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RESEARCH ARTICLE

High efficiency of agroinfiltration approach in *Phalaenopsis amabilis* (L.) Blume (moth orchid) with transient expression of green fluorescent protein reporter gene

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Abstract

Agroinfiltration has become a crucial technique in molecular biology, enabling the transfer of functional genes into target plants for rapid gene expression analysis. This study investigated the use of agroinfiltration for transient expression of the green fluorescent protein (*GFP*) reporter gene in *Phalaenopsis amabilis* (L.) Blume transformant leaves. The aim was to establish an efficient and rapid genetic transformation method for generating transformant plant candidates. Agroinfiltration was performed on the leaves using a suspension containing *Agrobacterium tumefaciens* strain GV3101, which harbored a T-DNA construct in the pRI101AN vector carrying the 35S::*GFP* and neomycin phosphotransferase II (*NPTII*) genes. Plant development post-infiltration was monitored microscopically with a fluorescence microscope and confirmed molecularly using PCR (polymerase chain reaction) with specific primers for *GFP* (736 bp), *NPTII* (550 bp) and *ACTIN* (114 bp). The results showed that green fluorescence, indicating GFP expression, was detected under a blue laser (450 - 480 nm) within 24 hr post-infiltration on both the adaxial and abaxial leaf surfaces, particularly along the midvein (primary veins) and leaf margins. Anatomical analysis of transverse and longitudinal free-hand sections further confirmed the rapid occurrence within the epidermis, mesophyll and vascular tissues. The accuracy of the data was supported and confirmed by the positive results of all three *P. amabilis* transformant plants that were analyzed by PCR and it was proven that all positive transformant plants carried the *GFP*, *NPTII* and *ACTIN* genes as positive controls. These results indicate agroinfiltration and GFP approach offering efficient, rapid and valuable prospects for accelerating genetic engineering applications in plant research.

Keywords: agroinfiltration; efficiency; green fluorescent protein; *Phalaenopsis amabilis*; reporter gene

Introduction

Orchidaceae, popularly known as orchids, are the largest family of flowering plants contains at least 28000 species over the world (1). The various hybrids, primarily of tropical origin, hold substantial horticultural importance. One of the genus, *Phalaenopsis*, a beautiful and popular orchid, comprises approximately 66 species (2). *Phalaenopsis* is named with “phalaina” for moth and “opsis” for appearance and is commonly known as “moth orchids” because of its white flowers shaped like a moth (3). The unique character of an antenna-like structure present at the end of its labellum, which always appears in the flower of this orchid’s hybrid, makes *P. amabilis* a significant ancestral species of *Phalaenopsis* hybrids and one of the most economically valuable orchid species in the Orchidaceae family (4). This species is highly recognized in the international market and ornamental plants industry, due to its long-lasting flowers and adaptability to indoor environments (5). This makes genetic engineering techniques crucial for development and increased production of ornamental plants (6). The application of genetic transformation allows the introduction of specific genes to generate desirable traits for consumer demand and appreciation for aesthetics and novelty.

Genetic transformation of plants is a powerful method used in a variety of molecular studies, such as gene function analysis, production of protein, interaction between protein and promoter activity in plants (7). This method can be carried out through indirect gene transfer such as *Agrobacterium*-mediated, floral dip, agroinfiltration and direct gene transfer like polyethylene glycol method (PEG)-mediated, particle bombardment (biolistic), microinjection and electroporation (8). The first transgenic orchid plant from *Dendrobium* was reported using particle bombardment (9), meanwhile *Agrobacterium*-mediated transformation by using callus or protocorm like bodies with immersion system has been done in *Dendrobium, Cymbidium, Oncidium* and *Phalaenopsis* (10). However, it takes relatively long time to get callus as plant material. *Agrobacterium* -mediated transformation by using 3-week-old intact protocorms then applied in *P. amabilis* (11-13) but the

transformation efficiency is relatively low. Furthermore, the immersion method takes a long time to ensure the integration of T-DNA into the cell and requires aseptic conditions to perform the genetic transformation process. To enhance genetic improvement in orchids, efficient and rapid gene delivery systems are urgently needed to overcome these limitations.

Recently, agroinfiltration has been widely utilized for the transient expression of genes, evaluation of vector constructs and production of recombinant proteins in plant cells (14). The agroinfiltration method for genetic transformation is frequently helpful for transient gene expression (within hours or days) without requiring time-consuming processes (15). It can also reveal important details about the functions of the coding region or gene components that influence the timing or intensity of expression (16). Otherwise, agroinfiltration can be done without aseptic condition. Many agroinfiltration techniques have been developed over time, such as syringe infiltration (agro-injection), vacuum infiltration and agro-drench (soil soaked with an *Agrobacterium* suspension next to the plant roots) (15). This technique has been successfully used in various plants such as citrus (15), tobacco (17), tomato (18), avocado (19) and ornamental plants (*Dendrobium* and *Phalaenopsis*) (20, 21).

One of the primary applications of this technique is the visualization of fluorescent protein expression, particularly green fluorescent protein (GFP), which is widely recognized for its fluorescence properties that facilitate the observation of biological processes in research. Green fluorescent protein was first discovered in marine organisms and has become an invaluable tool in biotechnology as a genetic marker in gene studies and cellular research (22). Studies indicate that optimized expression vectors and effective delivery methods can significantly enhance GFP signal intensity, providing better resolution for spatial and temporal expression analysis (23). One of the main advantages of using GFP is its stability under normal conditions, which facilitates visualization without the need for extensive substrates (24) and can be used as reporter gene for early detection of gene expression (25). In addition, GFP can tag specific locations of proteins in cells, allowing researchers to explore gene interactions and functions in more depth (26). This also allows for a detailed analysis of the gene's function within the context of leaf structure and function. With further optimization, this approach is useful for studying gene promoter properties, transcription factor activity and protein localization.

The effectiveness of agroinfiltration is influenced by the structure and composition of the expression vector used. Studies indicate that optimized vectors can significantly enhance GFP expression levels. The underlying mechanism of *Agrobacterium tumefaciens*-mediated agroinfiltration involves its ability to transfer DNA into plant cells. This study aims to establish a rapid agroinfiltration approach for *Phalaenopsis amabilis* leaves using a *GFP* reporter gene to enable early detection of gene expression and to evaluate the spatial distribution of gene expression across different leaf tissues. This study opens new avenues for application in developing valuable plant varieties, where this technique can be optimized for recombinant protein production in ornamental species (27).

Material and Methods

Plant materials

In this study, about 3-year-old *P. amabilis* as model plants were collected from "Rumah Anggrek Anik", Muntilan, Magelang Regency, Central Java, with the altitude approximately 354 m above mean sea level. The plants were cultivated in the greenhouse at 25–30 °C with natural sun lighting environment.

Plasmid vector and bacterial strain

The plasmid used was pRI101AN in *A. tumefaciens* strain GV3101 (Fig. 1) obtained from Laboratory of Plant Breeding, Faculty of Agriculture, Universitas Gadjah Mada (28). The T-DNA contains 35S::*GFP* and *NPTII* genes.

Agroinfiltration

The agroinfiltration method in this study follows the protocol of agroinfiltration for orchid plants that has received a patent certificate from the Ministry of Law and Human Rights of the Republic of Indonesia at the year 2024 (29). *Agrobacterium tumefaciens* strain GV3101 containing plasmid pRI101AN as a recombinant bacteria colony was cultured into a liquid Luria Bertani (LB) medium containing the antibiotics kanamycin (50 mg/L), rifampicin (100 mg/L) and gentamicin (12.5 mg/L). The cultures were incubated in the dark with shaking at 200 rpm at 28 °C for 2 days. *Agrobacterium tumefaciens* cells were harvested by centrifugation at 6000 rpm for 10 min. The supernatant was discarded and the pellet was resuspended in MMA buffer (10 mM MES pH 5.6, 10 mM $MgCl_2$, 200 µM acetosyringone) to an OD_{600} of 0.5-1.0. The culture is then incubated for 1-3 hr at room temperature. The abaxial parts of the midvein of *P. amabilis* leaves were injected with 100 µL of *A. tumefaciens* suspension using a 1 mL 26G × ½" syringe (Fig. 2). This injection was done in 3 plants as replication. Injected leaves were collected after infiltration at 24 hr to observe GFP expression using an Olympus BX53 (Japan) fluorescence microscope with an integrated camera. Green fluorescent protein expressions are indicated by green fluorescence within the cells.

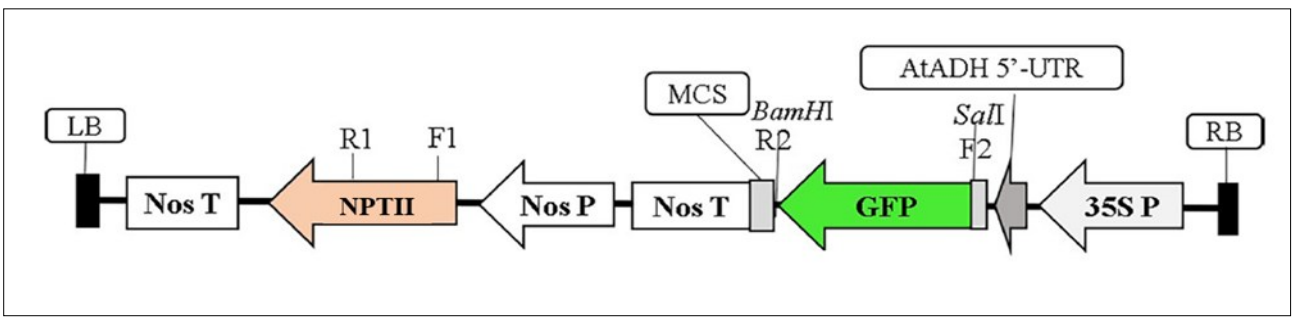

Fig. 1. T-DNA construct in pRI101AN containing *NPTII* and 35S::*GFP*; LB: Left border sequence; *NPTII*: Neomycin phosphotransferase II coding sequence; Nos P: Nopaline synthase (NOS) promoter; Nos T: NOS terminator; GFP: Green fluorescent protein; 35S P: Strong universal promoter 35S; RB: Right border.

Fig. 2. Agroinfiltration was performed on abaxial part of midvein of *P. amabilis* leaf.

Observation of GFP fluorescence using a fluorescence microscope

Injected leaf samples were cut transversely and longitudinally with free-hand section method. Leaf slices were placed on a glass slide, sludge with sterile distilled water and covered with cover glass. These leaf pieces were observed directly under a fluorescence microscope with bright field light and blue light laser (wavelength 450 – 480 nm) that would show fluorescence expression of GFP.

Genomic DNA extraction and confirmation of T-DNA in the infiltrated plants

Extraction of genomic DNA (gDNA) from leaves was completed following the modification (30). The gDNA purification was conducted by using PCI (phenol: chloroform: isoamyl alcohol; Sigma Aldrich, Germany) with a ratio of 25:24:1. The purified orchid's gDNA was checked using 1 % agarose gel electrophoresis and visualized with a UV transilluminator.

The gDNA of the infiltrated plants as transformant candidates was analyzed by PCR using MyTaq™ Red Mix PCR Kit (Bioline). Specific primers were used to detect the presence of the *GFP, NPTII* and *ACTIN* genes (Table 1). The PCR conditions used were pre-denaturation (95 °C for 3 min), denaturation (95 °C for 30 sec), annealing for 30 sec in the specific temperature (58 °C for *GFP*, 58 °C for *NPTII* and 51 °C for *ACTIN*), extension (72 °C for 1 min for *GFP* and *NPTII,* 10 sec for *ACTIN*) 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 (BioRad). The DNA amplification results were separated by electrophoresis using a 1 % agarose gel stained with RedSafe staining solution and visualized with a UV transilluminator.

Results and Discussion

In this study, the agroinfiltration technique was employed to introduce *A. tumefaciens* carrying the target gene into the leaf tissues of *P. amabilis*. This method enables rapid, specific and efficient DNA transfer. The leaf structure of orchids, which is thicker and waxier compared to model plants like *Nicotiana benthamiana*, poses a unique challenge for gene delivery (31). The infiltration process was conducted by injecting an *Agrobacterium* suspension into the midvein of leaf using a syringe to ensure uniform bacterial distribution within the plant tissue. Syringe infiltration is the simplest and most efficient method of agroinfiltration for gene function analyses (15). It allows the optimization of experimental settings that may affect the efficiency of protein expression by enabling the execution of several transient expression assays on a single leaf. Visual observations after infiltration were performed to evaluate the effectiveness of bacterial penetration and detect morphological changes in the leaves caused by the injection process. Fig. 3 (A & B) illustrates the differences in leaf conditions before and after infiltration, demonstrating the initial effects of this procedure.

The injection of *A. tumefaciens* suspension into *P. amabilis* leaves resulted in distinct visual changes. Before injection (Fig. 3A), the leaf surface appeared healthy, exhibiting a fresh green colour with no visible signs of injury or discoloration. This indicates that the leaves were in optimal condition for use as target tissues in this study. However, after injection (Fig. 3B), the infiltrated area showed significant changes, including the formation of a transparent zone, suggesting the successful penetration of the bacterial suspension into the leaf tissue.

This visual change, observed as a transparent zone at the infiltration site, serves as an indicator that the *Agrobacterium* suspension successfully entered the intercellular spaces of the leaf tissue. The formation of this transparent zone is presumed to result from osmotic shock caused by the influx of liquid and compounds from the bacterial suspension, leading to localized tissue swelling around the infiltration area. The successful penetration of *Agrobacterium* demonstrates that syringe-based agroinfiltration not only facilitates efficient bacterial entry into plant tissues but also provides a suitable environment for transient gene transfer and expression within leaf cells. However, the success of transient expression in agroinfiltration is influenced by the distribution of the bacterial suspension within the leaf tissue after it successfully penetrates the epidermal barrier. Ensuring effective spread is essential for optimal results.

These findings align with previous studies which have shown that injection-based agroinfiltration enhances gene transfer efficiency by reducing processing time and increasing the infected area (14). The successful infiltration observed in this study represents a crucial step in progressive gene expression, where *Agrobacterium*

Table 1. Primers used for PCR analysis of *GFP*, *NPTII* and *ACTIN* genes in infiltrated plants

Primer	Sequences	Size product (bp)
GFP_F	5'-GCTGTCGACATGCGTAAAGG-3'	736
GFP_R	5'-GCGCGGATCCTTA TTTGTATAG-3'	
NPTII_F	5'-GTCATCTCACCTTGCTCCTGCC-3'	550
NPTII_R	5'-GTCCGTTGGTCGGTCATTTCG-3'	
ACTIN_F	5'-GTATTCCCTAGGATTGTTGGT-3'	112
ACTIN_R	5'-CAGAGTGAGAATACCTCGTTG-3'	

Note: *GFP:* Green fluorescent protein*; NPTII:* Neomycin phosphotransferase II; F: Forward primer; R: Reverse primer.

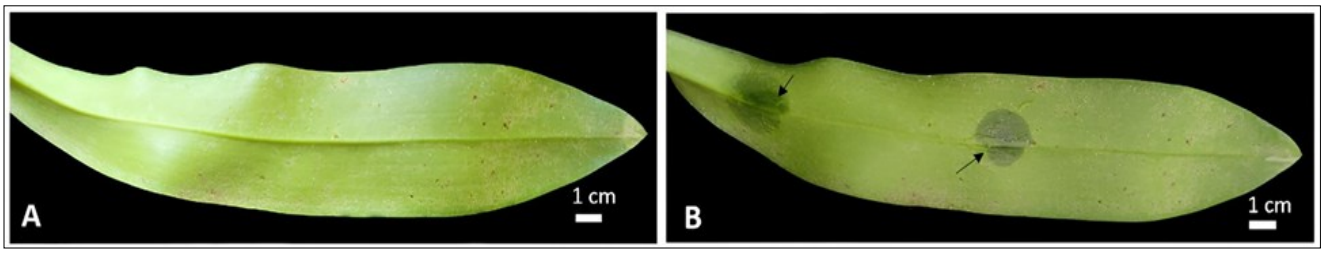

Fig. 3. Injected *P. amabilis* leaves by *Agrobacterium* suspension: (A) Before injection; (B) After injection.

delivers the target DNA into plant cells, enabling interactions and integration within the cellular system. Therefore, the visual observations not only depict the infiltration process's outcomes but also highlight this technique's potential in plant biotechnology applications, including recombinant protein production and functional genomics research.

To further validate the expression of the *GFP* gene at the cellular level, observation using fluorescence microscopy was performed at *P. amabilis* (Fig. 4 A-F). This analysis provides visual confirmation of GFP protein expression within plant tissues. Under blue light excitation, chloroplasts in the leaf tissues emitted red autofluorescence (32), while GFP expression was detected as a distinct green fluorescence signal. Observations of the adaxial (Fig. 4D) and abaxial (Fig. 4E) surfaces of injected leaves indicate that GFP fluorescence was directly detected and primarily localized in the midvein and marginal regions of agroinfiltrated leaves. Green fluorescent protein fluorescence in these areas suggests that agroinfiltration effectively introduced the target gene into the leaf tissue and facilitated the transient gene expression.

The fluorescence microscopy results in Fig. 4 provided evidence of GFP expression in specific leaf regions, particularly in the midvein and marginal cells. To further investigate the localization of GFP expression within leaf tissues at the histological level, free-hand sections were observed under bright-field and fluorescence microscopy. These observations allow for a more detailed assessment of transgene expression in different cellular structures, including the epidermis, mesophyll and vascular tissues. This phenomenon with agroinfiltration has been specifically proven to enhance GFP expression in *Phalaenopsis* hybrid orchid (33). Following infiltration with *A. tumefaciens* carrying the *GFP* gene, fluorescence can be detected within a short period. Microscopic observations have confirmed clear fluorescence on the outer leaf layers, indicating that this technique not only produces fluorescent proteins but also provides insight into gene distribution in plant tissues, including the adaxial and abaxial leaf surfaces, as well as the main veins and leaf margins. The mechanism by which agroinfiltration introduces recombinant DNA into plant cells through small pores explains the rapid and efficient expression of genes.

Free-hand sections of *P. amabilis* leaves were observed under fluorescence microscope with bright field light (Fig. 5 A - C) and blue laser light (Fig. 5 D - F). Bright-field images of transverse sections (Fig. 5 A & B) illustrate the overall tissue organization in both control and agroinfiltrated leaves, with clear differentiation between the epidermis (e), mesophyll (m) and vascular bundles (vb). The corresponding fluorescence images (Fig. 5 D & E) confirm that GFP expression is localized primarily in the vascular bundle and epidermal cells of agroinfiltrated leaves, as indicated by the presence of green fluorescence. This suggests that the introduced transgene was successfully expressed in these tissues. Previous research showed that bright-field imaging of transverse sections of both control and agroinfiltrated leaves provides insights into the cellular architecture, highlighting distinct layers such as the epidermis, mesophyll and vascular bundles (34). This delineation in tissue organization is essential for understanding where specific gene products, like GFP are localized.

Additionally, longitudinal sections (Fig. 5 C & F) further reveal the distribution of GFP expression in the epidermis, mesophyll and vascular bundle, reinforcing the findings from transverse sections.

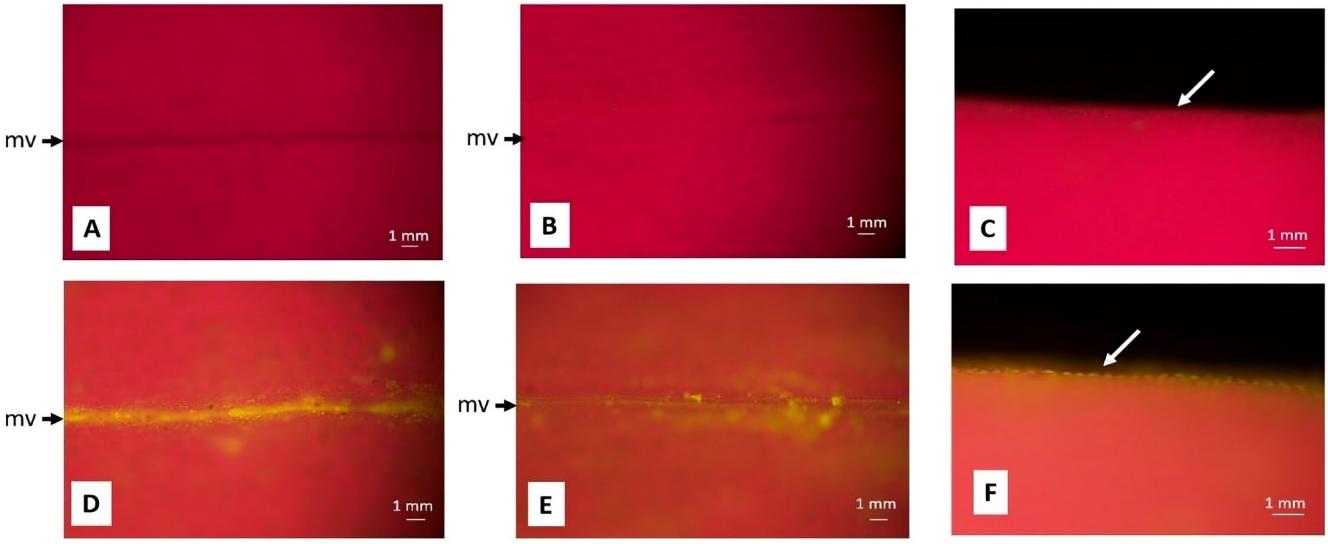

Fig. 4. Fluorescence microscopic view of adaxial and abaxial surfaces of *P. amabilis* leaves under blue laser excitation. (A-C): Control (without agroinfiltration); (D-F): Agroinfiltrated leaves; (A, D): Adaxial; (B, E): Abaxial; (C, F): Marginal leaf. GFP expression appeared as green fluorescence in midvein and marginal cells (D–F), while chloroplasts emitted red autofluorescence. mv: Midvein.

The absence of green fluorescence in the control samples confirms that the observed signals are specific to agroinfiltrated tissues. These results highlight the successful delivery and expression of the *GFP* transgene in multiple leaf cell types, demonstrating the effectiveness of agroinfiltration for transient gene expression in *P. amabilis*. The localization of GFP within vascular tissues suggests potential applications for the method in studying gene function related to plant vascular systems and systemic protein expression. In this study, the bacterial suspension was injected into the midvein of the leaf, which allowed the bacterial suspension to spread to the vascular bundles and throughout the other tissues. The fluorescence imaging is revealing a concentrated expression of *GFP* primarily in the vascular bundles and epidermal cells of the agroinfiltrated leaves, as indicated by the prominent green fluorescence observed (35, 36).

To further validate the effectiveness of agroinfiltration at the molecular level, an analysis of gDNA integrity was conducted. This analysis aimed to determine whether the infiltration process affects the structural integrity of plant DNA. The electrophoregram results illustrate the quality of gDNA extracted from both non transformant (control) and transformant leaves, providing insights into the impact of this method on genetic stability. The electropherogram of *P. amabilis* leaf gDNA (Fig. 6A) illustrates the results of genomic DNA extraction using agarose gel electrophoresis. Lane 1 represents a 1000 bp DNA marker, which serves as a reference for estimating the fragment sizes in the samples. Lane 2 corresponds to the gDNA extracted from *P. amabilis* leaves that were not subjected to agroinfiltration (non-transformant/ control plant), while lanes 3, 4 and 5 represent gDNA extracted from leaves that had undergone infiltration with *A. tumefaciens* (transformant plant).

The electrophoresis results indicate that all samples, including both control and agroinfiltrated leaves, exhibit DNA bands larger than 10000 bp. This finding suggests that the integrity of gDNA remained well-preserved, with no significant degradation following the agroinfiltration process. The presence of distinct and intense bands in lanes 3, 4 and 5 further confirms that the infiltration process did not lead to substantial DNA fragmentation, implying that this treatment does not directly disrupt the genomic stability of the plant. The control sample, without agroinfiltration, shows similar integrity to the samples that were infiltrated with *A. tumefaciens*, reinforcing the conclusion that agroinfiltration does not compromise genomic stability. Previous research indicates that agroinfiltration has been successfully utilized for transient gene expression without significant impacts on DNA integrity (21, 22).

Based on the present results, the gDNA extracted from *P. amabilis* leaves exhibited high integrity, with no significant degradation observed after agroinfiltration. To further validate the successful introduction of the target gene into the plant genome, PCR amplification was performed to detect the presence of the *GFP* and *NPTII* genes, using the *ACTIN* gene as a positive control (Fig. 6B, Table 2). The PCR results confirm the successful detection of the *GFP* gene (736 bp) and the *NPTII* gene (550 bp) in transformant plants (lanes 3, 4 and 5 respectively), while no amplification was observed in the non-transformant plant (lane 2). The distinct amplification of *GFP* and *NPTII* in the agroinfiltrated samples strongly indicates that *A. tumefaciens*-mediated agroinfiltration effectively delivered and transiently expressed the target genes in the leaves of *P. amabilis*. The successful detection of *GFP* and *NPTII* genes in *P. amabilis* transformant leaves through PCR analysis confirms the presence of the introduced transgenes at the molecular level. The presence of the *ACTIN* gene (114 bp) in all lanes, including the non-transformant plant, verifies the quality of the gDNA and ensures that the PCR reaction was performed correctly. *ACTIN* is recognized as an important housekeeping gene that functions as an internal control gene within cells. Internal control genes are constitutive in nature, meaning they are expressed consistently in both normal and

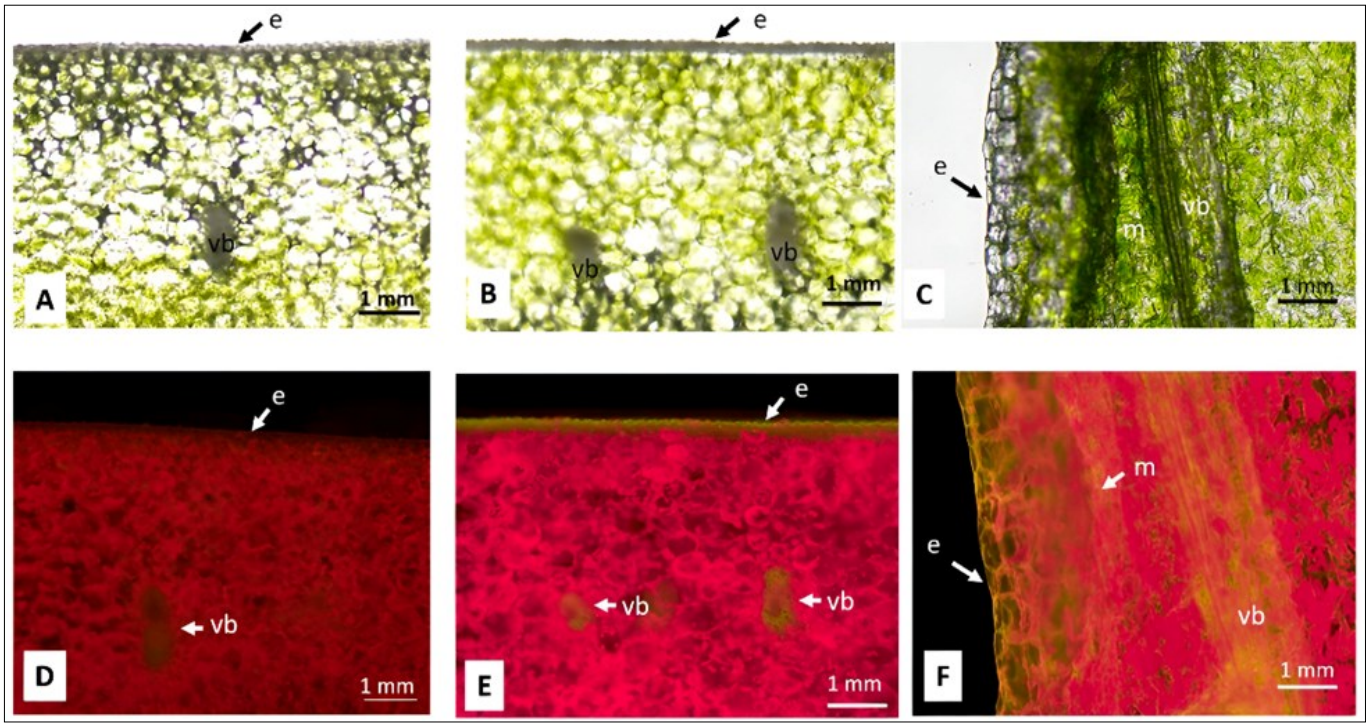

Fig. 5. Free-hand sections of *P. amabilis* leaves observed under bright field light and blue laser light of fluorescence microscopy. (A–C): Bright-field; (D–F): Blue laser; (A, D): Transverse section of control (without agroinfiltration); (B, E): Transverse section of agroinfiltrated leaf showing GFP expression in vascular bundles and epidermis; (C, F): Longitudinal section of agroinfiltrated leaf showing GFP expression in epidermis, mesophyll and vascular bundles. Chloroplasts appeared red under blue laser excitation. e: Epidermis; m: Mesophyll; vb: Vascular bundle.

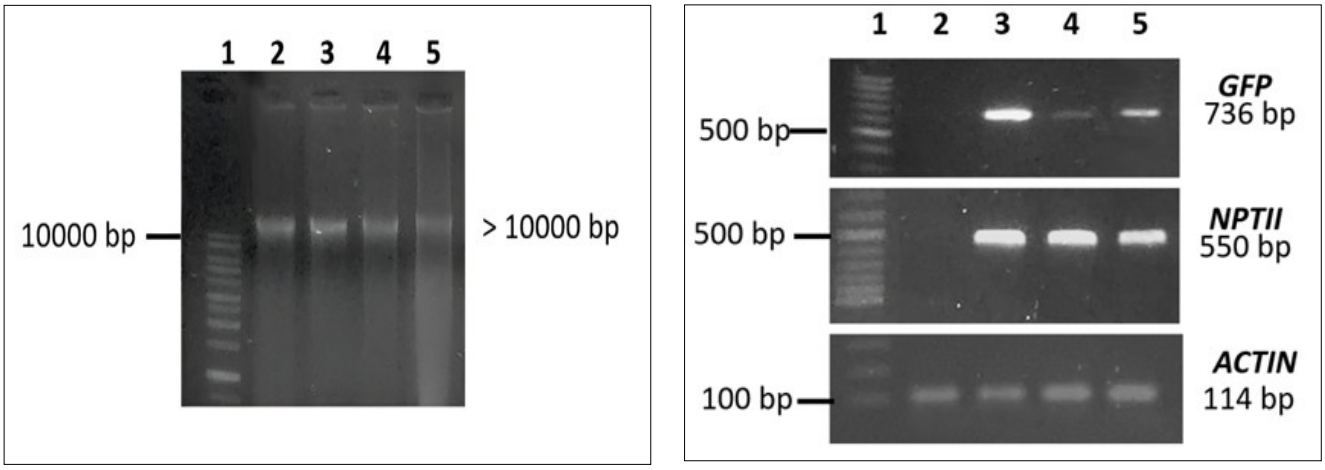

Fig. 6. Molecular detection of T-DNA construct in pRI101AN containing *NPTII* and 35S::*GFP*. (A): DNA genome visualization of *P. amabilis* leaves. Lane 1: DNA marker 1000 bp (Geneaid); Lane 2: Non transformant (control) plant; Lane 3: Transformant plant #1; Lane 4: Transformant plant #2; Lane 5: Transformant plant #3; (B): Detection of *GFP*, *NPTII* and *ACTIN* gene from gDNA of *P. amabilis* leaves. Lane 1: DNA marker 100 bp (Geneaid); Lane 2: Non transformant (control) plant; Lane 3: Transformant plant #1; Lane 4: Transformant plant #2; Lane 5: Transformant plant #3.

Table 2. Efficiency of agroinfiltration by using transient expression of *GFP* reporter gene

S. No.	Plants	Microscopic fluorescence of GFP	PCR result		
			***GFP* (736 bp)**	***NPTII* (550 bp)**	***ACTIN* (112 bp)**
1	Non transformant	-	-	-	+
2	Transformant #1	+	+	+	+
3	Transformant #2	+	+	+	+
4	Transformant #3	+	+	+	+

Note: PCR: Polymerase chain reaction; + Positive result; - Negative result.

challenging conditions across various living organisms. Their significant role in sustaining cellular functions is essential (37).

Research on gene stability and integration following *A. tumefaciens* injection is essential to ensure that the results can be applied on a broader scale, both in field applications and academic research. Moving forward, expanding the use of GFP for functional genomics in valuable ornamental species such as *P. amabilis* will not only enhance fundamental knowledge of gene function but also increase their economic value in the horticultural industry. With the appropriate strategies, agroinfiltration has the potential to become a primary method in modern plant breeding, offering a more efficient and sustainable approach.

Conclusion

Agroinfiltration using GFP transient expression was successfully performed for the rapid detection of *GFP* as a reporter gene carried by pRI101AN on *P. amabilis* transformant leaves. The results showed that GFP expression was rapidly detected within 24 hr post-infiltration. Observation under fluorescence microscopy using blue laser light revealed that GFP expression was directly detected in the adaxial and abaxial surfaces of the leaf, especially on the midvein and marginal leaf. The transverse and longitudinal free-hand cross-section of leaves also shows the GFP expression on the epidermis, mesophyll and vascular bundle. The accuracy of the data was supported and confirmed by the positive results of all three *P. amabilis* transformant plants that were analyzed by PCR and it was proven that all positive transformant plants carried the *GFP*, *NPTII* and *ACTIN* genes. Based on this result, the agroinfiltration and GFP approach can be applied for rapid genetic transformation for orchid species and also can be used for other plants.

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Authors' contributions

PDK collected plant materials, carried out agroinfiltration, molecular data analysis, anatomical slides preparation, analyzed data and wrote the manuscript. EG and YRH assisted in the wet lab activities and manuscript proofreading. P and M guided data collection and also reviewed the manuscript. ES designed the research, supervised the entire process. All authors have read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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