 | 15.00±0.00 | b | 100.00 |
| | | short | 100 | 10.00±4.24 | a | 66.67 |

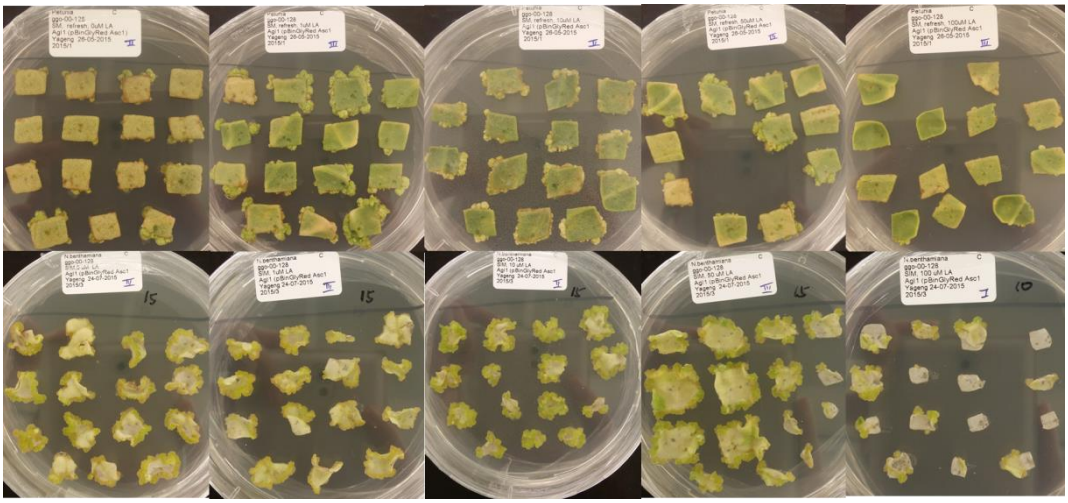


Figure 17 - Explants in petri dishes treated with lipoic acid with different concentrations. Upper line from left to right: *P.hybrida* treated with 0µM, 1µM, 10µM, 50µM and 100µM LA; lower line from left to right: *N.benthamiana* treated with 0µM, 1µM, 10µM, 50µM and 100µM LA.

## 3.3 The effect of AIP, PBZ, SA and LA on ultimate transformation efficiency

The effect of different compounds on the ultimate transformation efficiency (%RE) was determined by counting the number of explants/calli on which shoots regenerated on Km SM or SIM after 90 days (12 weeks). Due to time limitation, the %RE of *N.benthamiana* was calculated after 85 days. The following paragraphs shows the effect of AIP, PBZ, SA and LA on %RE separately. The result of examination of transgenic plants via fluorescence and/or PCR test was also display in the end of this section.

### 3.3.1 The effect of AIP on ultimate transformation efficiency

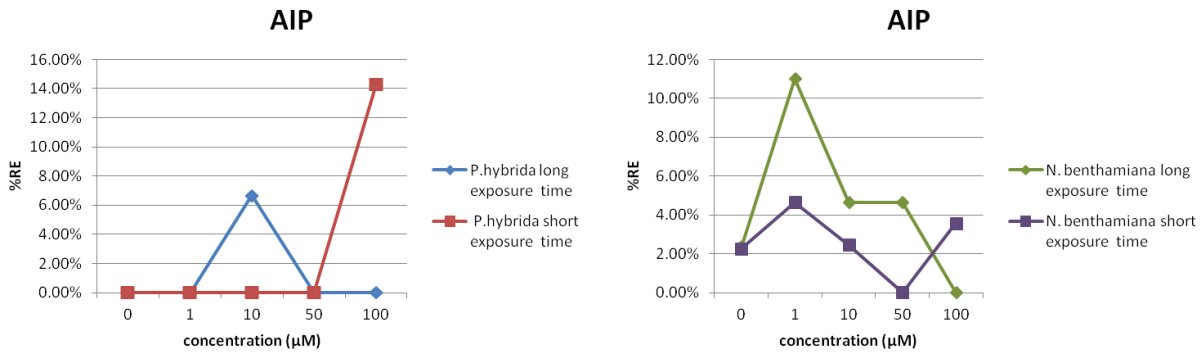



Figure 18 - The effect of AIP on ultimate transformation efficiency.

The Figure 18 above shows the effect of AIP on ultimate transformation efficiency. For *P.hybrida*, The highest %RE (14.29%) is obtained at 100µM with short exposure time. The second highest %RE (6.67%) is obtained at 10µM with long exposure time. All other concentration and exposure times did not yield any shorts, value 0.00%. For *N. benthamiana*, the highest %RE (10.00%) is obtained at 1µM with long exposure time. 10µM and 50µM with long exposure time and 1µM with short exposure time have the same %RE, value 4.65% (Figure 18).

### 3.3.2 The effect of PBZ on ultimate transformation efficiency

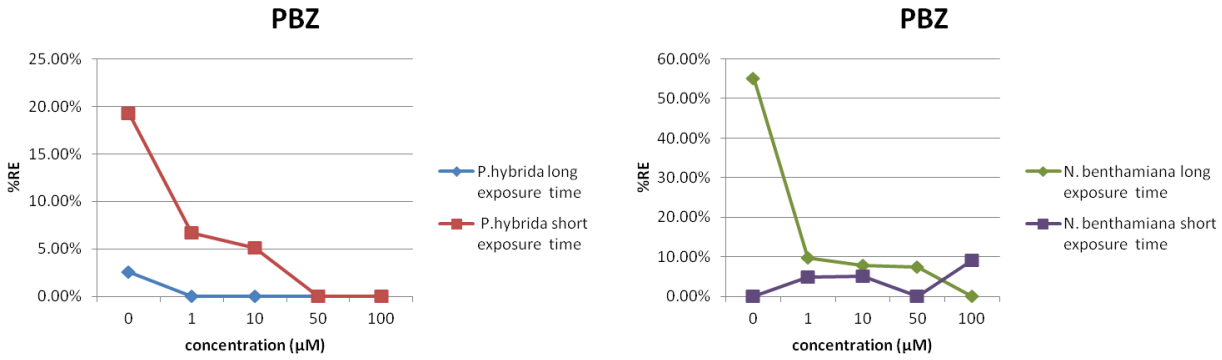



Figure 19 - The effect of PBZ on ultimate transformation efficiency.

As shown in Figure 19, *for P.hybrida*, the highest %RE (19.29%) is obtained at 0µM with short exposure time. For *N.benthamiana*, the highest %RE (55.17%) was obtained at long exposure time with 0µM PBZ. Interestingly, for *P.hybrida* with both long and short exposure time and *N.benthamiana* with long exposure time, the concentration level at 0µM of PBZ has higher %RE than other concentrations and they all have a similar trend except for *N.benthamiana* of short exposure time (100µM has higher %RE).

### 3.3.3 The effect of SA on ultimate transformation efficiency

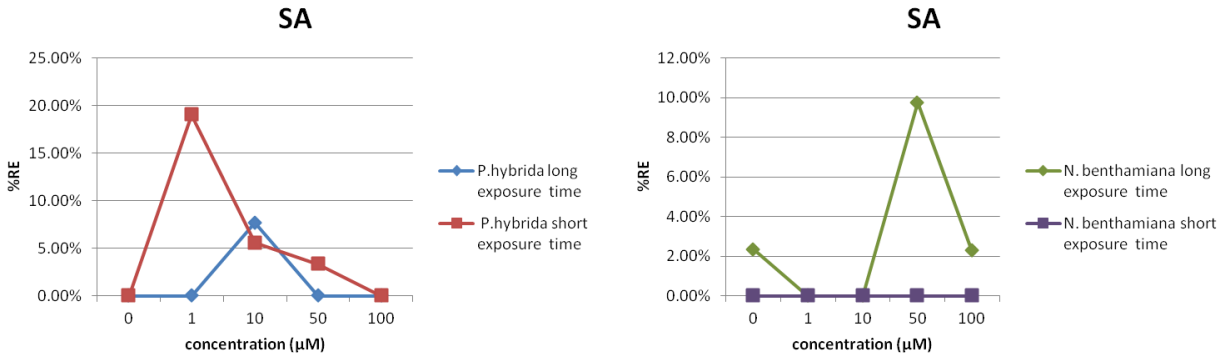



Figure 20 - The effect of SA on ultimate transformation efficiency.

As Figure 20 shows above, for *P.hybrida*, 1µM with short exposure time, the %RE yielded the highest number (19.05%), followed with the long exposure time with concentration level of 10µM (7.69%). For *N.benthamiana*, the highest %RE obtained at 50µM with long exposure time (9.76%) followed with 0µM with long exposure time (2.33%) and 100µM with long exposure time (2.27%). With short exposure time, there was no regeneration shoot appeared so the %RE was valued 0.00%.

### 3.3.4 The effect of LA on ultimate transformation efficiency

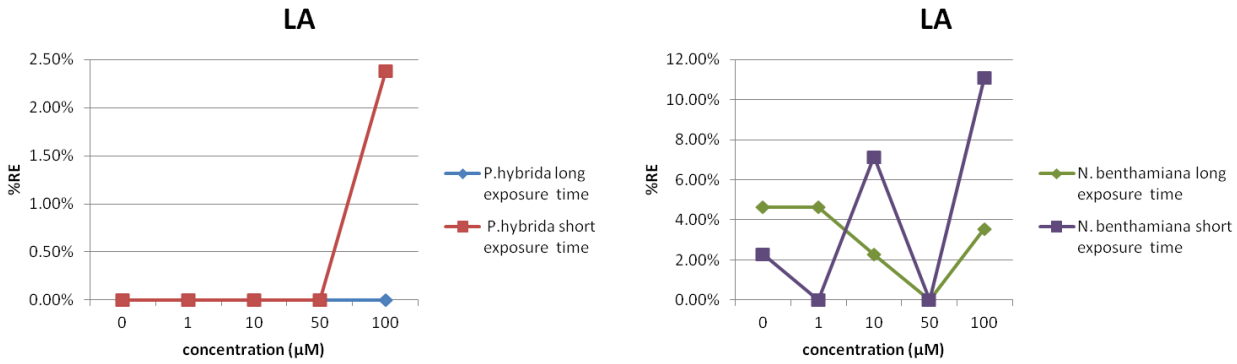



Figure 21 - The effect of LA on ultimate transformation efficiency.

Figure 21 above shows the effect of LA on ultimate transformation efficiency (%RE). For *N.benthaminana*. The highest %RE (11.11%) is obtained at 100µM LA with short exposure time, followed with 10µM LA with short exposure time, about 7.14%. With long exposure time, the concentration of 0µM and 1µM have same %RE (4.65%). For both species, the ultimate transformation efficiency treat with 50µM LA with two exposure time (long days and short days) were all zero. Furthermore, the %RE of *P. hybrida* was much lower than *N. benthamiana* for long exposure time as well as short exposure time, almost all 0.00%, except 100µM LA with short exposure time (2.38%).

### 3.3.5 Examine transgenic plant via fluorescence and/or PCR test

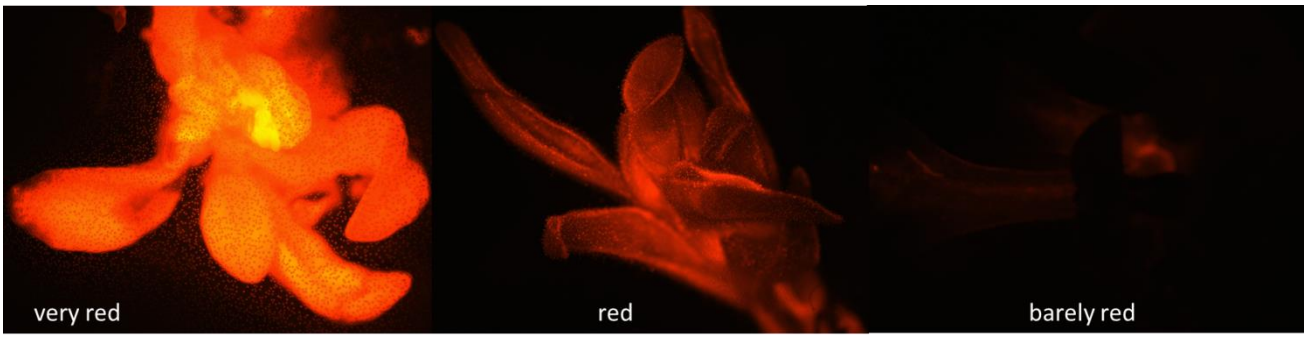



Figure 22 – Different levels of dsRed fluorescence in individual *P.hybrida* transgenic plants (t=90 days).

For *P. hybrida*, 26 plants (include one control) were test by dsRed fluorescence (Figure 22) and 24 plants out of those 26 plants were examined by PCR test (the PCR result shown in appendix 5). Because extraction of DNA failed in two plants (the result of extraction of DNA shown in appendix 4), these two were not further examined by PCR. To determine whether a plant was transgenic generally several parameters are used such as fluorescence and positive PCR test, but also the results from transient dsRed expression after 14 days grown on Km SM or SIM medium, TE, the survival ability in Km SM are considered. Based on those parameters mentioned above, the 25 tested plants (not including the control), 13 of them were confirmed to be transgenic plants (52.00%), 6 of them were not certain (24.00%), and the rest of them (24.00%) were confirmed to be non-transgenic plant or escapes (Appendix 7).


Figure 23 - Different levels of dsRed fluorescence in individual *N.benthamiana* transgenic plants (t=85 days).

For *N.benthamiana*, due to time limitations, PCR result are not available, and to decide whether a plant is transgenic thus depended on the results from 85 days dsRed fluorescence check at 85 days (Figure 23) and combined with results from transient dsRed expression after 14 days grown in SIM, TE and the survival ability on Km SM(appendix 8). The results showed that, of 58 tested plants (not including the control), 11 of them were confirmed as transgenic plants (18.97%), 3 of them were not certain (5.17%), and the rest of them (75.86%) are considered non-transgenic plants.

# 4. Discussion

In general, the transformation efficiency of *N. benthamiana* was higher than *P.hybrida*. One main reason was that the number of explants surviving in the Km SM medium of *P. hybrida* was much lower than of *N.benthamiana* due to contamination and/or bacteria overgrowth.

The hypothesis in this study was that SA inhibitors like AIP and PBZ can increase transformation efficiency. For *P.hybrida,* the transient dsRed expression in the presence of SA was lower with than AIP and PBZ with both exposure times at three days after co-cultivation (checking point t=3 days) but the optimum concentration was variable. This result may confirm the role of SA, which is effective against pathogen infection in plant defense mechanism. However, 14 days after co-cultivation (checking point t=14 days), the transient dsRed expression with SA was higher than AIP and PBZ. The %TE of AIP and PBZ with long exposure time was higher than SA, but with short exposure time, the %TE was lower than SA. The possible reason was that with long exposure in the Petri dish continually provide SA inhibitors to block the SA biosynthesis, which could promote gene transfer and gene integration. The %RE in the presence of SA was lower with than AIP and PBZ but the optimum concentration was variable. For both AIP and PBZ, more callus growth and less regeneration shoots were observed, which indicated the %RE was lower than %TE. For *N.benthamiana*, three days after co-cultivation (checking point t=3 days), AIP with long days exposure time, PBZ with short days exposure time has highest transient dsRed expression, which confirms the role of SA in plant defense system again. However, 14 days after co-cultivation (checking point t=14 days) with two exposure times, the transgenic dsRed expression of the treatment with SA was higher than with its inhibitors same as *P.hybrida*, which is an opposite result of the role of SA. The possible reason may reside in the long exposure time to the external SA from Km SM or SIM triggering the biosynthesis of some unclear compound which may help promoting gene uptake and integration. The %TE of *N. benthamiana* with SA or SA inhibitors showed no differences between exposure times and concentration levels, most explants have callus after 30 days which indicated that a faster callus growth in the presence of these compounds. The reason could be that the level/rate of differentiation and callus growth in *N.benthamiana* and *P.hybrida* are different and vary due to the physiology condition. The % RE of *N.benthamiana* in the presence of SA was lower with than AIP and PBZ with both exposure times but the optimum concentration was variable. However, the %RE of AIP and PBZ was not as high as expected, the highest %RE was obtained at 0µM PBZ with long exposure time. In general, for both these two plant species, the AIP and PBZ can increase the transformation efficiency, but more callus growth in the presence of these compounds and lower regeneration so more likely was that these two compounds induced callus at high frequency instead of regenerating shoots. This situation was also found in sweet potato treated with PBZ during *Agrobacterium*-mediated transformation (González et al., 2008).

Lipoic acid, was reported to be an antioxidant reducing the oxidative stress of plants and to enhance the transformation of soybean, tomato, wheat and cotton (Dan et al., 2009). In this report, the average number of dsRed fluorescent spots depended on the LA concentration. The highest transient dsRed expression was obtained at 10µM and 50µM with short exposure time in *P.hybrida*; 1µM and 10µM with short exposure time in *N.benthamiana* at two checking point. This indicated that for *P.hybrida*, 10µM and 50µM were the optimal concentrations on gene uptake by explants and gene integration; for *N.benthamiana*, 1µM and 10µM were the optimal concentrations on gene uptake and gene integration. The highest %TE on LA is obtained at 0µM with short exposure time in

*P.hybrida* and no significant different between concentrations and exposure times were found in *N.benthamiana*. This result suggested that LA was not efficient at improve %TE, but calluses were vigorous and greener compared with other compounds. The highest %RE is obtained at 100µM with short exposure time (Figure 21) for *N.benthamiana*. This result suggests that the in *N.benthamiana* transformation efficiency can be increased by adding this compound to the medium. Same result was observed on *P.hybrida* with short exposure time. But with long exposure time of *P.hybrida*, the LA did not enhance the transformation efficiency. This observation may suggest that for *P.hybrida* LA may need short exposure time as a role in enhance transformation efficiency. In addition, with 1µM, 10µM and 50µM, the explants were more vigorous and greener and calluses were healthier compare with the explants without LA (Figure 17) in both two plant species. This observation confirms the role of LA in anti-oxidative activity and overcoming the problem of browning and necrosis after inoculation with *Agrobacterium*.

In some of the transformant shoots fluorescence by UV light was observed, but no gene was detected by the PCR test. The possible explanation could be the concentration of template was high or the template was contaminating when set up PCR. Some other transformant shoots did not show fluorescence by UV light under microscope but did have the dsRed fluorescing gene present according to the PCR test. A possible explanation could be that the dsRed fluorescing gene was silenced and its expression reduced, therefore fluorescence cannot be observed under microscope but using PCR, the target gene being present was amplified. The remaining shoots did not have fluorescence and also no dsRed gene was detected, hence they were confirmed being non-transgenic plants. Interestingly, in *P.hybrida*, there was one transformant shoot showing very clear fluorescence in the cutting point from the stem, and no fluorescence in the leaves (Figure 24). PCR tests confirmed this plant as a transgenic plant, which could be evidence for the dsRed fluorescence gene being silenced but activated again by cutting. In *N.benthamiana*, a flower bud was checked for fluorescence together with leaves by accident. An interesting phenomenon was found: three transformant shoots with three flower buds were tested and in all fluorescence was observed, however only the leaves from two out of three transformant shoots showed fluorescence, the other one did not have fluorescence in its leaves (Figure 25). One possible reason could be that the transformant shoot was chimera, which composed of genetically distinct cells and result in differential gene expression.

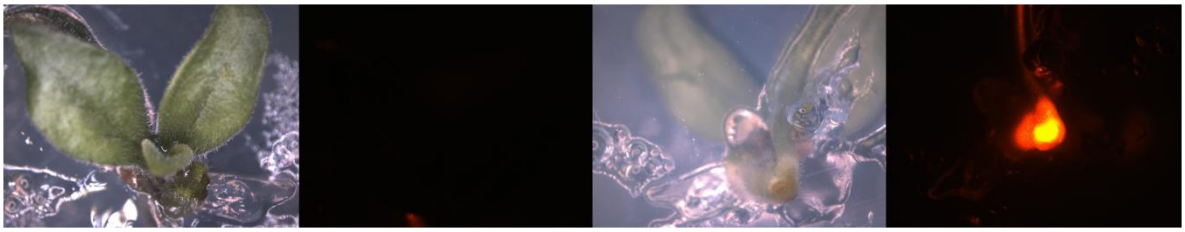


Figure 24 - Transformants shoots in individual under microscope with normal light and UV light from *P.hybrida*

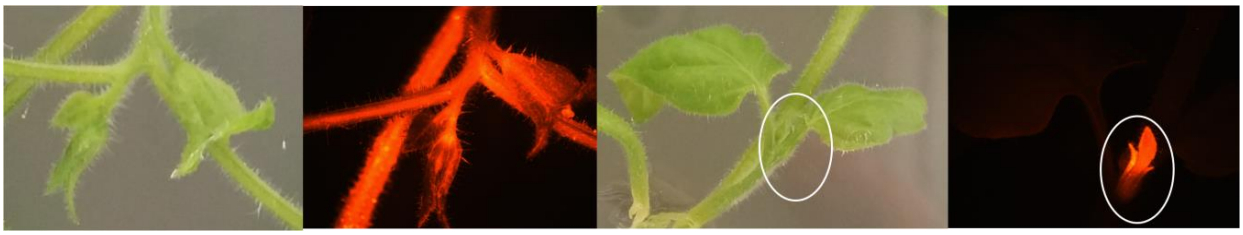


Figure 25 - Transformant shoots in individual under microscope with normal light and UV light from *N.benthamiana.*

## 5. Conclusion and future research

In summary, the efficiency of *Agrobacterium*-mediated transformation depends on a balanced interaction between plant and *Agrobacterium*. In order to introduce the transfer (-T) DNA and integrate it into the plant genome, the plant must be susceptible to *Agrobacterium*. The data from these experiments here show that *Agrobacterium*-mediated transformation efficiency was decreased by adding SA which confirms the role of SA in plant defense against pathogen infection. SA inhibitors like PBZ and AIP can block SA biosynthesis and increase the *Agrobacterium*-mediated transformation efficiency. The optimum concentration and exposure time that worked were variable in *P.hybrida* and *N. benthamiana*. Explants treated with LA have less browning and necrosis and probed to be more vigorous in two plant species. For *N. benthamiana*, LA increased the transformation efficiency at the concentration level of 100µM. But for *P.hybrida*, the LA has less efficiency. Repeating this experiment is necessary to get more data. A transformant shoot with observed fluorescence could be transgenic, but a transformant shoot without fluorescence could also be transgenic plant due to gene silencing.

For the future research, it could be better to keep the two species in the same sterile environmental conditions, e.g. both sowing and growing *in vitro* to reduce the contamination and bacterial overgrowth therefore to decrease the standard deviation. For evaluation of the transient dsRed expression it might be better to standardize the dsRed spot counting in order to reduce the standard deviation and to get more precise results, for instance, by using software to counting dsRed spots by setting a universal standard on luminance. Although, this paper proved that SA inhibitors like AIP and PBZ can enhance transformation efficiency in *P. hybrida* and *N. benthamiana*, in other species the optimum exposure time will need to be established again. Moreover, the usefulness of SA inhibitors like AIP and PBZ should be investigated further to confirm the influence of SA biosynthesis by doing an experiment in which internal SA levels in these two plant species before and after adding SA inhibitors will be determined. Last, combine the SA inhibitors with lipoic acid, such as 10µM PBZ combine with 100µM LA with short exposure time for *P.hybrida* and 1µM AIP with long exposure time combine with 100M LA with short exposure time, might also improve the transformation efficiency since these compound were confirmed in increase the transformation efficiency separately in this paper.

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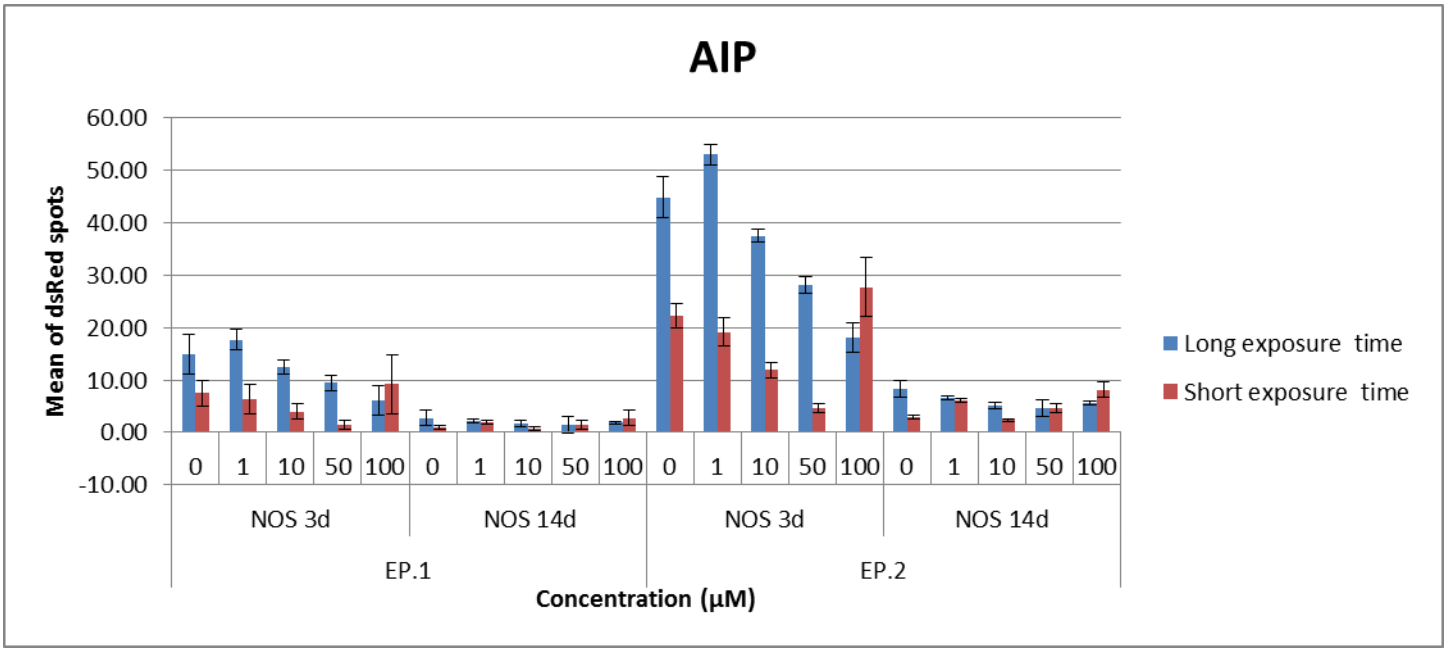
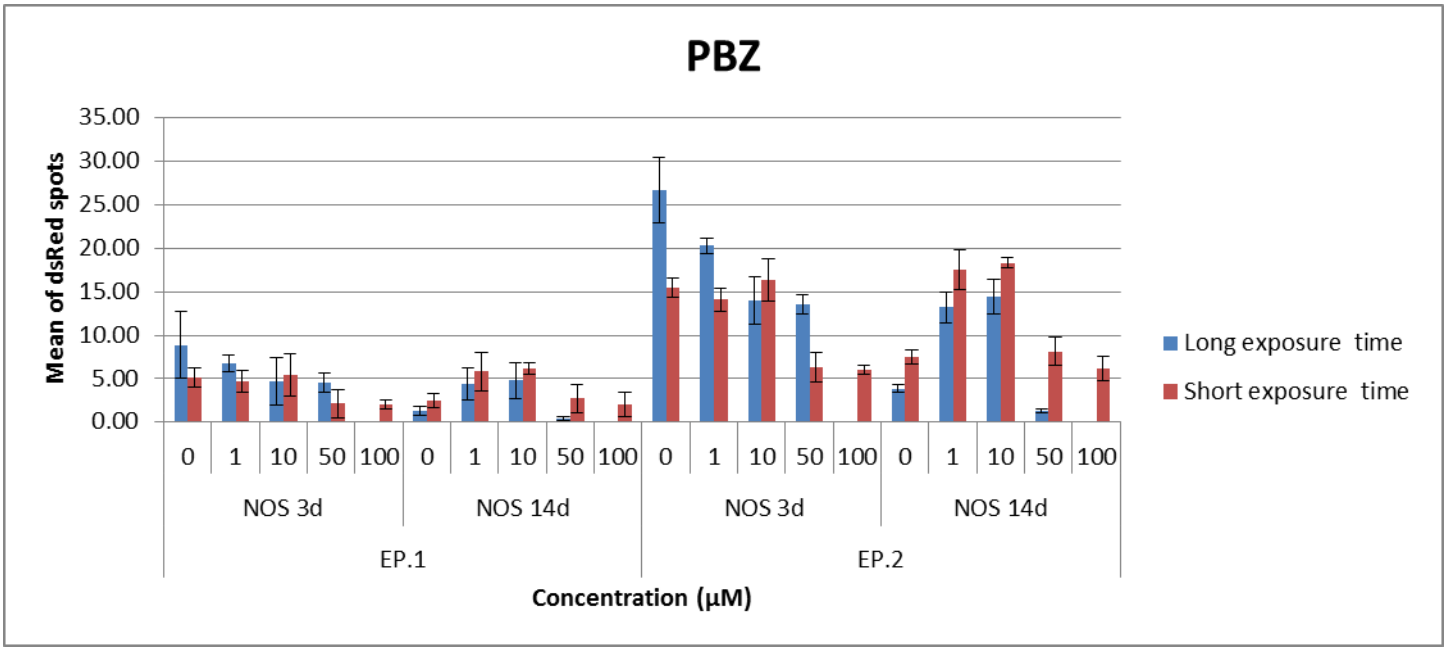
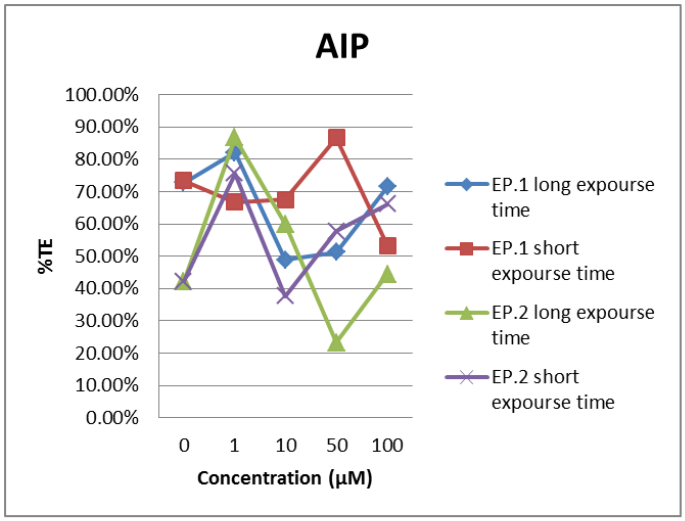
**Appendix 1 – Compare of the effect of AIP and PBZ on dsRed expression of *Petunia hybrida* at experiment 1 and experiment 2.**

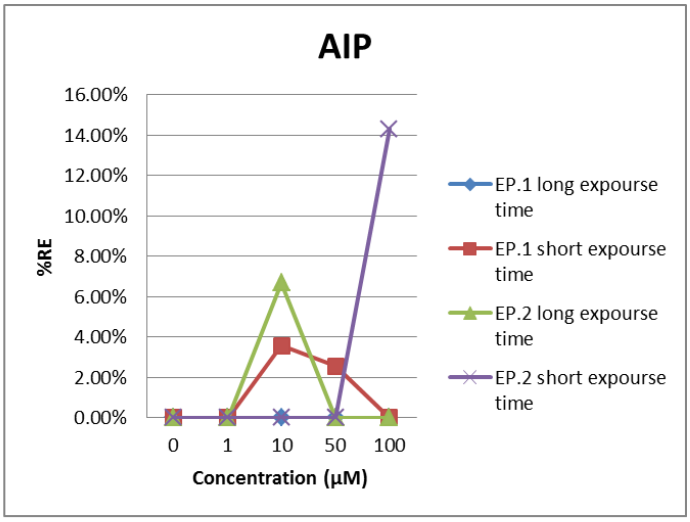


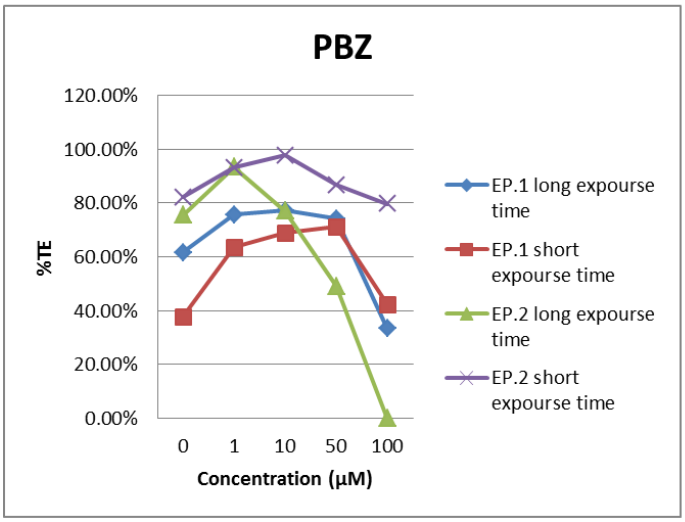



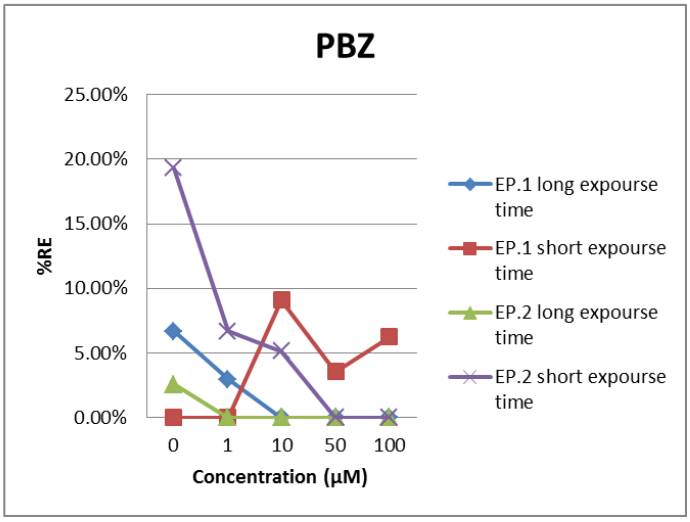

Ep.1 = first time expreiment, EP.2 = repeat expreiment

**Appendix 2 – Compare of the effect of AIP and PBZ on stable transformation efficiency and ultimate transformation efficiency of *Petunia hybrida* in experiment 1 and experiment 2**




Ep.1 = first time expreiment, EP.2 = repeat expreiment

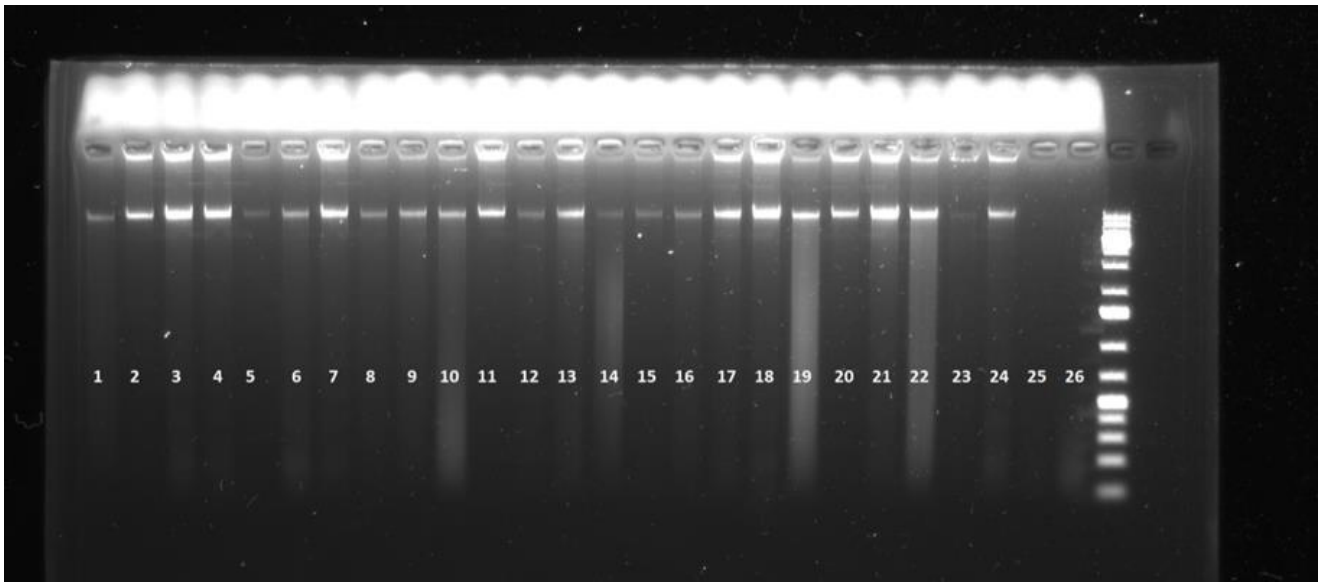
**Appendix 3 – Genstat result of transient dsRed expression and transformation efficiency (long exposure time) of *Petunia hybrida* and *N.benthamiana***

| Cpd. | Plant | Conc. (µM) | NOS3 | | NOS14 | | NOEcalli4W | |
|---|---|---|---|---|---|---|---|---|
| AIP | *P. hybrida* | 0 | 14.93 | cd | 2.78 | a | 3.33 | a |
| | | 1 | 17.67 | d | 2.20 | a | 6.33 | ab |
| | | 10 | 12.51 | bc | 1.67 | a | 13.00 | c |
| | | 50 | 9.40 | ab | 1.51 | a | 9.00 | b |
| | | 100 | 6.00 | a | 1.87 | a | 6.67 | ab |
| | *N.benthamiana* | 0 | 10.20 | abc | 1.82 | ab | 14.00 | a |
| | | 1 | 10.62 | bc | 0.58 | a | 14.67 | b |
| | | 10 | 12.09 | c | 1.24 | ab | 15.00 | b |
| | | 50 | 5.64 | a | 2.20 | b | 15.00 | b |
| | | 100 | 6.47 | ab | 2.56 | b | 15.00 | b |
| PBZ | *P. hybrida* | 0 | 8.89 | c | 1.29 | ab | 11.33 | c |
| | | 1 | 6.76 | bc | 4.40 | bc | 14.00 | c |
| | | 10 | 4.64 | b | 4.80 | c | 11.33 | c |
| | | 50 | 4.51 | b | 0.42 | a | 6.67 | b |
| | | 100 | 0.00 | a | 0.00 | a | 0.00 | a |
| | *N.benthamiana* | 0 | 4.16 | a | 0.91 | a | 15.00 | a |
| | | 1 | 1.27 | a | 1.11 | a | 15.00 | a |
| | | 10 | 1.53 | a | 0.73 | a | 14.33 | a |
| | | 50 | 2.62 | a | 0.93 | a | 15.00 | a |
| | | 100 | 1.96 | a | 2.00 | b | 14.67 | a |
| SA | *P. hybrida* | 0 | 0.07 | a | 0.82 | a | 3.67 | a |
| | | 1 | 0.02 | a | 0.19 | a | 0.67 | a |
| | | 10 | 0.09 | a | 1.27 | a | 3.00 | a |
| | | 50 | 0.96 | b | 0.50 | a | 1.50 | a |
| | | 100 | 1.16 | b | 0.67 | a | 2.67 | a |
| | *N.benthamiana* | 0 | 2.73 | a | 2.51 | a | 15.00 | a |
| | | 1 | 1.60 | a | 3.62 | ab | 14.67 | a |
| | | 10 | 0.76 | a | 4.96 | bc | 15.00 | a |
| | | 50 | 0.69 | a | 5.40 | c | 14.67 | a |
| | | 100 | 0.22 | a | 2.93 | a | 15.00 | a |
| LA | *P. hybrida* | 0 | 0.02 | a | 7.11 | a | 7.67 | a |
| | | 1 | 0.96 | a | 6.33 | a | 14.98 | a |
| | | 10 | 8.20 | b | 6.76 | a | 12.67 | a |
| | | 50 | 0.44 | a | 2.69 | a | 8.33 | a |
| | | 100 | 2.84 | a | 2.93 | a | 5.33 | a |
| | *N.benthamiana* | 0 | 5.04 | bc | 1.87 | a | 15.00 | c |
| | | 1 | 7.33 | bc | 4.64 | b | 15.00 | bd |
| | | 10 | 7.56 | c | 4.44 | b | 15.00 | bd |
| | | 50 | 4.38 | ab | 5.38 | b | 14.33 | b |
| | | 100 | 1.90 | a | 4.60 | b | 9.50 | a |

**Appendix 4 – Genstat result of transient dsRed expression and transformation efficiency (short exposure time) of *Petunia hybrida* and *N.benthamiana***

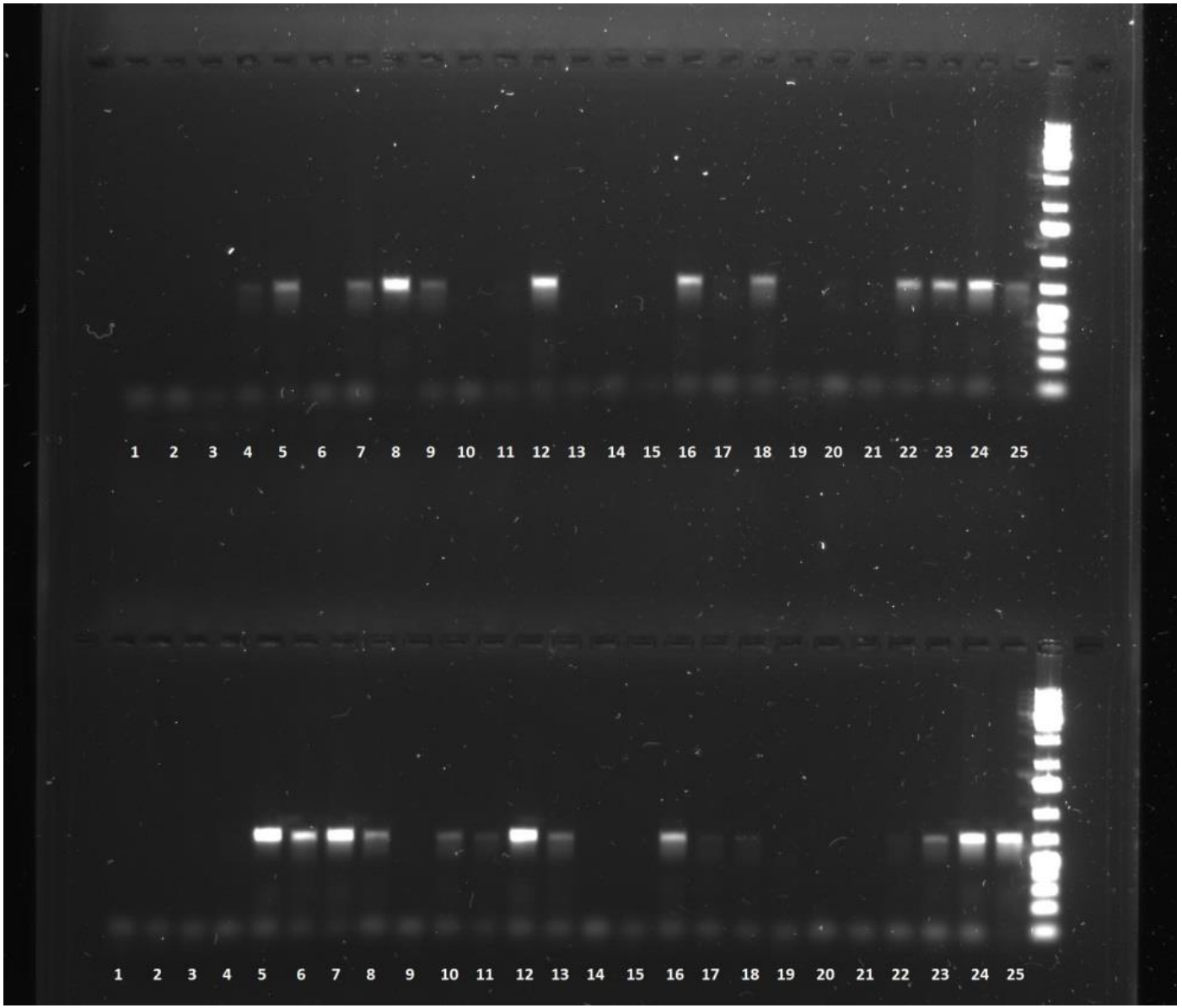
| Cpd. | Plant | Conc. (µM) | NOS3 | | NOS14 | | NOEcalli4W | |
|---|---|---|---|---|---|---|---|---|
| AIP | *P. hybrida* | 0 | 7.44 | ab | 0.98 | a | 6.33 | ab |
| | | 1 | 6.38 | ab | 2.02 | a | 11.33 | c |
| | | 10 | 3.96 | ab | 0.77 | a | 5.00 | a |
| | | 50 | 1.53 | a | 1.53 | a | 8.67 | bc |
| | | 100 | 9.22 | b | 2.71 | a | 9.67 | c |
| | *N.benthamiana* | 0 | 4.29 | a | 0.96 | a | 15.00 | a |
| | | 1 | 8.11 | b | 0.67 | a | 14.33 | a |
| | | 10 | 7.60 | ab | 0.93 | a | 14.67 | a |
| | | 50 | 11.36 | c | 0.67 | a | 12.00 | a |
| | | 100 | 7.27 | ab | 0.33 | a | 14.50 | a |
| PBZ | *P. hybrida* | 0 | 5.16 | b | 2.51 | a | 12.33 | a |
| | | 1 | 4.69 | ab | 5.84 | b | 13.67 | a |
| | | 10 | 5.44 | b | 6.11 | b | 14.67 | a |
| | | 50 | 2.11 | a | 2.71 | a | 13.00 | a |
| | | 100 | 2.00 | a | 2.07 | a | 11.67 | a |
| | *N.benthamiana* | 0 | 11.42 | a | 1.04 | a | Multiple comparisons cannot be calculated for Conc. as its standard errors are zero. | |
| | | 1 | 11.16 | a | 1.09 | a | | |
| | | 10 | 12.84 | a | 1.44 | a | | |
| | | 50 | 9.64 | a | 1.33 | a | | |
| | | 100 | 13.42 | a | 1.93 | a | | |
| SA | *P. hybrida* | 0 | 1.07 | a | 11.47 | b | 15.00 | a |
| | | 1 | 8.58 | a | 16.18 | c | 14.00 | a |
| | | 10 | 6.44 | a | 6.80 | a | 13.00 | a |
| | | 50 | 4.76 | a | 6.93 | a | 11.33 | a |
| | | 100 | 3.00 | a | 9.74 | ab | 13.01 | a |
| | *N.benthamiana* | 0 | 8.38 | c | 1.87 | a | Multiple comparisons cannot be calculated for Conc. as its standard errors are zero. | |
| | | 1 | 3.58 | b | 4.64 | b | | |
| | | 10 | 2.93 | b | 4.44 | b | | |
| | | 50 | 0.89 | a | 5.38 | b | | |
| | | 100 | 0.76 | a | 4.60 | b | | |
| LA | *P. hybrida* | 0 | 1.42 | a | 9.11 | a | 14.67 | a |
| | | 1 | 4.71 | ab | 7.76 | a | 12.33 | a |
| | | 10 | 12.49 | c | 10.46 | a | 11.67 | a |
| | | 50 | 7.13 | b | 10.62 | a | 11.33 | a |
| | | 100 | 9.47 | bc | 9.82 | a | 11.33 | a |
| | *N.benthamiana* | 0 | 7.76 | cd | 1.40 | ab | 15.00 | b |
| | | 1 | 8.38 | d | 2.51 | bc | 15.00 | b |
| | | 10 | 3.36 | ab | 2.93 | c | 15.00 | b |
| | | 50 | 4.87 | bc | 2.33 | bc | 15.00 | b |
| | | 100 | 0.57 | a | 0.90 | a | 10.00 | a |

## Appendix 5 – DNA extraction results for *Petunia hybrida*




| Gel No. | Exposure time | Compound | Conc. (µM) | Replication | Plant No. | DNA |
|---|---|---|---|---|---|---|
| 1 (control) | Control without Kanamycin | | | | | + |
| 2 | long | AIP | 10 µM | II | 6 | + |
| 3 | short | AIP | 100 µM | III | 3-a | + |
| 4 | short | AIP | 100 µM | III | 3-b | + |
| 5 | short | AIP | 100 µM | III | 13 | + |
| 6 | short | AIP | 100 µM | III | 15 | + |
| 7 | long | PBZ | 0 µM | I | 2 | + |
| 8 | short | PBZ | 0 µM | I | 4 | + |
| 9 | short | PBZ | 0 µM | II | 3 | + |
| 10 | short | PBZ | 0 µM | II | 9 | + |
| 11 | short | PBZ | 1 µM | III | 13 | + |
| 12 | short | PBZ | 10 µM | II | 5 | + |
| 13 | short | PBZ | 10 µM | III | 10 | + |
| 14 | short | SA | 1 µM | II | 3-a | + |
| 15 | short | SA | 1 µM | II | 3-b | + |
| 16 | short | SA | 1 µM | III | 8-a | + |
| 17 | short | SA | 1 µM | III | 8-b | + |
| 18 | short | SA | 10 µM | I | 6 | + |
| 19 | short | SA | 10 µM | III | 14 | + |
| 20 | short | SA | 50 µM | I | 11 | + |
| 21 | long | LA | 10 µM | I | 7-a | + |
| 22 | long | LA | 10 µM | I | 7-b | + |
| 23 | long | LA | 10 µM | III | 8 | + |
| 24 | short | LA | 100 µM | II | 15 | + |
| 25 | short | PBZ | 1 µM | II | 11 | - |
| 26 | short | PBZ | 1 µM | II | 1 | - |
| "+" : extraction of DNA successes; "-" : extraction of DNA failed | | | | | | |

**Appendix 6 – PCR results for *Petunia hybrida* at 58℃ (upper) and 60℃ (lower).**



| Gel No. | Exposure time | Compound | Conc. (µM) | Replication | Plant No. | dsRed PCR | |
|---|---|---|---|---|---|---|---|
| | | | | | | 58℃ | 60℃ |
| 1 | Control without Kanamycin | | | | | - | - |
| 2 | long | AIP | 10 µM | II | 6 | - | - |
| 3 | short | AIP | 100 µM | III | 3-a | - | - |
| 4 | short | AIP | 100 µM | III | 3-b | - | - |
| 5 | short | AIP | 100 µM | III | 13 | + | ++ |
| 6 | short | AIP | 100 µM | III | 15 | - | ++ |
| 7 | long | PBZ | 0 µM | I | 2 | + | ++ |
| 8 | short | PBZ | 0 µM | I | 4 | ++ | + |
| 9 | short | PBZ | 0 µM | II | 3 | + | - |
| 10 | short | PBZ | 0 µM | II | 9 | - | + |
| 11 | short | PBZ | 1 µM | III | 13 | - | - |
| 12 | short | PBZ | 10 µM | II | 5 | ++ | ++ |
| 13 | short | PBZ | 10 µM | III | 10 | - | + |
| 14 | short | SA | 1 µM | II | 3-a | - | - |
| 15 | short | SA | 1 µM | II | 3-b | - | - |
| 16 | short | SA | 1 µM | III | 8-a | ++ | ++ |
| 17 | short | SA | 1 µM | III | 8-b | - | - |
| 18 | short | SA | 10 µM | I | 6 | + | - |
| 19 | short | SA | 10 µM | III | 14 | - | - |
| 20 | short | SA | 50 µM | I | 11 | - | - |
| 21 | long | LA | 10 µM | I | 7-a | - | - |
| 22 | long | LA | 10 µM | I | 7-b | ++ | - |
| 23 | long | LA | 10 µM | III | 8 | ++ | + |
| 24 | short | LA | 100 µM | II | 15 | ++ | ++ |
| 25 | NPT-2 positive control | | | | | + | ++ |
| “++” = Very clear band; “+” = Clear band ; “-” = no band | | | | | | | |

## Appendix 7 – *Agrobacterium*-mediated transformation efficiency in *Petunia hybrida*

| No. | Ept (days) | Cpd | Conc. (μM) | Rep | Plant No. | 14 days [a] dsRed check | 30 days calli check [b] | Survival in Km medium [c] | 90 days [d] dsRed check | PCR (90 days) [e] | | Summary [f] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | 58°C | 60°C | |
| 1 | Control | | | | | / | / | / | / | / | / | / |
| 2 | long | AIP | 10 μM | II | 6 | ++ | + | + | ++ | - | - | X/O |
| 3 | short | AIP | 100 μM | III | 3-a | ++ | + | + | + | - | - | X/O |
| 4 | short | AIP | 100 μM | III | 3-b | ++ | + | + | + | - | - | X/O |
| 5 | short | AIP | 100 μM | III | 13 | ++ | + | + | ++ | + | ++ | O |
| 6 | short | AIP | 100 μM | III | 15 | ++ | + | + | ++ | - | ++ | O |
| 7 | long | PBZ | 0 μM | I | 2 | ++ | + | + | ++ | + | ++ | O |
| 8 | short | PBZ | 0 μM | I | 4 | + | + | + | - | ++ | + | O |
| 9 | short | PBZ | 0 μM | II | 3 | + | + | + | + | + | - | O |
| 10 | short | PBZ | 0 μM | II | 9 | ++ | + | + | - | - | + | O |
| 11 | short | PBZ | 1 μM | III | 13 | ++ | + | + | - | - | - | X |
| 12 | short | PBZ | 10 μM | II | 5 | ++ | + | + | - | ++ | ++ | O |
| 13 | short | PBZ | 10 μM | III | 10 | ++ | + | + | + | - | + | O |
| 14 | short | SA | 1 μM | II | 3-a | ++ | + | + | - | - | - | X |
| 15 | short | SA | 1 μM | II | 3-b | ++ | + | - | - | - | - | X |
| 16 | short | SA | 1 μM | III | 8-a | ++ | + | + | - | ++ | ++ | O |
| 17 | short | SA | 1 μM | III | 8-b | ++ | + | + | - | - | - | X |
| 18 | short | SA | 10 μM | I | 6 | + | + | + | + | + | - | O |
| 19 | short | SA | 10 μM | III | 14 | ++ | + | + | ++ | - | - | X/O |
| 20 | short | SA | 50 μM | I | 11 | ++ | + | + | ++ | - | - | X/O |
| 21 | long | LA | 10 μM | I | 7-a | + | + | + | - | - | - | X |
| 22 | long | LA | 10 μM | I | 7-b | + | + | + | + | ++ | - | O |
| 23 | long | LA | 10 μM | III | 8 | + | + | - | - | ++ | + | O |
| 24 | short | LA | 100 μM | II | 15 | ++ | + | + | + | ++ | ++ | O |
| 25 | short | PBZ | 1 μM | II | 11 | ++ | + | - | - | / | / | X |
| 26 | short | PBZ | 1 μM | II | 1 | ++ | + | - | + | / | / | X/O |

a: “++” = Very clear dsRed spot; “+” = Clear dsRed spot; “-” = no dsRed spot

b: “+” = Explant with calli; “-” = Explant without calli

c: “+” = Survival in Km medium; “-” = Not survival in Km medium

d: “++” = Very red; “+” = red; “-” = barely or not red

e: “++” = Very clear band; “+” = Clear band; “-” = no band

f: “X” = non transgenic plant; “O” = transgenic plant; “X/O” = Uncertain transgenic plant

## Appendix 8 – *Agrobacterium*-mediated transformation efficiency in *Nicotiana benthamiana*

| No. | Ept (days) | Cpd | Conc. (μM) | Rep | Plant No. | 14 days [a] dsRed check | 30 days calli check [b] | Survival in Km medium [c] | 85 days [d] dsRed check | Summary [e] |
|---|---|---|---|---|---|---|---|---|---|---|
| 0 | Control "-" | | | | | / | / | / | / | / |
| 1 | long | PBZ | 0 | I | 5 | ++ | + | + | - | X |
| 2 | long | PBZ | 0 | I | 10 | - | + | + | - | X |
| 3 | long | PBZ | 0 | I | 15 | - | + | + | - | X |
| 4 | long | PBZ | 0 | II | 2 | - | + | + | - | X |
| 5 | long | PBZ | 0 | II | 5 | - | + | - | - | X |
| 6 | long | PBZ | 0 | II | 6 | + | + | + | - | X |
| 7 | long | PBZ | 0 | II | 7 | - | + | - | - | X |
| 8 | long | PBZ | 0 | II | 11 | + | + | + | - | X |
| 9 | long | PBZ | 0 | III | 9 | - | + | + | ++ | O |
| 10 | long | PBZ | 0 | III | 12 | - | + | + | - | X |
| 11 | long | PBZ | 0 | III | 14 | - | + | - | - | X |
| 12 | long | PBZ | 10 | I | 5 | - | + | + | ++ | O |
| 13 | short | AIP | 0 | II | 15 | + | + | + | - | X |
| 14 | short | AIP | 100 | I | 13 | - | + | + | ++ | O |
| 15 | short | PBZ | 1 | III | 8 | - | + | + | - | X |
| 16 | short | PBZ | 10 | II | 8 | - | + | + | ++ | O |
| 17 | short | PBZ | 100 | III | 2 | + | + | + | - | X |
| 18 | short | PBZ | 100 | III | 9 | ++ | + | + | - | X |
| 19 | short | PBZ | 100 | III | 12 | - | + | + | ++ | O |
| 20 | long | PBZ | 0 | I | 1 | - | + | + | - | X |
| 21 | long | PBZ | 0 | I | 7 | + | + | + | - | X |
| 22 | long | PBZ | 0 | I | 11 | ++ | + | + | - | X |
| 23 | long | PBZ | 0 | III | 6 | - | + | + | - | X |
| 24 | long | PBZ | 0 | III | 10 | - | + | + | - | X |
| 25 | long | PBZ | 1 | II | 14 | + | + | + | - | X |
| 26 | long | PBZ | 1 | III | 4 | - | + | + | + | X/O |
| 27 | long | PBZ | 10 | II | 1 | - | + | + | - | X |
| 28 | long | PBZ | 10 | II | 15 | - | + | + | - | X |
| 29 | long | PBZ | 50 | II | 11 | ++ | + | + | - | X |
| 30 | long | PBZ | 50 | II | 15 | ++ | + | + | ++ | O |
| 31 | long | PBZ | 50 | III | 3 | ++ | + | + | ++ | O |
| 32 | long | AIP | 0 | III | 7 | - | + | + | - | X |
| 33 | long | AIP | 10 | II | 7 | - | + | + | + | X/O |
| 34 | long | AIP | 10 | III | 3 | - | + | + | - | X |
| 35 | long | AIP | 50 | I | 9 | - | + | + | - | X |
| 36 | long | AIP | 50 | I | 11 | - | + | + | - | X |
| 37 | short | PBZ | 1 | III | 12 | + | + | + | - | X |
| 38 | short | PBZ | 10 | III | 14 | ++ | + | + | - | X |

| No. | Ept (days) | Cpd | Conc. (μM) | Rep | Plant No. | 14 days [a] dsRed check | 30 days calli check [b] | Survival in Km medium [c] | 85 days [d] dsRed check | Summary [e] |
|---|---|---|---|---|---|---|---|---|---|---|
| 39 | short | PBZ | 100 | II | 12 | ++ | + | + | - | X |
| 40 | short | AIP | 1 | III | 2 | - | + | + | - | X |
| 41 | short | AIP | 1 | III | 12 | - | + | + | - | X |
| 42 | short | AIP | 10 | II | 2 | - | + | + | - | X |
| 43 | long | SA | 0 | I | 7 | - | + | + | - | X |
| 44 | long | SA | 50 | I | 14 | - | + | + | - | X |
| 45 | long | SA | 50 | I | 15 | ++ | + | + | - | X |
| 46 | long | SA | 50 | II | 1 | ++ | + | + | ++ | O |
| 47 | long | SA | 50 | II | 14 | + | + | + | - | X |
| 48 | long | SA | 100 | II | 10 | + | + | - | - | X |
| 49 | long | LA | 0 | I | 10 | + | + | + | ++ | O |
| 50 | long | LA | 0 | III | 10 | + | + | + | - | X |
| 51 | long | LA | 1 | I | 2 | - | + | + | - | X |
| 52 | long | LA | 1 | III | 9 | + | + | + | ++ | O |
| 53 | long | LA | 10 | II | 11 | - | + | + | - | X |
| 54 | long | LA | 100 | I | 9 | - | + | + | ++ | O |
| 55 | short | LA | 0 | I | 1 | ++ | + | + | - | X |
| 56 | short | LA | 100 | I | 2 | - | + | + | - | X |
| 57 | short | LA | 100 | I | 11 | + | + | + | + | X/O |
| 58 | short | LA | 100 | II | 1 | - | + | + | - | X |

a: “++” = Very clear dsRed spot; “+” = Clear dsRed spot; “-” = no dsRed spot

b: “+” = Explant with calli; “-” = Explant without calli

c: “+” = Survival in Km medium; “-” = Not survival in Km medium

d: “++” = Very red; “+” = red; “-” = barely or not red

e: “X” = non transgenic plant; “O” = transgenic plant; “X/O” = Uncertain transgenic plant