

Research Article

Stable Transformant of *Phalaenopsis amabilis* Somatic Embryo Carrying *35S::AtRKD4* Develops Into Normal Phenotype of Transgenic Plant

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ABSTRACT

Phalaenopsis amabilis (L.) Blume orchid is an Indonesian national flower. The number of these orchids in their natural habitat is very limited, therefore plant propagation efforts are needed. One of the promising methods is plant propagation by inserting embryo gene *AtRKD4* from a model plant *Arabidopsis thaliana* into the orchid genome to produce many somatic embryos. From previous research, we have obtained 28 plant *P. amabilis* transformants carrying the *AtRKD4* gene, however, it was unknown whether these plants have normal phenotypes and growth similar to their parents. Therefore, descriptions on growth and morphology are needed. This research aimed to evaluate the phenotype of *P. amabilis* carrying *35S::AtRKD4* the transformants grown in greenhouse. To achieve it, *AtRKD4* gene integration stability on transformants genome was analyzed. Morphology and cross-section anatomy structure on transformant and non-transformant plants were described. The stability of *AtRKD4* gene integration in the plant genome was confirmed by amplification of the *AtRKD4* gene from genomic DNA with Polymerase Chain Reaction (PCR) using a specific primer for *AtRKD4* and *ACTIN* genes as the internal control. The quantitative data from morphology and anatomy measurements were analyzed statistically using ANOVA. The results showed that *AtRKD4* was stably integrated into the genome of *P. amabilis* transformants and all transformant plants showed similar morphology and anatomy characteristics as non-transformant plants. The *AtRKD4* embryo gene was stably integrated into the orchid genome and the transformant plants grow normally without significant changes in phenotype.

Keywords: *Arabidopsis*, *AtRKD4*, Orchid, *Phalaenopsis amabilis* (L.) Blume, Somatic embryogenesis

INTRODUCTION

Orchid is one of the commercial plants in the field of floriculture (Pamarthi et al. 2019) and has high economic value (Palma et al. 2010). One of the important orchids is *Phalaenopsis amabilis* (L.) Blume, which is commonly called the Moth Orchid, which produces beautiful flowers shaped like a moth. The beauty of *P. amabilis* flowers has made it called "Puspa Pesona"

which is used as one of Indonesia's national flowers, along with jasmine and *Rafflesia arnoldii* (Schuiteman 2010; Semiarti et al. 2018). The uniqueness of this *P. amabilis* flower is the presence of an antenna-like structure at the end of its labellum, which always appears in the flower of this orchid's hybrid so that *P. amabilis* is commonly used as a parental species for breeding purposes to produce superior *Phalaenopsis* hybrid in the world (Semiarti et al. 2011).

Generative/sexual propagation depends on the success of the pollination process. It is ineffective because it takes a long time to produce a new plant (Shekarriz et al. 2014; Mursyanti et al. 2015; Saputro et al. 2018). Somatic embryogenesis is an effective method for orchid propagation. It can produce large numbers of uniform plants in a relatively short time (Feng & Chen 2014; Mose et al. 2017; Yang et al. 2020). The development of methods through genetic transformation could be beneficial for increasing crop production and conservation (Semiarti et al. 2007; Hsing et al. 2016; Zulwanis et al. 2020).

It is well known that plant development is controlled by gene regulation. The *RKD4* gene is a member of the RWP-RK protein family in a model plant *Arabidopsis thaliana* encoding transcription factors for gene expression in early plant development (Waki et al. 2011), promotes zygote extension (Jeong et al. 2011), induces somatic embryogenesis (Zimmerman 1993) and induces cell proliferation and expression of an egg cell (Kószegi et al. 2011). The insertion of *AtRKD4* gene from *A. thaliana* succeeded in inducing somatic embryo in the *Phalaenopsis* "Sogo Vivien" orchid hybrid (Mursyanti et al. 2015) and *Dendrobium phalaenopsis* (Setiari et al. 2018). So far, there has never been comprehensive studies regarding the influence of ecological factors on the growth of transformant plants during the acclimatization period after being transferred from *in vitro* condition which is strictly controlled to *ex vitro* condition, in which various environmental factors highly influence it. In fact, the process of transformant plants going through the acclimatization period to be able to grow normally in *ex vitro* condition is crucial. Therefore, we need to ensure that the transformant plants produced by somatic embryo induction from *AtRKD4* expression can grow and develop similarly as non-transformant plants.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The plant materials used in this study were ten non-transformant of wild type *P. amabilis* (Yogyakarta Forms) plants age 8-10 months and 10 plants of *P. amabilis* transformants, carrying the 35S::*AtRKD4* gene, obtained from previous research results of the Orchid Research Team Faculty of Biology UGM. Each plant was planted in pots with a combination of fern and wood charcoal (1: 1), they were maintained in a greenhouse under natural sun lighting environment (28-31°C) and relative humidity ranges 74-80%.

Detection of *AtRKD4* gene in the genome of *P. amabilis* transformants

Isolation of the whole genome DNA of wild type and transformants *P. amabilis* was performed by the standard method of plant genome DNA isolation as described by Semiarti et al. (2007) by using Cetyltrimethyl Ammonium Bromide (CTAB). Detection of the existence of *AtRKD4* gene in the transformants was performed using *Polymerase Chain Reaction* (PCR) with KOD FX NEO PCR Kit (Toyobo) and specific primers for *AtRKD4* gene and *Phalaenopsis* actin gene (*ACT4*) (Yuan et al. 2014) as the internal control (Table 1).

PCR reactions for both genes were performed in 3-step-cycles: an initial denaturation 94°C for 2 min, denaturation at 98°C for 10 seconds,

annealing at 58 °C for *AtRKD4* and 51°C for *ACT4* for 30 seconds, and extension at 68°C for 6 sec seconds using a Thermal Cycler (T100, Biorad). PCR steps were performed in 35 cycles. The amplified DNA fragments were checked with 0.8% agarose gel (Agarose Type II, SIGMA).

Table 1. Primers used in this study.

Primer name	Primer direction	Sequence (5' to 3')	Product size (bp)
<i>AtRKD4</i>	<i>forward</i>	GTTCA [*] TTTCAT [*] TTGGAGAGGACG	198
	<i>reverse</i>	CTTCCATATCTAGGAGAGAATCAAG	
<i>ACT4</i>	<i>forward</i>	GTA [*] TTCCTAGCAT [*] TGTTGGT	114
	<i>reverse</i>	CAGAGTGAGAATACCTCGT [*] TTG	

Morphological Analysis

Plant growth was observed every week for eight weeks to get detailed similarities and differences in growth between *P. amabilis* transformant and non-transformant plants, ten plants each for both non-transformant and transformant plants. Plant morphological data covering the total number of leaves, leaf length, leaf width, as well as the total number of roots, root length and root diameter were observed and measured weekly. The phenotypic analysis includes the shape and color of leaves, roots, and survival ability. The observations were documented using a digital camera (EOS M100, Canon), color characteristic was determined using RHS color chart (The Royal Horticultural Society, London).

Anatomical Structure Analysis of Roots and Leaves

Anatomical preparations of roots and leaves cross-section were conducted using the paraffin embedding method according to Sutikno (2016) and stained by 1% safranin in 70% alcohol, the sample was fixed with FAA solution (Formalin: glacial acetic acid: 70% alcohol = 5: 5: 90). Observations of the anatomy slides were performed by using a light microscope (CX22LED, Olympus) and documented using a digital microscope camera (Optilab Advance V2, Miconos).

Statistical analysis

Quantitative data is processed using Microsoft Excel 2013 applications and Raster 3 image, then statistically analyzed using IBM SPSS Statistics 26.0 to find out whether there are growth and phenotype differences. Analysis of Variance (ANOVA) was conducted to test the significance difference of the growth by both groups with significance level 0.05. Qualitative data was descriptively analyzed.

RESULTS AND DISCUSSION

P. amabilis transformants and non-transformants growth

Growth of *P. amabilis* transformant and non-transformant plants were observed for 8 weeks. The phenotypes of *P. amabilis* transformant and non-transformant plants showed very similar growth patterns (Figures 1 and 2). Observation was conducted as a first step to determine morphological characteristics of the two groups.

During acclimatization, *P. amabilis* non-transformant and transformant orchids at week 0 (Figures 1A and 2A) had compact green leaves (RHS Green group 138A) and compact green roots (RHS Green group 145A). At

week one (Figures 1B and 2B) the leaves began to undergo chlorosis at the tips. *P. amabilis* non-transformant orchids began to adapt in *ex vitro* environment at week 4 (Figure 1D), which marked by new root and leaf growth after 5-week acclimatization (Figure 1E). Whereas, *P. amabilis* (I) at week 2 (Figure 2C) have shown the ability to adapt in *ex vitro* environment characterized by the growth of new roots and leaves on week 4 (Figure 2E). Mirani et al. (2017) reported a similar phenomenon in *Dendrobium nobile* acclimatization. Maintenance of humidity and temperature around the plantlets during the initial days of acclimatization is of utmost importance. During *in vitro* culture, plantlets were grown in high humidity conditions in jars. Meanwhile, in greenhouse or field has much lower humidity than *in vitro* condition. Therefore, rapid changes in environmental conditions, especially in terms of temperature and humidity, could led to wilting and chlorosis. *P. amabilis* non-transformant and transformant have an obovate leaf shape, where the widest part at the tip of the leaf blade. The shape of the leaf tip is retuse and the leaf edge is integer (Figures 1 and 2).

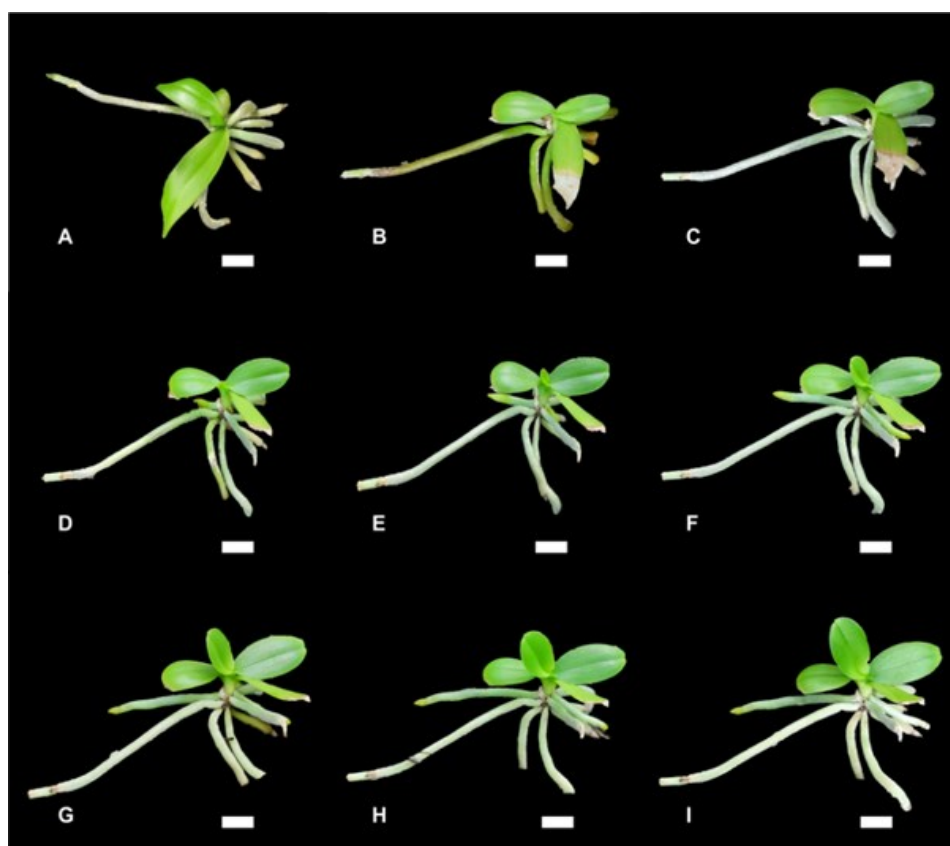


Figure 1. Non-transformant *P. amabilis* growth during acclimatization was observed each week from (A) week 0 to (I) week 8.

The growth of *P. amabilis* non-transformant and transformant plants was statistically analyzed to determine significant growth differences. The viability percentage of *P. amabilis* non-transformant and transformant plants from week 0 to 8 during acclimatization showed that both of them were successfully adapting in *ex vitro* environment. Total number of leaves of *P. amabilis* non-transformant and transformant plants decreased during week 1 of acclimatization but showed a positive trend starting from week 2 of acclimatization. The leaf length of *P. amabilis* non-transformant plants decreased until week 2 of acclimatization, then showed an increase in trend until week 8. Whereas the *P. amabilis* (I) plants decreased slightly at week 1 then showed an increase in trend until week 8. Leaf width, the total number of roots, root length and diameter of the root of *P. amabilis* non-transformant

and transformant plants from week 0 to 8 of acclimatization showed an increase in trend.



Figure 2. Transformant *P. amabilis* carrying *AtRKD4* gene during acclimatization was observed each week from week 0 (A) to week 8 (I).

A significance level above 0.05 indicates no difference in growth between *P. amabilis* non-transformant and transformant plants. As shown in Table 2, *P. amabilis* non-transformant and transformant plants had

Table 2. Comparison of the growth rate between *P. amabilis* non-transformant and transformant plants using ANOVA.

		Sum of Squares	df	Mean Squares	F	Signifi-cance
Total Number of Leaf	Beetwen Groups	0,251	1	0,251	0,844	0,370
	Within Groups	5,352	18	0,297		
	Total	5,603	19			
Leaf Length	Beetwen Groups	4,990	1	4,990	3,812	0,067
	Within Groups	23,560	18	1,309		
	Total	28,550	19			
Leaf Width	Beetwen Groups	0,305	1	0,305	4,351	0,051
	Within Groups	1,262	18	0,070		
	Total	1,567	19			
Total Number of Roots	Beetwen Groups	4,901	1	4,901	4,382	0,051
	Within Groups	20,131	18	1,118		
	Total	25,031	19			
Root Length	Beetwen Groups	4,418	1	4,418	1,698	0,209
	Within Groups	46,834	18	2,602		
	Total	51,252	19			
Root Diameter	Beetwen Groups	0,006	1	0,006	1,199	0,288
	Within Groups	0,092	18	0,005		
	Total	0,098	19			

Note: significance level 0.05

significance levels above 0.05 in all parameters. The results of this study are in line with Semiarti et al. (2018) who compared the vegetative growth between *Phalaenopsis* “Sogo Vivien” carrying *35S::Gal4::AtRKD4::GR* and the non-transformant plants that showed no change in plant phenotype and growth condition.

Anatomic structure of roots and leaves of *P. amabilis* transformants and non-transformants

Cross-section of root anatomical structure (Figure 3) and leaf anatomic structure (Figure 4) shows high similarities in the shape and size of the leaf and root cells between transformant and non-transformant plants. There were no significant differences between transformant and non-transformant plants on the anatomic structure of roots and leaves.

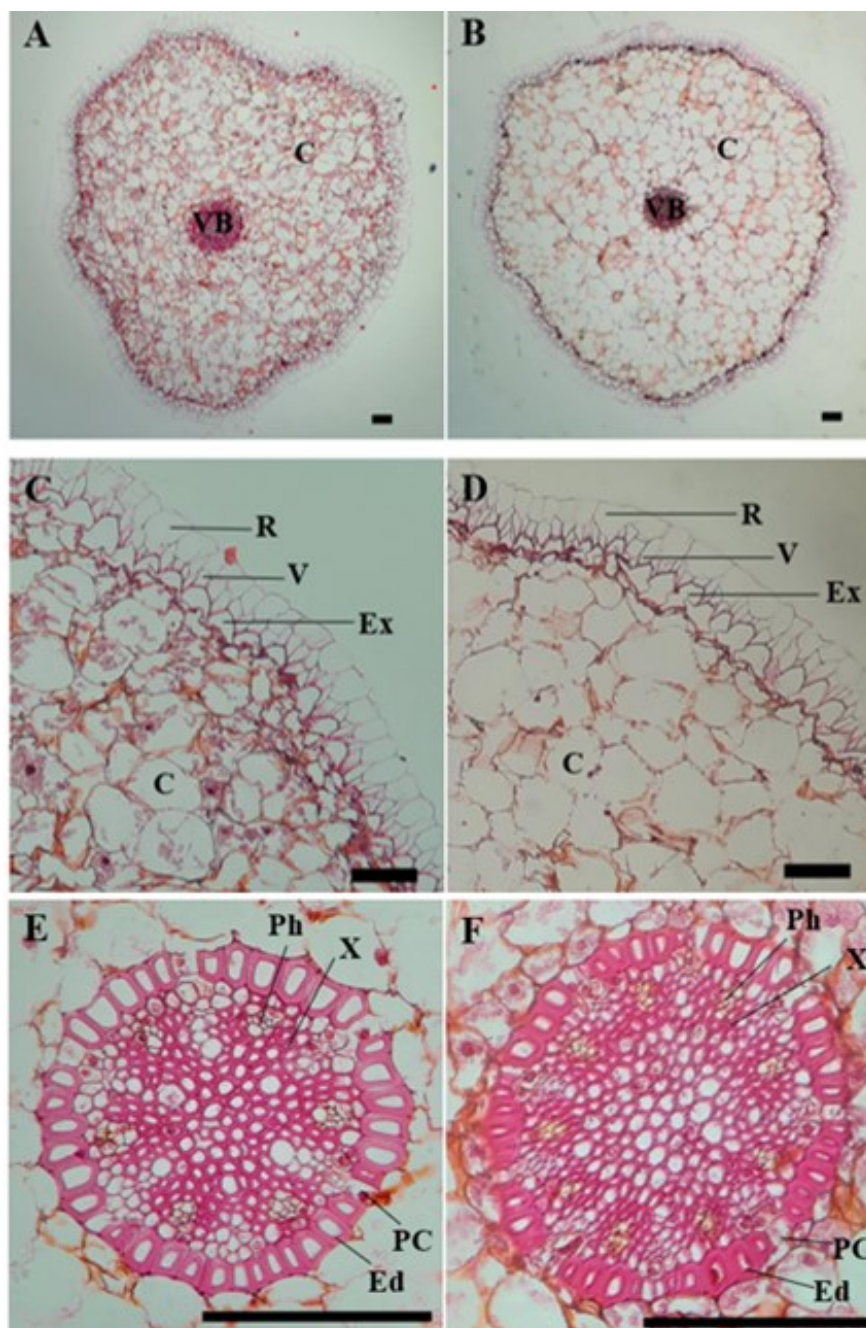


Figure 3. Anatomic structure of root cross-section of *P. amabilis*. (A, C, E) *P. amabilis* non transformant; (B, D, F) *P. amabilis* transformant. (A, B) Full cross-sectional view of the roots; (C, D) cortex and exodermis; and (E, F) stele. C, Cortex; Ed, Endodermis; Ex, Exodermis; Ph, Phloem; PC, Passage Cells; R, Rhizodermis; V, Velamen; VB, Vascular Bundle; X, Xylem. Bars: 50 μ m.

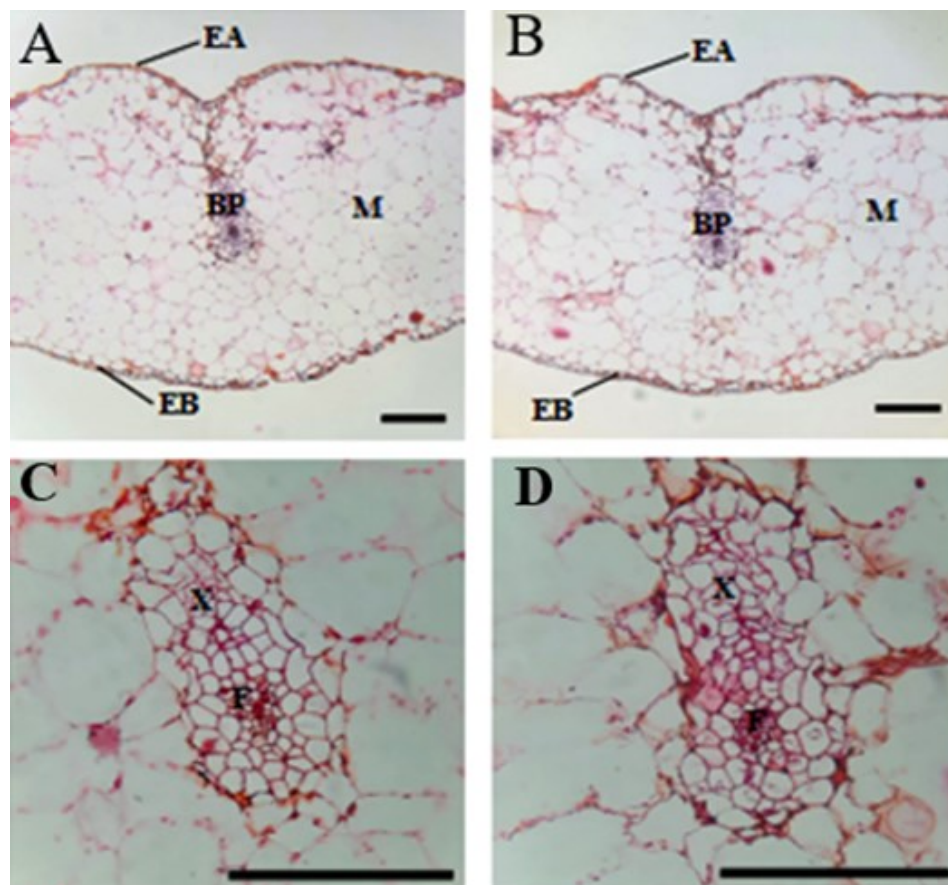


Figure 4. Anatomic structure of leaves cross-section of *P. amabilis*. (A,C) *P. amabilis* non transformant; (B,D) *P. amabilis* transformant. (A,B) Cross-section of the leaves; and (C,D) vascular bundle in the center part of the leaves. AbE, Abaxial Epidermis; AdE, Adaxial Epidermis; M, Mesophyll; Ph, Phloem; VB, Vascular Bundle; X, Xylem. Bars: 50 μ m.

The cross-section of roots of *P. amabilis* orchid transformant and non-transformant showed a very similar anatomical structure consisting of Rhizome, Cortex and Stele (Figure 3). Table 3 shows that the size of the length and width of these cells did not show a significant difference of Rhizome, Cortex and Stele with ANOVA significance of 5%. The anatomical structure of the leaves (Figure 4) also did not show significant differences in both the structure and size of cells Epidermis, vascular bundle (Xylem and Phloem), and parenchyma (Table 4). This phenomenon indicated that the activity of the *AtRKD4* gene in somatic embryo initiation did not affect on the growth and further development of the orchid somatic embryos, only at an early stage for the initiation of somatic cells into embryos. The *AtRKD4* gene, therefore, has no impact on the activity of growth genes to continue the growth and development of embryos into complete plants. This fact is highly expected by genetic engineers for *P. amabilis* orchid's mass propagation as a conservation effort as well as for producing large number of plants for agribusiness purposes.

Detection of the integration of *AtRKD4* gene in the genome of *P. amabilis* transformant

Positive amplification results of genomic DNA with specific primers for *AtRKD4* gene and primers for housekeeping gene *ACTIN* as an internal control for PCR reaction showed that a 114 bp of *ACTIN* fragment was amplified from the genome of both *P. amabilis* non-transformant and transformant, whereas 198 bp of *AtRKD4* fragment was amplified only from the genome of *P. amabilis* transformants (Figure 5). This data indicated that

Table 3. Root anatomical parameters of *P.amabilis* non-transformant and transformant.

Parameters	<i>P. amabilis</i> orchid		Significance
	non-transformant	transformant	
Rhizodermis Length (µm)	13,85 ± 2,40	13,27 ± 2,13	0,575
Rhizodermis Width (µm)	17,82 ± 1,94	17,36 ± 1,55	0,571
Velamen Length (µm)	7,13 ± 1,77	7,29 ± 1,20	0,810
Velamen Width (µm)	9,57 ± 1,77	9,29 ± 1,40	0,704
Exodermis Length (µm)	14,78 ± 1,58	14,56 ± 2,19	0,805
Exodermis Width (µm)	12,70 ± 2,09	12,39 ± 1,41	0,695
Cortex Diameter (µm)	49,69 ± 8,04	52,95 ± 8,02	0,377
Endodermis Length (µm)	6,11 ± 0,64	6,15 ± 0,55	0,894
Endodermis Width (µm)	9,60 ± 1,01	9,12 ± 0,79	0,262
Passage Cell Length (µm)	5,41 ± 0,51	5,58 ± 0,51	0,463
Passage Cell Width (µm)	7,32 ± 0,77	7,32 ± 0,80	1,000
Xilem Diameter (µm)	2,93 ± 0,20	2,96 ± 0,13	0,761
Phloem Diameter (µm)	1,88 ± 0,34	1,86 ± 0,19	0,899

Significant level 0,05

Table 4. Leaf anatomical parameters of *P.amabilis* non-transformant and transformant.

Parameters	<i>P. amabilis</i> orchid		Significance
	non-transformant	transformant	
Adaxial Epidermis Length (µm)	18,00 ± 1,26	18,58 ± 1,20	0,307
Adaxial Epidermis Width (µm)	9,97 ± 1,22	9,92 ± 1,14	0,921
Mesophyll Diameter (µm)	35,89 ± 3,25	35,95 ± 2,38	0,961
Xilem Diameter (µm)	6,13 ± 0,44	6,13 ± 0,38	0,983
Phloem Diameter (µm)	1,97 ± 0,14	1,92 ± 0,09	0,460
Abaxial Epidermis Length (µm)	17,38 ± 1,50	17,28 ± 1,36	0,877
Abaxial Epidermis Width (µm)	9,94 ± 1,03	9,79 ± 0,83	0,716

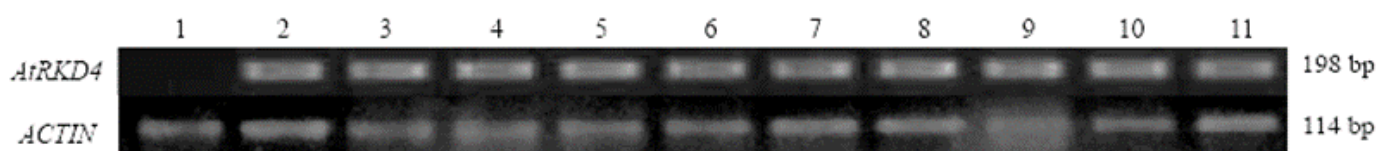


Figure 5. Detection of *ACTIN* and *AtRKD4* genes integration in the genome of non-transformant and transformant *P. amabilis* orchid lane 1: *P. amabilis* non-transformant; lane 2-11: *P. amabilis* transformant. Fragment of *AtRKD4* gene (198 bp) was amplified on *P. amabilis* transformant but not in non-transformant. Fragment of *ACTIN* gene 114 bp was amplified from genom of both *P. amabilis* non-transformant and transformant.

the *AtRKD4* gene is stably integrated in the genome of *P. amabilis* transformants while the transformant plants grow and develop similarly as non-transformant plants.

Waki et al. (2011) reported that in the *Arabidopsis thaliana* plant, the *AtRKD4* gene produced the RKD4 protein triggers gene expression and pattern formation in early embryogenesis. This is also supported by research data on somatic embryogenesis induction on *Phalaenopsis* 'Sogo Vivien' from Mursyanti et al. (2016) and research by Setiari et al. (2018) in somatic embryogenesis of *Dendrobium phalaenopsis*. This is also in accordance with Mose et al. (2020), who succeeded in inducing somatic embryogenesis by treating growth regulators thidiazuron and NAA from protocorms and *P. amabilis* leaf pieces as explants.

CONCLUSION

P. amabilis transformant plants remain stable on carrying *AtRKD4* gene and grew normally as the same as non-transformant plant. It has great potential to be used as an explant donor for the propagation of *P. amabilis* plants for various purposes, including production of seedlings for both conservation and agribusiness purposes. Overall, morphological data, anatomical data, and molecular data support the use of the *AtRKD4* gene for orchid propagation for the conservation of *P. amabilis* orchids whose population is decreasing in nature, as well as orchid production for economic purposes.

AUTHORS CONTRIBUTION

N.G.A.P collected and analyzed the data and wrote the manuscript, W.M idea of the experiment and wrote the manuscript. M.D.L revised and finalized the manuscript, J.G.M founder of T-DNA construct, E.S. designed the research and supervised all process.

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CONFLICT OF INTEREST

The authors declare there is no competing interest.

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