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Improving transient gene expression and agroinfiltration-based transformation effectiveness in Indonesian orchid *Phalaenopsis amabilis* (L.) Blume

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ABSTRACT Transient gene expression is an approach used to study transient genes across various species, with infiltration by *Agrobacterium tumefaciens* (agroinfiltration) being a commonly used method. Agroinfiltration offers a simple and effective means of delivering transgenes into the plant genome. An alternative method for enhancing the quality and productivity of orchids as ornamental plants is genetic modification through agroinfiltration. Although *Agrobacterium*-mediated genetic transformation by immersion has been used on the *Phalaenopsis amabilis* (L.) Blume species of orchid, transformation efficiency using the immersion technique remains relatively low and the method itself is challenging due to its requirement for aseptic handling. The application of agroinfiltration in *P. amabilis* has not previously been reported. This study investigates the impact of the injection site, acetosyringone concentration, bacterial density (OD_{600}), and injection volume to determine the optimum conditions for agroinfiltration on *P. amabilis*. The results demonstrated that injection site had a noticeably distinct impact on transformation effectiveness, with the abaxial position of the leaf being the optimal site for *Agrobacterium* culture suspension injection. While adjustments in acetosyringone concentration, bacterial density (OD_{600}), and injection volume did not significantly affect transformation efficiency, they did influence the peak time of GFP fluorescence. Acetosyringone at a concentration of 200 µM, an OD_{600} of 1.0 for *Agrobacterium* culture, and an injection volume of 500 µL effectively accelerated GFP expression duration.

KEYWORDS *Agrobacterium tumefaciens*; Agroinfiltration; *Green Fluorescent protein*; *Phalaenopsis amabilis*

1. Introduction

Orchids are ornamental plants with a high market value due to their beauty flowers. *P. amabilis* is one orchid that has the potential to be developed. However, there are now several challenges facing the development of *P. amabilis*, including a lack of superior seeds and outdated technologies. Micropropagation is a culture technique that has been used by many researchers and successfully developed through *in vitro* culture. However, this method is influenced by several variables, including explant size and section, plant genotype, media composition, and growth regulators, all of which heavily depend on the culture environment. Genetic transformation is a technique that has also been used to modify plant genomes so that it can regenerate into transgenic plants by introducing genes from other plants or from the transformed plant itself.

One of the most effective methods of genetic transformation is *Agrobacterium*-mediated transformation. In the previous study, the genetic transformation of *P. amabilis* and produced a transformation efficiency of 1.7% (Semiarti et al. 2007, 2010). Based on the research that has been done, it is known that the transformation efficiency is still relatively low. In addition, the genetic transformation technique by using immersion method in *Agrobacterium* culture requires quite a lot of time and must be carried out under aseptic conditions. The genetic transformation technique using the immersion method is a gene insertion process with *Agrobacterium* carried out through an *in vitro* process in the infection process (Dwiyani et al. 2016).

Agoinfiltration is a technique used to introduce a gene construct into a plant for the purpose of analyzing transient gene expression. Agroinfiltration is one of the methods for studying transient gene expression in plant system through *Agrobacterium* mediated transformation that uses in conjunction with instruments, such as syringes (Vargas-Guevara et al. 2018). Several studies that have been conducted with the agroinfiltration method are tobacco, tomato, coffee, grape, potato, and other food crops (Matsuo et al. 2016; Vargas-Guevara et al. 2018; Mba'u et al. 2018; Norkunas et al. 2018); and medicinal plants (Faizal and Geelen 2012; Mirzaee et al. 2016). In this study, agroinfiltration method in *P. amabilis* was carried

out to increase the efficiency of genetic transformation and analyze candidate-resistant genes that could be expressed transiently in transformant plants (Mba'u et al. 2018).

The success of genetic transformation by agroinfiltration in *P. amabilis* was influenced by the OD_{600} of *Agrobacterium* culture, the volume of the suspension of *Agrobacterium* culture, and the injection site. Another orchid species that has successfully applied the agroinfiltration method is *Dendrobium* Sonia "Earsakul" using several parameters, such as OD_{600} of *Agrobacterium* culture of 0.5–2.0, *Agrobacterium* suspension culture volume of 0.5 mL and the injection target is the flower (Pinthong et al. 2014), with the T-DNA construct used being CaMV35S:GUS. However, there is no reports on the agroinfiltration method in *P. amabilis* orchids. Therefore, optimization of this method was conducted to develop an agroinfiltration for *P. amabilis* for efficiency of genetic transformation. To visualize transient gene expression in the transformant cells through agroinfiltration method, green fluorescent protein (GFP) was used as a marker (Ihsani et al. 2023).

2. Materials and Methods

2.1. Plant materials

P. amabilis, plants that were collected from CV. Widarakandang, Yogyakarta were used in this study. The plants have been cultivated in the greenhouse at 25–30 °C with natural light for 5 years. In this research, 25 plants were used. Leaves, stems, and roots were the parts employed as injection targets.

2.2. Plasmid vector and bacterial strain

The plasmid used was p2KI with the T-DNA rolC::GFP construct from *Agrobacterium tumefaciens* strain GV3101 obtained from Dr. Yekti Asih Purwestri, M.Si (Faculty of Biology, Gadjah Mada University) (Figure 1). The T-DNA contains two components: the *hygromycin phosphotransferase* (*HPT*) resistant gene and the rolC promoter.

2.3. Agroinfiltration

One colony of recombinant bacteria was inoculated into a liquid LB medium containing the antibiotics hygromycin (50 mg/L) and kanamycin (50 mg/L). Subsequently, the cultures were incubated in a shaker incubator at 150 rpm at 28 °C for 2 days. In the next step, 1 mL of bacterial culture was inoculated into 100 mL of liquid LB medium supplemented with the appropriate antibiotic and incubated at 28 °C, 150 rpm, for 14 h. *A. tumefaciens* cells were harvested by centrifugation (6000 rpm for 10 min), and the supernatant formed was discarded. The bacterial pellet was resuspended in MMA buffer (10 mM MES pH 5.6, 10 mM $MgCL_2$, without acetosyringone and/or 200 µM acetosyringone) to an OD_{600} of 0.5–1.0. The culture is then incubated for 1–3 h at room temperature. The abaxial parts of the leaves, stems, and roots of *P. amabilis* were injected with *A. tumefaciens* suspension using a 1 mL 26G x ½" syringe. The volume of *A. tumefaciens* suspension injected was 100 µL and 500 µL. Leaf samples were collected after infiltration at 24, 48, and 72 h to observe GFP expression using a Carl Zeiss/Axio Observe confocal microscope. 21; Basic for LSM 800. Each experiment was repeated three times.

2.4. Observation of GFP fluorescence using a confocal microscope

Following agroinfiltration, plants were cultivated for 24, 48, and 72 h before being cut for examination under a confocal microscope (Carl Zeiss/Axio Observe.21; Basic for LSM 800). At a site close to the place where the needles entered contact with the leaf, it was severed. The leaf pieces are cut transversely and longitudinally, placed on a glass slide, sludge with sterile distilled water, and covered with a cover glass. The microscope table was inverted and filled with brand-new preparations. A laser with a wavelength of 509 nm was used in confocal microscopy to detect the GFP glow. Three pictures were captured of each plant tissue sample. GFP expression efficiency is expressed as a percentage calculated from the number of transformed plants containing the GFP gene divided by the total number of infiltrated plants.

2.5. Genomic DNA isolation

By using the CTAB method, isolation was completed following the modified method of Murray and Thompson (1980). DNA was isolated from the leaves, stems, and roots of candidate transformants and control plants in amounts up to 100 mg. The sample was put in a sterile 1.5 mL microtube, to which 500 µL of 3% CTAB buffer was then added, and the tube was smoothed out. Using a water bath, the extract mixture was incubated at 65 °C for 30 min. Chloroform in the volume of 500 µL was added, inverted, and shaker for 30 min, and then centrifuged at 12000 rpm for 10 min. The top layer is gradually taken off and put into a fresh, sterile 1.5 mL microtube. The amount of supernatant collected was determined. Up to one tenth volume of Na-acetate or twice the volume of cold EtOH was added, then inverted 6 times and incubated at –20 °C

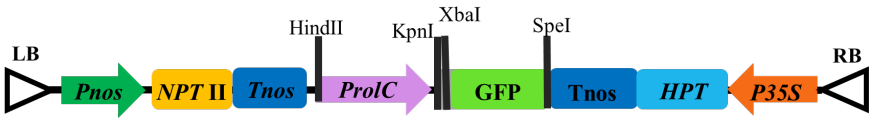


FIGURE 1 p2KI-GFP plasmid T-DNA construct. LB, T-DNA left border sequence. Pnos, Nopaline Synthase (NOS) promoter. NPTII, Neomycin Phoshotransferase coding sequence. Tnos, NOS terminatior. ProlC, Promoter rolC. GFP, Green Flourescent Protein, HPT, Hygromycin phosphotransferase. P35S, Strong promoter 35S.

for 60 min. Samples were centrifuged at 14000 rpm for 5 min, and the supernatant formed was discarded. The pellet was washed with 70% ethanol and centrifuged at 10000 rpm for 5 min and the supernatant was discarded. The pellets were air-dried and resuspended in 25–30 µL 10T-0.1E. DNA samples were stored at –20 °C.

2.6. Polymerase chain reaction analysis of transformant/PCR test for transformant candidates

The genomic DNA of the transformant candidates was analyzed by PCR using MyTaq™ Red Mix PCR Kit (Bioline). Specific primers used to detect the presence of the *HPT* gene are: HPTF 5′-TCGGACGATTGCGTCGCATC-3′; HPTR 5′-AGGCTATGGATGCGATCGCTG-3′, a specific primer for the *ACTIN* gene is: ACTF 5′-CACTCGTGAGAAGATGACCC-3′; ACTR 5′-GTCCATCAGGAAGCTCGTAGC-3′ (Mursyanti et al. 2016). The PCR conditions used were pre-denaturation (95 °C for 1 min), denaturation (95 °C for 15 s), annealing (61.2 °C for *HPT* and 51 °C for *ACTIN* for 10 s), extension (72 °C for 10 s), and final extension (72 °C for 5 min). The number of cycles used is 35. The PCR process was carried out using a T100™ Thermal Cycler (Bio-Rad). The DNA amplification results were separated by electrophoresis using 1% agarose gel stained with Florosafe staining solution and visualized with a UV transilluminator. Efficiency of transformation was determined as the number of transformant plants containing the GFP gene divided by the number of plant undergo agroinfiltration, and expressed as percentage

2.7. Data analysis

For Each treatment variation - injection site, OD_{600}, acetosyringone concentration, and volume of *Agrobacterium* suspension was performed three times so that 24 plants were used. The completely randomized design (CRD) factorial treatment design with Univariate ANOVA was used to analyze the data. The outcomes shown are the mean and standard deviation. With a 95% confidence level, an analysis of Duncan's Multiple Range Test (DMRT) results will show significant changes between treatments. The SPSS 15.0 software was used to conduct the analysis.

3. Results and Discussion

3.1. Observation of GFP fluorescence in Agrobacterium tumefaciens GV3101 carrying the rolC::GFP construct

In this study, the *A. tumefaciens* recombinant strains GV 3101 were used. At the beginning, a confirmation test was conducted to confirm the presence of the GFP gene in *A. tumefaciens* bacteria that carry the GFP gene (Figure 2).

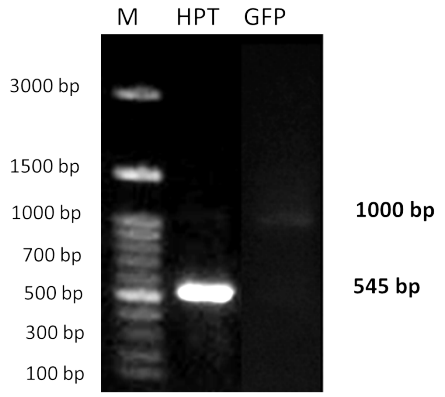


FIGURE 2 Amplification of HPT and GFP fragments in *A. tumefaciens* GV3101 carrying the rolC::GFP construct. The *HPT* gene was amplified with a length of 545 bp and *GFP* gene was amplified with a length of 1000 bp. M = 100 bp marker.

3.2. Determination of the agroinfiltration method on the leaves, stems and roots of P. amabilis sub

In this study, adult plants *P. amabilis* were used as infiltration targets. This orchid was infected by *A. tumefaciens* containing p2kI-GFP using a syringe. There are three parts of the injected orchid: roots (Figure 3a), stems (Figure 3b), and leaves (Figure 3c). This is done to determine efficient plant parts as infiltration targets. The density of bacteria used at this stage was 1.0 and the volume of the suspension injected was as much as 100 µL. After three days, the roots, stems, and leaves were taken to isolate the genomic DNA, while observations were continued for 4 weeks to see any morphological changes.

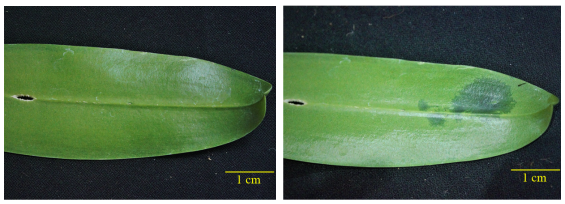
One plant, four stems, and 24 leaves out of the total number of plants successfully tested for the presence of the *hygromycin phophotransferase* (*HPT*) gene in the injection site treatment. This yields a genetic transformation efficiency of 4.17% in roots, 12.49% in stems, and 100% in leaves (Table 1). The p2KI plasmid contains the *HPT* gene, which indicates the selection of resistance to the hygromycin antibiotic (Tamaki et al. 2007).

The agroinfiltration injection on the leaves is known to have the maximum efficiency of genetic transformation. Abaxial, or the leaf's underside, was the area that received the injection of the *Agrobacterium* suspension. Stomata of an anomocytic type, or kidney-shaped stomata guard cell form, are slightly larger than those on the top surface of the leaf of *P. amabilis*. An essential component of a plant, stomata serve as a point of entry for CO_2 during the photosynthetic process. Additionally, stomata serve as a location for the processes of respiration and transpiration, making them crucial tissues in the metabolic processes of plants

Based on Table 1, it is also known that only six plants were able to survive after injection in the roots, and seven plants were able to survive after injection in the stem. This shows that many plants wilted at first and ultimately perished following injections in the roots and stems. Different case with plants with infiltration treatment on the leaves.

(a) (b) (c)

FIGURE 3 Application of genetic transformation of the agroinfiltration method on *P. amabilis* (a) roots, (b) stems, and (c) leaves.



(a) (b)

FIGURE 4 *Agrobacterium* suspension injected using a syringe on *P. amabilis leaves*. (a) Before injection, (b) After injection.

The plants that had been given injections all survived and did not wilt. This data showed that for the agroinfiltration technique on *P. amabilis*, the agrobacterium solution was more successfully injected into the leaves than into the roots and stems.

The number of plants that died after injection in the roots and stems was related to tissue necrosis. Browning and necrosis in tissues after exposure to *A. tumefaciens* are common. One of the earliest defense mechanisms activated after exposure to an *A. tumefaciens* infection is the production of reactive oxygen species (ROS), which can trigger programmed cell death (Tiwari et al. 2022). When plants are exposed to biotic or abiotic stress, the first response is an oxidative burst followed by the production of phenolic compounds and other secondary metabolites (Baker et al. 2020).

Agrobacterium is one of the most pathogenic bacteria (Mansfield et al. 2012). Molecular patterns in the

TABLE 1 Effect of injection site of *Agrobacterium* on *P. amabilis* survival and transformation efficiency.

	Σ Plant tested	Σ Survival plants	% Survival plants	Σ Wilted plants	% Wilted plants	Detection		Efficiency of transformation (%)
						ACTIN+	*HPT+*	
Root	24	6	25	18	75	24	1	4,16a
Stem	24	7	29,17	17	70,83	24	4	12,49a
Leaf	24	24	100	0	0	24	24	100b

TABLE 2 Transformation Efficiency of *P. amabilis* on leaves using the agroinfiltration technique.

Treatment			Treatment Label	Σ Infiltrated plants	Σ survival plants	PCR Detection		Efficiency transformation (%)
Acetosyringone (µM)	OD_{600}	Volume Injection (µl)				*ACTIN+*	*HPT+*	
0	0,5	100	Vp1	3	3	3	3	100a
		500	Vp2	3	3	3	3	100a
	1	100	Vp3	3	3	3	3	100a
		500	Vp4	3	3	3	3	100a
200	0,5	100	Vp5	3	3	3	3	100a
		500	Vp6	3	3	3	3	100a
	1	100	Vp7	3	3	3	3	100a
		500	Vp8	3	3	3	3	100a

case of pathogen-induced infections are called 'pathogen-associated molecular patterns' (PAMPs). PAMPs are part of the elicitors found in a large group of pathogenic bacteria that play a role in microbial pathogenesis. PAMP signals during pathogen attack can be detected by plants using a cell surface receptor called 'pattern recognition receptor' (PRR) (Gust et al. 2017).

PAMP recognition by PRR is the first level of defense called PAMP-triggered immunity (PTI). PTI controls plant resistance at an early level that affects basal resistance (Tang et al. 2017). Once the PAMP signal recognition is captured and PTI is activated, there is immediate inhibition of the infection process at an early stage. ROS are the products that arise due to the infection process (Noman et al. 2019).

The second level of defense in plants is determined by 'effector triggered immunity' (ETI). Activation of ETI will cause hypersensitive reactions in plant tissues (Janda et al. 2019). Hypersensitive reaction is one of the plant defense responses and is generally characterized by local cell death around the infection site that leads to cell necrosis (Kuta and Tripathi 2005). Necrosis that occurs in roots and stems is more severe than that which occurs in leaves. The tissue around the injection site in the roots and stems did not recover, namely there were holes formed, while in the leaves no holes were formed.

3.3. Determination of agroinfiltration on P. amabilis leaves with variations in acetosyringone concentration, bacterial density, suspension volume

The results of the first experiment showed that the effective injection site for *P. amabilis* was in the leaves, so it was continued with the addition of various treatments, namely acetosyringone concentration (0 µM and 200 µM), bacterial density (0.5 and 1.0), and suspension injection volume (100 µL and 500 µL) with a total of 8 treatment variations (Table 2). Each treatment was repeated three times.

Twenty-four plants in total that had received injections of various treatments displayed a 100% transformation efficiency. The outcomes of the molecular study that verified the *HPT* gene's presence in the transformed plants' genome verify this. Leaf samples were taken three days post-infiltration, and DNA was extracted using the CTAB technique. *ACTIN* and *HPT*-specific primers were used in a polymerase chain reaction (PCR) after the genomic DNA was recovered. One of the housekeeping genes, *ACTIN* serves as an internal regulatory gene in cells. Internal control genes are constitutive genes that are expressed in both normal and stressful conditions in living things and play a role in sustaining cellular function (Din Mufti et al. 2015).

The detection of *ACTIN* and *HPT* genes in infiltrating altered plants provided insight into the effectiveness of genetic modification. *HPT* genes 545 bp and *ACTIN* 114 bp were found in a total of 24 plants made up of 8 treatments (Vp1, Vp2, Vp3, Vp4, Vp5, Vp6, Vp7, and Vp8), each of which was repeated three times (Figure 5). On plant genomes that had not been contaminated, PCR and electrophoresis procedures were performed as a negative control, and the outcome was the presence of the 114 bp *ACTIN* gene but not the *HPT* band. These results demonstrate the successful integration of the T-DNA construct into the *P. amabilis* genome.

3.3.1 Effect of acetosyringone on transformation efficiency

Most plants with the highest brightnes GFP fluorescence were found in the treatment with the addition of acetosyringone at the 24th-hour post-infiltration, but their number gradually dropped until the 72nd hour. This contrasted with the treatment without acetosyringone, in which most plants began to exhibit the brightest GFP fluorescence at the 72nd-hour post infiltration (Table 3). This suggests that the treatment, including the addition of acetosyringone to the suspension solution injected into the plant, results in the highest GFP expression. This also applies to the co-cultivation period, during which *A. tumefaciens* will be

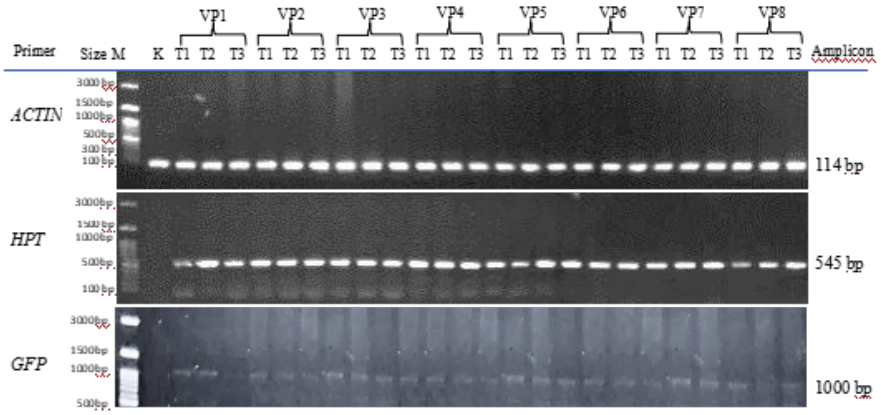


FIGURE 5 Amplification of *ACTIN*, *HPT*, and *GFP* fragments. The *ACTIN* gene was amplified at 114 bp in length, the *HPT* gene was amplified at 545 bp in length, and the *GFP* gene was amplified at 1000 bp in length.
Description: Vp1 = Treatment Variation 1, Vp2 = Treatment Variation 2, Vp3 = Treatment Variation 3, Vp4 = Treatment Variation 4, Vp5 = Treatment Variation 5, Vp6 = Treatment Variation 6, Vp7 = Treatment Variation 7, and Vp8 = Treatment Variation 8, T1 = plant 1, T2 = plant 2, T3 = plant 3

TABLE 3 Effect of acetosyringone, optical density, and volume of injection on co-cultivation time of GFP fluorescence in *P. amabilis*.

Treatment			Label	Σ Infiltrated plants	Σ survived plants	Σ PCR positive plants			Transformation efficiency (%)	Plant with GFP fluorescence			GFP expression efficiency (%)
Aceto-syringone (µM)	OD_{600}	Injection volume (µl)				*ACTIN+*	*HPT+*	GFP+		24h	48h	72h	
0	0,5	100	Vp1.a	1	1	1	1	1				v	
			Vp1.b	1	1	1	1	1	100			v	100
			Vp1.c	1	1	1	1	1				v	
		500	Vp2.a	1	1	1	1	1			v		
			Vp2.b	1	1	1	1	1	100			v	100
			Vp2.c	1	1	1	1	1				v	
	1	100	Vp3.a	1	1	1	1	1				v	
			Vp3.b	1	1	1	1	1	100			v	100
			Vp3.c	1	1	1	1	1				v	
		500	Vp4.a	1	1	1	1	1		v			
			Vp4.b	1	1	1	1	1	100	v			100
			Vp4.c	1	1	1	1	1		v			
200	0,5	100	Vp5.a	1	1	1	1	1		v			
			Vp5.b	1	1	1	1	1	100	v			100
			Vp5.c	1	1	1	1	1				v	
		500	Vp6.a	1	1	1	1	1				v	
			Vp6.b	1	1	1	1	1	100		v		100
			Vp6.c	1	1	1	1	1				v	
	1	100	Vp7.a	1	1	1	1	1			v		
			Vp7.b	1	1	1	1	1	100	v			100
			Vp7.c	1	1	1	1	1			v		
		500	Vp8.a	1	1	1	1	1		v			
			Vp8.b	1	1	1	1	1	100		v		100
			Vp8.c	1	1	1	1	1		v			

detected and bound to plant cells. For *A. tumefaciens* to transmit T-DNA to plant cells, the addition of acetosyringone will initiate the process of recognition and interaction between virulence proteins in *A. tumefaciens* and plant cells (Mba'u et al. 2018). The expression of GFP demonstrates that the T-DNA has been integrated into the plant genome, resulting in no appreciable difference in the efficiency of genetic transformation between the two treatments at 100%.

Acetosyringone as phenolic subtances serves an attractant for *A. tumefaciens* to infect plant tissues (Gnasekaran and Subramaniam 2015). Injured plant cells can trigger transcription of the *Vir* genes on the *A. tumefaciens* plasmid-Ti by producing the compound acetosyringone. Through the use of vir A, *A. tumefaciens* will collect the released phenolic chemicals. When vir A is autophosphorylated, vir G receives the phosphoryl group. As a result, vir G becomes activated, stimulating the transcription of additional vir proteins such as C, D, E, B, F, and H. (Krenek et al. 2015).

3.3.2 Effect of *A. tumefaciens* GV3101 culture's OD_{600} value on transformation efficiency

At 24 h after injection, GFP expression started to show in both treatments. However, in the OD_{600} 0.5 treatment, the greatest proportion of plants with the brightest GFP fluorescence appeared at the 72nd-hour post-infiltration, whereas in the OD_{600} 1.0 treatment, it appeared at the 24th-hour post-infiltration (Table 3). Wroblewski et al. (2005), explained that the density of cell suspension has a significant impact on the temporary expression of a gene. Bacterial cell densities with OD_{600} values of 1.0 produce low transient expression. The findings demonstrated that OD_{600} 0.5 treatment resulted in lower expression and lasted comparatively longer than OD_{600} 1.0 treatment.

Bacterial density measures how many bacteria are present in a medium, the greater the bacterial density value, the more *A. tumefaciens* are present in the medium (Dewanto and Suhandono 2016). The most active logarithmic phase of *Agrobacterium* growth is represented by the OD_{600} value, which has a significant impact on the transformation procedure (Yadav et al. 2012). Logarithmic phase of *A. tumefaciens*, which ranges from optical density 0.5 to 1.0, is particularly useful for transformation and can enhance plant infection (Nishimura et al. 2007). Since the T-DNA has been integrated into the plant genome, the GFP fluorescence results show that the transformation efficiency is not materially different from 100%.

3.3.3 Effect of injected suspension volume on transformation efficiency

For each injection treatment with a different solution volume, 12 plants in total were observed. In the 100 µL injection volume treatment, the greatest proportion of plants

exhibited the brightest GFP fluorescence at the 72nd hour after infiltration, whereas in the 500 µL injection volume treatment, it was seen in the 48th hour (Table 3). According to these findings, plants injected with 500 µL express GFP more quickly than plants injected with 100 µL. The T-DNA is successfully transported and integrated into the plant genome, as shown by the presence of GFP fluorescence in both treatments, indicating that the transformation efficiency, which is 100%, is not significantly different. Using a syringe, infiltration only needs a modest amount of *Agrobacterium* suspension (Leuzinger et al. 2013).

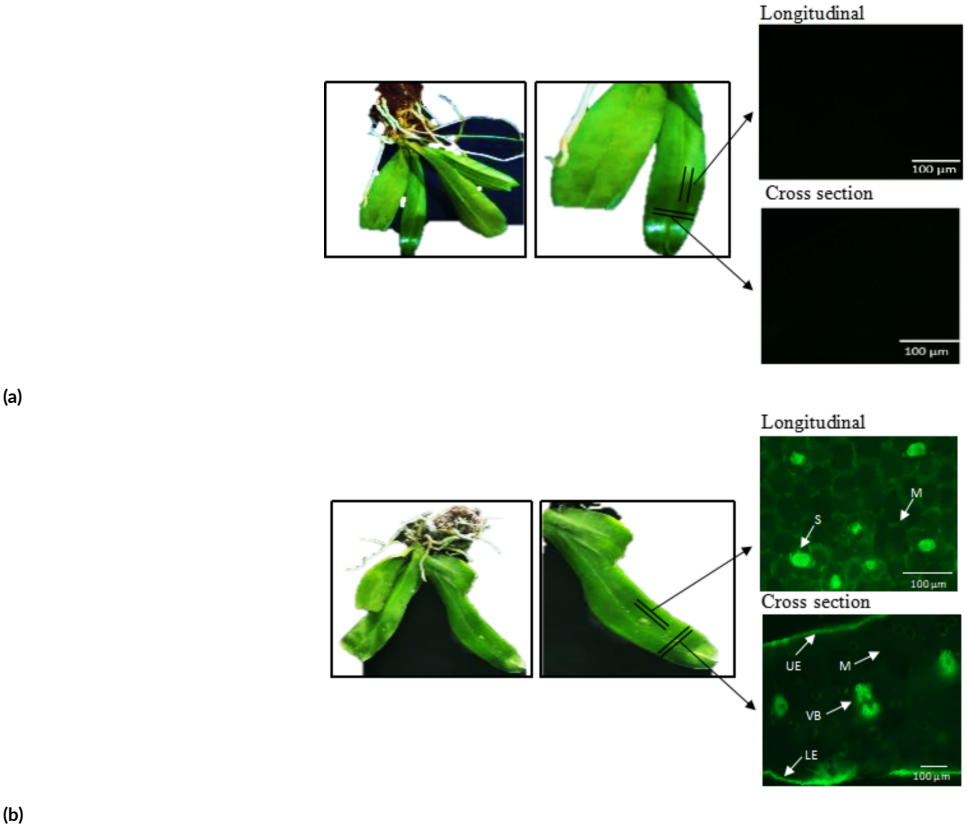


FIGURE 6 Anatomical structure of *P. amabilis* leaves exposed to UV light using confocal microscopy. a) Negative control *P. amabilis* leaf; b) Leaf of *P. amabilis* transformant. M= Mesophyll, S= Stomata, UE= Upper epidermis, VB= Vascular bundle, LE= Lower epidermis.

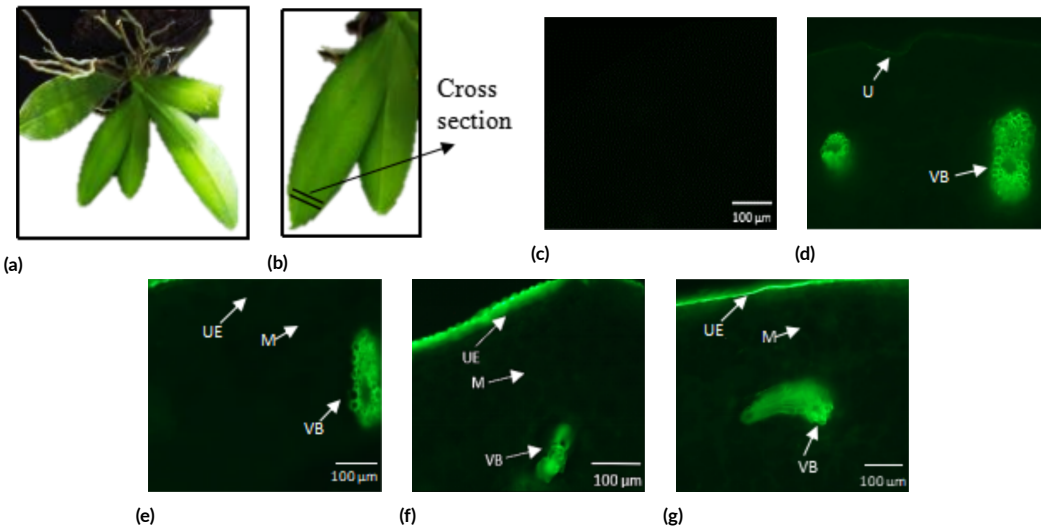


FIGURE 7 GFP expression in *P. amabilis* leaf tissues a) *P. amabilis* plants; b) *P. amabilis* leaf samples for microscopic observation of GFP expression; c) 0 hour; d) 4th hour; e) 24th hour; f) 48th hour; g) 72nd hour. M= Mesophyll, S = Stomata, UE= Upper epidermis, VB= Vascular bundle, LE=Lower epidermis

3.4. Transient expression of GFP from P. amabilis leaves

P. amabilis leaves that had been infiltrated with *A. tumefaciens* harboring p2KI-GFP was collected at 24, 48, and 72 h post-infiltration to view GFP expression using confocal microscopy. GFP expression was observed in all infiltrated regions. Previous research found that the frequency of transgenes in leaves varies depending on the area infiltrated. The part of the leaf with the most GFP expression was detected on the lower surface of the leaf (abaxial) (Yamamoto et al. 2018). In this experiment, GFP was successfully expressed in *P. amabilis* leaves, as indicated by the presence of GFP fluorescence in the vascular bundle and mesophyll (Figure 6).

GFP expression in the leaves revealed fluorescence of stomata and cell walls in longitudinal cross-section preparations of leaves as well as fluorescence of transport bundles and epidermis in cross-section preparations of leaves, in contrast to the negative control, which had no fluorescence in any part (Figure 7). The infiltrating *A. tumefaciens* was quickly made available to the mesophyll tissue between the upper and lower epidermis through the stomata (Ben-Amar et al. 2013). This additional area in the intercellular matrix is used by *A. tumefaciens* to infect cells in the mesophyll tissue. As a result, the transport bundles in the mesophyll tissue light up to demonstrate that GFP is expressed there.

The expression pattern of GFP in plant tissues was also identified using the data. Following the invasion, several components flash at various intervals. At the first hour after infiltration, there was no light discernible in the leaf tissue, but by the fifth hour, it was seen that illumination had begun to form on the transport bundle (Figure 7). During the 24^{th} and 48^{th} h, fluorescence appeared in the transport bundle, and it began to appear in some regions of the upper epidermis and some regions of the mesophyll tissue. At 72 h, the mesophyll tissue's GFP fluorescence is increasingly spreading (Figure 7). This is consistent with the finding reported by Malabadi et al. (2008) that the GFP fluorescence signal can be seen within a few hours after infiltration and will decrease within a few days.

When the post-infiltration incubation hours increased, the fluorescence first appeared in the transport bundle and then expanded to the upper epidermis and mesophyll (Figure 7). This is connected to the dispersion of the GFP gene-inserted *A. tumefaciens* that was used in this investigation. According to Sufianto (2019), bacteria can spread across the network of transport vessels in plants, specifically the xylem and phloem.

4. Conclusions

The abaxial region of *P. amabilis* leaves is the best position for an effective injection site in agroinfiltration technique. To accelerate the expression of GFP in plant cells, acetosyringone concentration, OD_{600} value, and injection volume that produced 100% genetic transformation efficiency are needed 200 µM, 1.0, and 500 µL respectively.

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Authors' contributions

ES oversaw the research's design and paper writing. Agroinfiltration, genomic DNA extraction, PCR, electrophoresis, data compilation, anatomical analysis, and manuscript writing were all carried out by DHP. YP offered suggestions and input for this research. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare no competing interest.

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