Journal of Tropical Biodiversity and Biotechnology
Volume 08, Issue 02 (71211): jtbb71211
DOI: 10.22146/jtbb.71211

Research Article

Detection of *AtRKD4* Gene and Induction of Somatic Embryo in Transformant of *Phalaenopsis amabilis* Carrying *35S::GR::AtRKD4*

Dika Sundari[1], Naufal Ghozi Aditya Perdana[2], Windi Mose[3], Jose Gutierrez-Marcos[4], Endang Semiarti[1,2]*

1)Graduate School of Magister Biology, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia
2)Department of Tropical Biology, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia
3)Faculty of Mathematics and Natural Sciences, Universitas Pattimura, Maluku, Indonesia
4)School of Life Science, University of Warwick, England
* Corresponding author, email: endsemi@ugm.ac.id

Keywords:
AtRKD4
embryo gene
Arabidopsis thaliana
Phalaenopsis amabilis
somatic embryo
Submitted:
14 December 2021
Accepted:
14 February 2023
Published:
21 July 2023
Editor:
Ardaning Nuriliani

ABSTRACT

Phalaenopsis amabilis (L.) Blume is a native of Indonesian orchid that plays important role in the breeding of orchid's hybrid worldwide. The high consumer demand causes a decline in the population of orchids for commercial trade. Plant propagation through induction of somatic embryogenesis will be very beneficial, because plants can be obtained in large numbers and uniforms. *AtRKD4* gene is an important gene in the model plant *Arabidopsis thaliana* which functions very early in development stage to initiate embryo formation. The *AtRKD4* gene has been inserted into the *P. amabilis* orchid and several transformants have been obtained. This study goals to determine the integration stability of the *AtRKD4* gene in the transformant genome of *P. amabilis* and to induce somatic embryo formation on transformant orchids. Plantation of leaf explants from *P. amabilis* transformants on hormone-free New Phalaenopsis basic medium induced somatic embryo formation by 20%. Anatomical analysis showed that there is no difference stage between anatomy of somatic embryo development pattern in *P. amabilis* transformant and somatic embryo development pattern of monocot plants in general, four weeks old-somatic embryos were analysed by PCR analysis using AtRKD4 specific primers that showed the embryos still contained 198 bp fragments of the *AtRKD4* gene. In conclusion, the *AtRKD4* gene is stably integrated in the genome of *P. amabilis* and can continuously induce the formation of somatic embryo from somatic cells of orchid transformants.

INTRODUCTION

Phalaenopsis amabilis (L) Blume is one of Indonesia's native orchids (Rahayu 2015) and has been designated as one of the national flowers since 1991 (Semiarti 2018). These orchids are often used as brood stock in breeding to produce hybrid orchids with superior traits (Semiarti et al. 2007). In orchids, the morphological character of the flower is the character used as a marker to distinguish plant groups (Pangestu et al. 2014). *P. amabilis* (Figure 1) produces beautiful flowers shaped like a moth and has a uniqueness of the antenna-like structure at the end of its labellum (Semiarti et al. 2011). The high consumer demand causes these orchids to

be widely cultivated and taken from their natural habitat (Rahayu 2015) resulting in over-harvesting for trade/commerce (Semiarti 2018). Germination of orchids from seeds is difficult to do (Mondal et al. 2016) making it ineffective for producing large numbers of seeds (Shekarriz et al. 2014). The right propagation method to produce orchid seeds quickly and in large numbers is needed for both commercial market and orchid conservation.

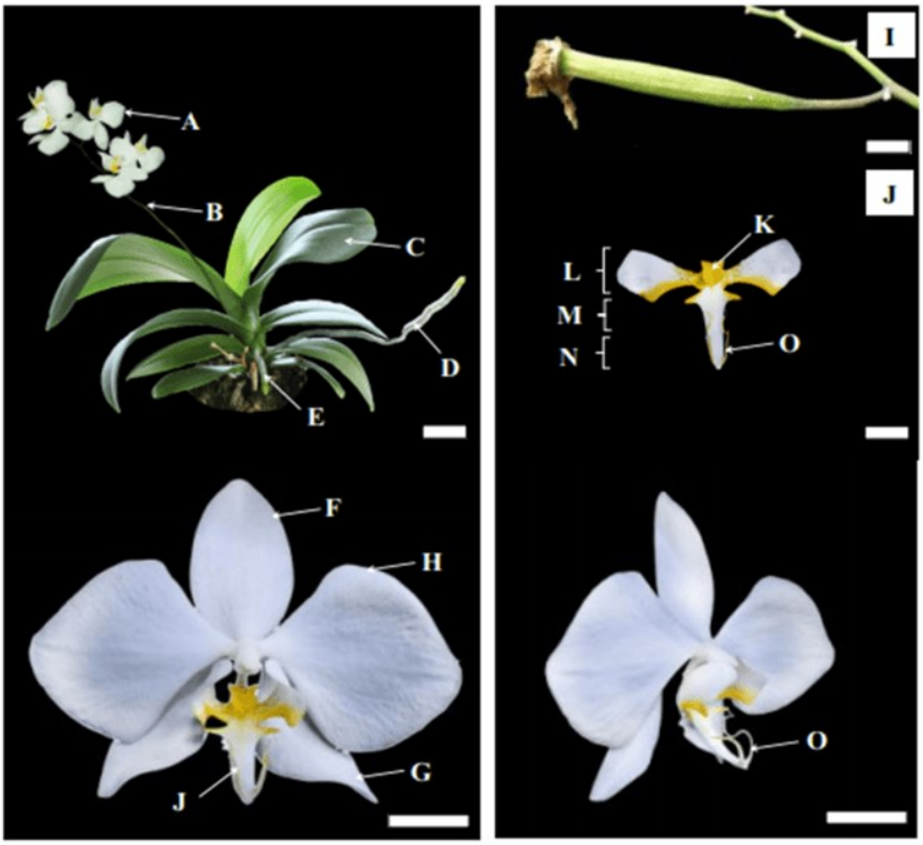


Figure 1. Morphology of *Phalaenopsis amabilis*. (A) flower), (B) flower stalks, (C) leaves, (D) air roots, (E) adhesive roots, (F) dorsal sepallum, (G) lateral sepallum, (H) lateral petallum, (I) fruit, (J) labellum, (K) callus, (L) hipochillium, (M) mesochillium, (N) epichillium, (O) antenna/mouth. Bars: 1 cm.

Somatic embryo formation can be induced with a molecular approach by introducing new traits into the plant genome. One of the genes that induces the change of somatic cells into embryogenic cells is the *RKD4* gene (Setiari et al. 2018) which encodes a protein with the RWP-RK motif (Chardin et al. 2014). The *RKD4* gene has the potential to initiate somatic embryo induction because it can activate other specific genes that have a role in the development of embryo. *RKD4* gene encodes a transcription factor protein with RWP-RK motif in Arabidopsis which expressed in early zygotic embryogenesis and required for embryonic pattern formation (Chardin et al. 2014). This gene encodes a transcription factor protein that expressed very early in zygotic embryogenesis (Waki et al. 2011).

Somatic embryogenesis is one of the methods to produce mass orchid propagation rapidly and efficiently (Hsing et al. 2016). Somatic embryogenesis is a model system to understand physiological, biochemical, and molecular conditions that occur during the development of plant embryo (Karami et al. 2009). Somatic cells contain all of the genetic information to form a functional and complete of new plant (Yang & Zhang 2010). Somatic cells are very unique because they are able to maintain

their developmental plasticity and totipotency in a differentiated state and have ability to differentiate, reproduce, and regenerate into adult plants within appropriate culture conditions (Neelakandan & Wang 2012).

The insertion of the *AtRKD4* gene in orchids has successfully induced ectopic somatic embryo formation in leaves and protocorms (Mose 2019). The somatic embryo has been successfully formed from leaves of the transformant orchids which contain *AtRKD4* gene after induced by DEX. *P. amabilis* transformant plant used in this study carrying T-DNA that contain glucocorticoid induction system (GR). T-DNA also carries the hygromycin phosphotransferase (*HPT*) gene, which is a resistance gene to hygromycin,

The success of genetic transformation was confirmed in the genome of the *Dendrobium phalaenopsis* transformant carrying 35S::GR::*AtRKD4* gene by amplifying the AtRKD4 gene. The construction of T-DNA carrying 35S::GR::*AtRKD4* found in plasmid pTA7002. Similar research on the integration detecting of gene carrying 35S::GR::AtRKD4 in the genome of orchid plants has been successfully conducted by Mursyanti et al. (2015) on Phalaenopsis hybrid orchids "Sogo Vivien".

The formation of somatic embryos involves a process characterized by a set of morphological changes that direct to plant regeneration. The stability integration of *AtRKD4* gene in the transformant *P. amabilis* genome needs to be studied. This study aims to analyse the stability integration level of *AtRKD4* gene in *P. amabilis* transformants, to compare the morphological and anatomical characters in these orchids, and to maintain the induction of somatic embryos from transformant plants.

MATERIALS AND METHODS

Materials

This study used 18 months old - non transformant (NT) plantlets and transformant (T) plantlets carrying 3S::GR::*AtRKD4* construct obtained from our previous research (Mose 2019). The cultures were maintained under continuous light at 20°C. Total sample of 36 explants randomly were divided into 2 groups, transformant dan non transformant, put in 6 Petri dish. Each Petri dish consisted of 6 explants.

Methods

Detection of Stability Integration of *AtRKD4* Gene

Detection of stability integration of *AtRKD4* gene was carried out to confirm the presence of *AtRKD4* gene in the *P. amabilis* genome carrying T-DNA with the construct of 35S::GR::AtRKD4 (Mursyanti et al. 2015) (Figure 2). Detection was carried out by isolating plant genomic DNA, then amplifying the DNA genome with specific primers, there are *HPT* and *AtRKD4* gene. *ACTIN* gene primers were used as positive controls because this gene is a house-keeping gene that owned by most of the plant cells.

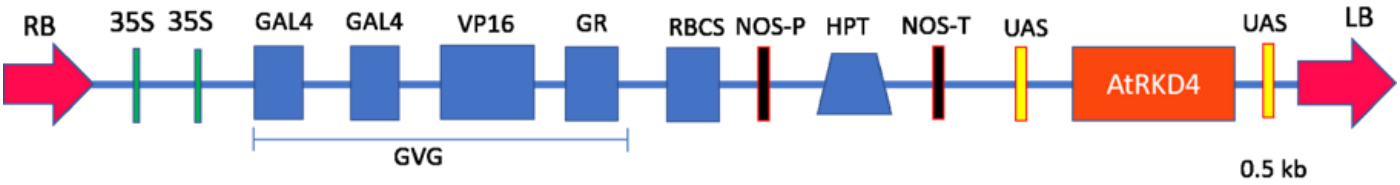


Figure 2. Construction of T-DNA carrying 35S::GR::*AtRKD4* found in plasmid pTA7002 (Mursyanti et al. 2015).

Regeneration of Positive Transformant

Plants that were positive in the detection for containing *AtRKD4* gene (transformant plants) were grown on somatic embryo induction media. The media used was *New Phalaenopsis* (NP). The part of the plant used as explants are roots and leaves. Each root and leave were cut into 3 parts as independent explants. The explants were cultured for eight weeks, maintained the growth, observed carefully whether there was growth of new cells, propagule formation or somatic embryos occurred.

Analysis of Morphological and Anatomical Structure of Somatic Embryos of *P. amabilis* Non-Transformant and Transformant

Morphological analysis was carried out by observing the orchid's growth and compare the morphology between transformant and non-transformant orchid. Morphological analysis was performed by calculating the formation of somatic embryos from *P. amabilis* orchid transformant that maintained on NP0 medium for eight weeks. The DNA genome of the somatic embryos was isolated and amplified with AtRKD4 primers to detect the stability of the integration of *AtRKD4* genes in the somatic embryos.

Anatomical preparation was conducted by using embedding method according to Sutikno (2016). Explants from somatic embryos were fixed using FAA solution (Formalin: Glacial acetic acid: 70% alcohol) and dehydrated using graded alcohol. Then explants were dealcoholized using alcohol and xylol mixture. The samples were filtered with pure paraffin. The paraffin blocks were cut using microtomes with a size of 10-20 µm. The cut explant samples were given double staining using 1% safranin and 1% fast green, then it was observed by using a light microscope to see the anatomical differences between transformant and non-transformant plants. Anatomical analysis was performed by comparing the stage of anatomical somatic embryo of transformant to non-transformant *P. amabilis*.

RESULTS AND DISCUSSION

Detection the Stability Integration of AtRKD4 Gene in *Phalaenopsis amabilis* Transformant Plants

Aoyama & Chua (1997) reported that 35S promoter of *cauliflower mosaic virus* (CaMV) is a strong promoter and expressed in almost all tissues in almost all of plant species at various stages of development. GR induction system is one of the gene expression systems that is widely used in plants. In this system, the transcription factor (TF) chimeric GVG contains of yeast GAL4 binding domain (BD), Herpes simplex VP16 activation domain (AD), and glucocorticoid receptor of mice. The presence of a GR domain determines the induction. The chimeric protein GVG in the cytoplasm is bound by a complex of 90 kDa Heat Shock Protein (HSP90) and cannot enter the nucleus without an inducer such as DEX (Picard 1993). After the addition of DEX, the chimeric protein is released and can be localized into nucleus as a transcription factor. Isolated genomic DNA from transformant plants was used as DNA template for detection stability integration of the *AtRKD4* gene in the plant genome by using PCR with specific primers of *AtRKD4* gene.

The PCR process works well with the ACTIN gene amplification in all plants, both transformant dan non transformant. The existence of 198 bp DNA fragments of *AtRKD4* gene and 545 bp DNA fragments of HPT gene indicated the integration of T-DNA in the *P. amabilis* transformants genome, and 114 bp band *ACTIN* supported the results as internal control indicating that the PCR reaction were running well

(Figure 3 lanes 3-10).

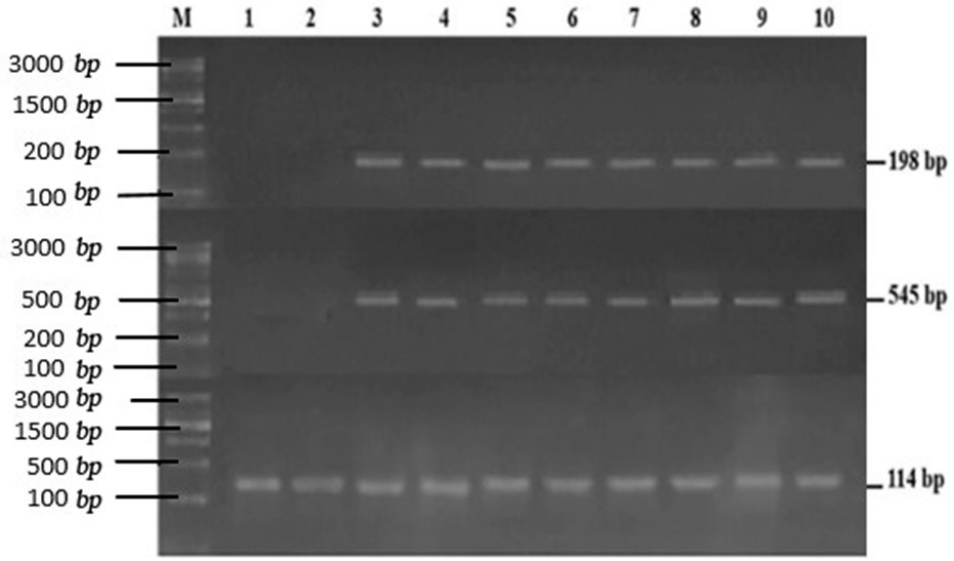


Figure 3. Detection of *AtRKD4* gene stability integration in the *Phalaenopsis amabilis* transformants genome. M: 100 bp GeneRuler markers, Line 1-2: non-transformant *P. amabilis* orchids, and Row 3-10: transformant orchid plants carrying the 35S::GR::*AtRKD4* gene. The 114 bp fragments of *ACTIN* gene were amplified from *P. amabilis* non-transformant and transformant plants carrying the *AtRKD4* gene. The 545 bp fragments of *HPT* and 198 bp fragment of *AtRKD4* gene were amplified from *P. amabilis* transformant.

The *ACTIN* as a housekeeping gene which detected almost in all plant cells was used as an internal control. (Semiarti et al. 2007). The *HPT* is the hygromycin resistant gene that used as selection marker of transformants to hygromycin antibiotics. According to Ouwerkerk et al. (2001) plants that survived on hygromycin-containing media showed that these plants were resistant to hygromycin. The 545 bp of *HPT* gene was amplified in the *P. amabilis* orchid transformant genome indicating that the plant contained a hygromycin resistant gene. The success of genetic transformation was confirmed by the amplification of the AtRKD4 in the genome of the *P. amabilis* transformant. Similar research on the detection of AtRKD4 gene integration in the orchid genome has been successfully carried out by Mursyanti et al. (2015) on the hybrid orchid Phalaenopsis "Sogo Vivien" and Setiari et al. (2018) on the *Dendrobium phalaenopsis*.

The morphological characters of *P. amabilis* orchids are shown in the Figure 4. Morphological characters can be observed and measured both qualitatively and quantitatively. Morphological observations of the samples were carried out to determine whether there were differences in the characters of the two plants. The samples have similar morphological characters. There is no significant difference between them. Observation data of *P. amabilis* orchid parameters used independent t-test with $\alpha = 0.05$ to determine the significant difference in growth between the two plants. The following Table 1. showed the parameters observed in plants aged 18 months including number, length, width of leaves and roots.

The data in the Table 1. showed that there are significant differences between non transformant and transformant plants in the growth of length and width of leaves. There is also significant differences between the number of roots of the sample. Meanwhile, in the number of leaves, length and width of roots, there were no significant difference in growth. The average growth of each parameter for 8 weeks can be seen in the following Figure 4.

Table 1. The growth of *Phalaenopsis amabilis* non-transformant and transformant for eight weeks.

Parameter	Average						
	Initial Measurement		Growth Difference		Percentage Growth (%)		Sig (2 tailed test)
	NT	T	NT	T	NT	T	
Number of leaves	4	3.75	0	0	0	0	0.730
Length of leaves (mm)	38.68	24.47	0.86	0.2	2.2	0.8	0.008
Width of leaves (mm)	9.16	8.16	0.59	0	6.5	0	0.001
Number of roots	4	6.5	0	0	0	0	0.032
Length of roots (mm)	27.05	30.34	0.56	1.87	2.1	6.2	0.642
Width of roots (mm)	3.08	3	0.63	0.05	20.5	1.5	0.230

*p < 0.05 means significant difference

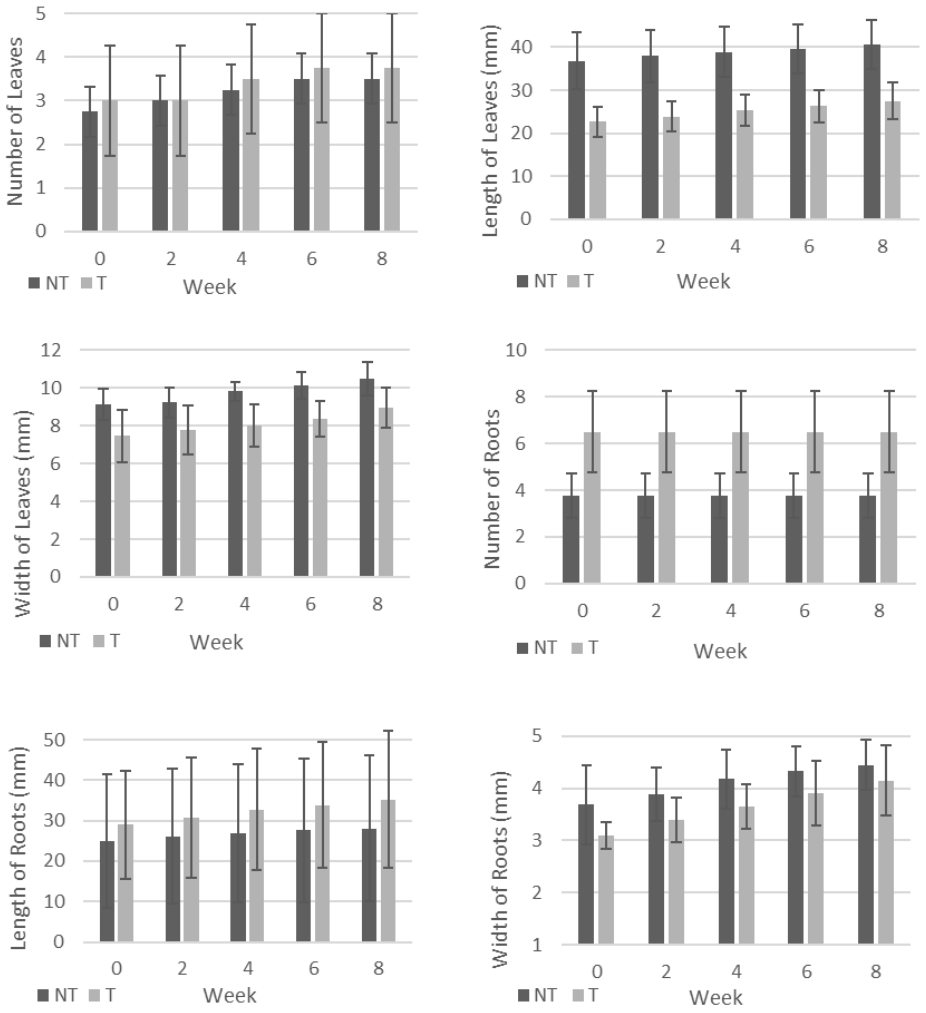


Figure 4. Growth graph of non-transformant (NT) and transformant (T) *P. amabilis* plants carrying construction 35S::GR::*ATRKD4* for 8 weeks.

From the graph, the growth rate in the number of leaves and roots of transformant plant explants was higher than non-transformant plant explants which indicates a higher regeneration ability of transformant plants (Dwiyani et al. 2015). Meanwhile, the root length of the transformant plants was also longer than the non-transformant plants. Based on the growth parameters of leaf length, leaf width, and root width, transformant plants had lower growth performance than non-transformant plants. Auxin in *in vitro* culture is able to make cells in plant tissues undergo a process of division, enlargement, and encourage root formation (Sulasiah et al. 2015). Auxin is a hormone that plays a role in the formation of cell polarity, cell enlargement and elongation, cell

asymmetric division, as well as initiation of root formation (Deo et al. 2010; Gutiérrez-Mora et al. 2012). The addition of auxin in transformant plants will activate the expression of the *AUXIN RESPONSE FACTOR* gene (*ARF7* and *ARF19*). ARF will then act as a transcription factor to activate downstream targets in the form of *LATERAL BOUNDARIES 29* (*LBD29*) and *KRYPTONITE* (*KYP/SUVH4*) genes. *LBD29* and *KYP/SUVH4* are key genes for *in vivo* development and *in vitro* dedifferentiation of *Arabidopsis* cells (Deng et al. 2015).

Induction of somatic embryos from *P. amabilis* transformant using hormone-free medium

The explants used for somatic embryo induction were from 1 year and 6-month-olds *P. amabilis* plantlets cultured on solid hormone free-NP medium for eight weeks. The emergence of propagules from wounded parts of leaf explants of *P. amabilis* transformant indicates that the formation of somatic embryogenesis has initiated. The injury in the middle of the explant aims to trigger the explant response to initiate embryogenesis. The percentage of somatic embryo (SE) emergence increases because of an injury in the middle of the explant. The injury is caused by a response from the uninjured cell near the injured cell that will activate and initiate cell proliferation (Iwase et al. 2011). Orchids transformants grown on NP0 medium formed somatic embryos directly from wounded area of the leaves and produced about 20% from the total 5 explants grown in hormone-free NP medium. Von Arnold et al. (2002) added that younger, meristematic explants had a higher ability to induce somatic embryogenesis than differentiated plant cells. Somatic embryo formation began to appear in the second week (Table 2).

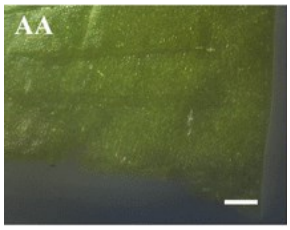
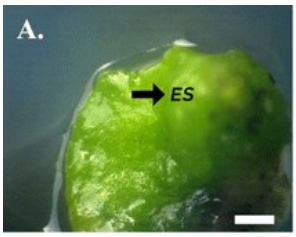
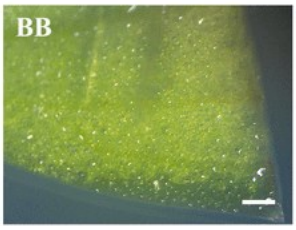
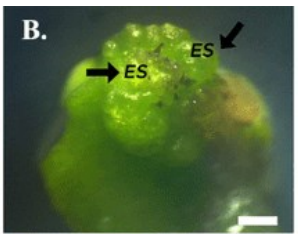
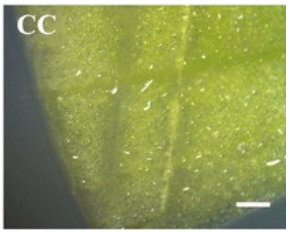
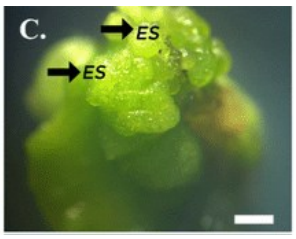
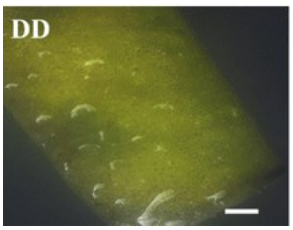
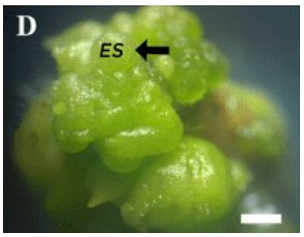
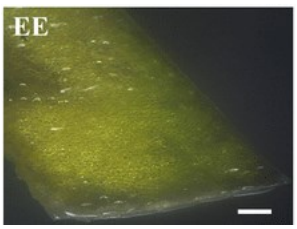
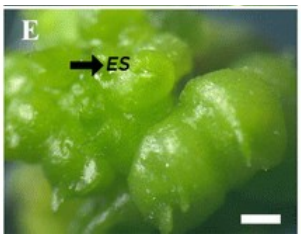
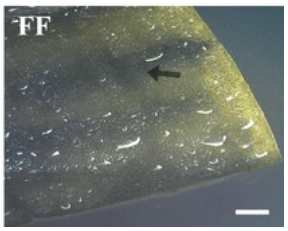
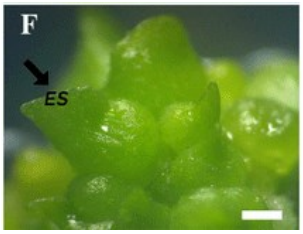
P. amabilis non-transformant did not produced somatic embryos when it cultured on NP0 medium. Although it showed the formation chlorosis of explants and then browning after being cultured for 4 weeks. Browning is caused by wounding on the explant when it is being cut. When the plant is injured, a burst of reactive oxygen occurs on the explants which affect the integrity of the membrane (Cascia et al. 2012) and result in loss of cellular compartmentalization of cells. Browning is caused by phenolic compounds in the explants (Di Guardo et al. 2013). The phenolic compounds were caused by wounding act as signaling molecules and promote increased levels of PPO (polyphenol oxidase). In this condition, PPO expression and PPO protein levels are up and cause browning to appear (Deng et al. 2015).

The histological analysis used to see the anatomical structure and origins of the somatic embryos structure that surrounding the embryo (Figure 5).

Steeves and Sussex (1989) stated that the stages of somatic embryo formation begin with the formation of proembryos in the explant epidermal tissue. The proembryo consists of small cells with a large, darker coloured nucleus. Then a globular embryo is formed which is characterized by the enlargement of the proembryo and the formation of a protoderm-like. This structure consists of a layer of cells, organized frequently and tightly. The globular embryo then grows lengthwise and forms a suspensor-like structure at its baseline. Suspensors function for nutrient transport for the embryo, especially in the beginning stages of embryogenesis. The appearance of the suspensors also indicates that the somatic embryos originates from a single cell. The basal structure of the embryo reveals the origin of an embryo. The embryo was derived from a single cell which resembles suspensor in its basal and has been determined since initial division (Horstman et al. 2017). Then, the embryo formed scutellar

notch which mark the conformation of the shoot apical meristem (SAM) and represent the early stages of coleoptile development (Alcantara et al. 2014). Then shoot meristem (sm) is formed from the scutellar notch. The embryo elongated formed a root meristem and two leaf primordia covered with coleoptile which named coleoptilar embryo. Histologically, the somatic embryo development of *P. amabilis* orchids transformant showed that the somatic embryo development pattern is the same as the somatic embryo development pattern of general monocot plants, which includes the stages of proembryo, globular embryo, scutellar embryo, and coleoptilar embryo.

Table 2. Induction of somatic embryos from transformant and non-transformant *P. amabilis* leaf explants.

Week	Non transformant *P. amabilis*	Transformant *P. amabilis*
0		
2		
3		
4		
6		
8		

Notes: (A-F) Formation of somatic embryos (SE) of leaf explants from

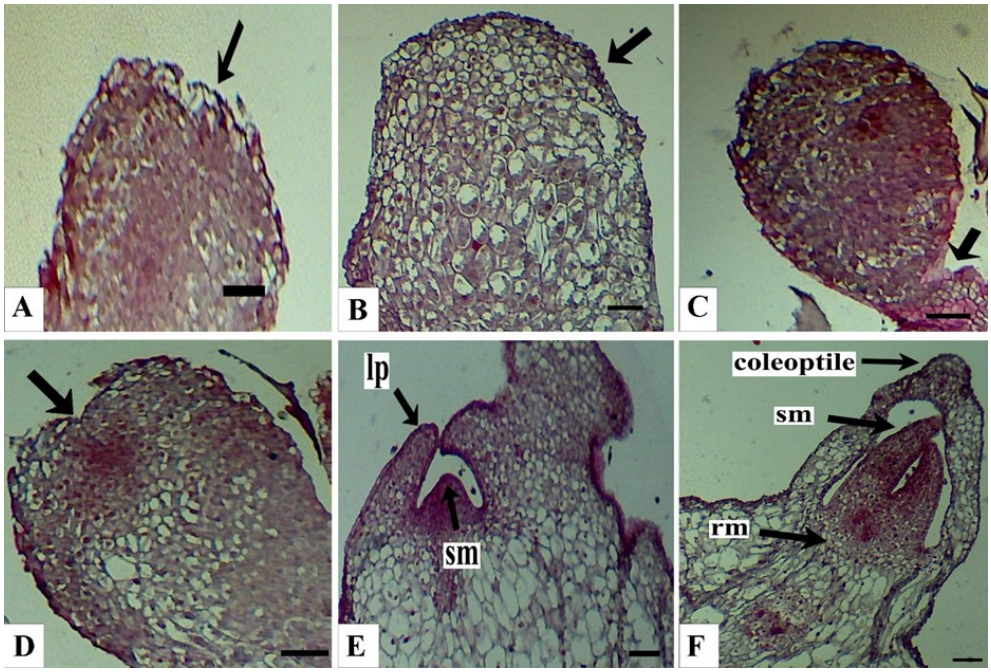


Figure 5. Anatomy of somatic embryos from the leaf explants of *P. amabilis* transformant. (A) The structure resembles to proembryo (arrow) derived from epidermal. (B) Globular embryo, enclosed by protoderm (arrow). (C) Globular embryo with an extended suspensor (arrow) on the basal region. (D) The formation of a scutellar notch in the apical (arrow). Arrows in A, B, and D indicate the position of somatic embryo initiation. Arrow in C indicates an extended suspensor on the basal region of SE. (E) The formation of shoot apical meristem (sam) and leaf primordia (lp). (F) SE contains shoot meristem (sm), leaf primordia (lp), enclosed by coleoptile, and root meristem (rm). Bars = 1000 µm.

35S::GR::*AtRKD4* transformants at the cutting site. (A) SE (arrows) formed from the surface area of the wounding section (B) early stages of somatic embryogenesis starting with formed of proembryo. (C) Proembryo developed into globular embryo. (D) The number of somatic embryos enlarged. (E) The globular embryo developed into a scutellar embryo (F) the embryo begins to form a bud (arrow). (AA-FF) Induction of SE in leaf explants from non-transformant plants. (FF) Arrows indicate browning in explants. (Bars: 5mm)

CONCLUSION

The *AtRKD4* gene is stably integrated in the *P. amabilis* transformants genome. There is no difference between the growth of *P. amabilis* transformant and non-transformant based on the number of leaves and the length and width of the roots. Somatic embryos can be induced directly from leaves of *P. amabilis* transformant cultured on hormone-free NP medium that produced about 20% somatic embryos, but not from non-transformant leaves. There is no significant difference on anatomic characters between somatic embryo of *P. amabilis* transformant and non-transformant plants.

AUTHORS CONTRIBUTION

D.S.: collecting data, preparing, analysing, and writing article from the data. N.G.A.P.: analysing data. W.M.: previous researcher of transformant plant. J.G.M: founder of T-DNA construct. E.S.: advisor of work, analysing, and critical review.

ACKNOWLEDGMENTS

This research was granted by research grants from the Ministry of Research and Technology/ National Agency for Research and Innovation for funding the Master's Thesis Research (PTM) with Contract No. 2011/UN1/DITLIT/DIT-LIT/PT/2020 given to ES and DS.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

REFERENCES

Alcantara, G.B. et al., 2014. Plant regeneration and histological study of the somatic embryogenesis of sugarcane (Saccharum spp.) cultivars RB855156 and RB72454. *Acta Scientiarum - Agronomy*, 36(1), pp.63–72. doi: 10.4025/actasciagron.v36i1.16342.

Aoyama, T. & Chua, N.H., 1997. A glucocorticoid-mediated transcriptional induction system in transgenic plants. *Plant Journal*, 11(3), pp.605–612. doi: 10.1046/j.1365-313X.1997.11030605.x.

Von Arnold, S. et al., 2002. Developmental pathways of somatic embryogenesis. *Plant Cell, Tissue and Organ Culture*, 69(3), pp.233–249. doi: 10.1023/A:1015673200621.

Cascia, G. et al., 2012. Investigation of ascorbate metabolism during inducement of storage disorders in pear. *Physiologia Plantarum*, 147 (2), pp.121–134. doi: 10.1111/j.1399-3054.2012.01641.x.

Chardin, C. et al., 2014. The plant RWP-RK transcription factors: Key regulators of nitrogen responses and of gametophyte development. *Journal of Experimental Botany*, 65(19), pp.5577–5587. doi: 10.1093/jxb/eru261.

Deng, Z. et al., 2015. Molecular cloning, expression profiles and characterization of a glutathione reductase in Hevea brasiliensis. *Plant Physiology and Biochemistry*, 96, pp.53–63. doi: 10.1016/j.plaphy.2015.07.022.

Deo, P.C. et al., 2010. Factors affecting somatic embryogenesis and transformation in modern plant breeding. *The South Pacific Journal of Natural and Applied Sciences*, 28(1), p.27. doi: 10.1071/sp10002.

Dwiyani, R. et al., 2015. Micropropagation of orchid carrying knottedl-like from arabidopsis thaliana (Knatl) gene. *Plant Tissue Culture and Biotechnology*, 25(1), pp.13–20. doi: 10.3329/ptcb.v25i1.24121.

Di Guardo, M. et al., 2013. A Multidisciplinary Approach Providing New Insight into Fruit Flesh Browning Physiology in Apple (*Malus x domestica* Borkh.). *PLoS ONE*, 8(10), e78004. doi: 10.1371/journal.pone.0078004.

Gutiérrez-Mora, A. et al., 2012. Plant Somatic Embryogenesis: Some Useful Considerations. In *Embryogenesis*. Mexico. doi: 10.5772/2143.

Horstman, A., Bemer, M. & Boutilier, K., 2017. A transcriptional view on somatic embryogenesis. *Regeneration*, 4(4), pp.201–216. doi: 10.1002/reg2.91.

Hsing, H.X. et al., 2016. Efficient and heritable transformation of Phalaenopsis orchids. *Botanical Studies*, 57(1), pp.1–12. doi: 10.1186/s40529-016-0146-6.

Iwase, A. et al., 2011. The AP2/ERF transcription factor WIND1 controls cell dedifferentiation in arabidopsis. *Current Biology*, 21(6), pp.508–514. doi: 10.1016/j.cub.2011.02.020.

Karami, O., Aghavaisi, B. & Mahmoudi Pour, A., 2009. Molecular aspects of somatic-to-embryogenic transition in plants. *Journal of Chemical Biology*, 2(4), pp.177–190. doi: 10.1007/s12154-009-0028-4.

Mondal, T., Aditya, S. & Banerjee, N., 2016. Role of plant growth regulators on asymbiotic seed germination and seedling development of Vanda coerulea Griff. ex Lindl. an endangered orchid. *Indian Journal of Fundamental and Applied Life Sciences*, 6(3), pp.36–41.

Mose, W., 2019. *Induksi Embriogenesis Somatik Tanaman Anggrek Phalaenopsis amabilis (L.) Blume dengan Zat Pengatur*. Universitas Gadjah Mada.

Mursyanti, E. et al., 2015. Induction of Somatic Embryogenesis through Overexpression of ATRKD4 Genes in Phalaenopsis "Sogo Vivien." *Indonesian Journal of Biotechnology*, 20(1), pp.42–53. doi: 10.22146/ijbiotech.15276.

Neelakandan, A.K. & Wang, K., 2012. Recent progress in the understanding of tissue culture-induced genome level changes in plants and potential applications. *Plant Cell Reports*, 31, pp.597–620. doi: 10.1007/s00299-011-1202-z.

Ouwerkerk, P.B. et al., 2001. Glucocorticoid-inducible gene expression in rice. *Planta*, 213, pp.370–378. doi: 10.1007/s004250100583.

Pangestu, F., Aziz, S.A. & Sukma, D., 2014. Karakterisasi Morfologi Anggrek Phalaenopsis Hibrida Morphological Characterization of Phalaenopsis Hybrid. *Indonesian Horticultural Journal*, 5(1), pp.29–35.

Picard, D., 1993. Steroid-binding domains for regulating the functions of heterologous proteins in cis. *Trends in Cell Biology*, 3(8), pp.278–280. doi: 10.1016/0962-8924(93)90057-8.

Rahayu, E.M. Della, 2015. Konservasi anggrek bulan (Phalaenopsis spp.) di Pusat Konservasi Tumbuhan Kebun Raya -LIPI, Bogor. *Prosiding Seminar Nasional Masyarakat Biodiversitas Indonesia*, 1(8), pp.1847–1850. doi: 10.13057/psnmbi/m010816.

Semiarti, E. et al., 2007. Agrobacterium-mediated transformation of the wild orchid species Phalaenopsis amabilis. *Plant Biotechnology*, 24(3), pp.265–272. doi: 10.5511/plantbiotechnology.24.265.

Semiarti, E. et al., 2011. Agrobacterium -Mediated Transformation of Indonesian Orchids for Micropropagation. In *Genetic Transformation*. pp. 215–240.

Semiarti, E., 2018. Orchid biotechnology for Indonesian orchids conservation and industry. In *AIP Conference Proceedings*. pp. 1–6. doi: 10.1063/1.5050118.

Setiari, N. et al., 2018. Microporopagation of Dendrobium phalaenopsis Orchid Through Overexpression of Embryo Gene AtRKD4. *Journal of Agricultural Science*, 40(2), pp.284–294. doi: 10.1017/S0021859618000163.

Shekarriz, P. et al., 2014. Coconut Water and Peptone Improve Seed Germination and Protocorm Like Body Formation of Hybrid Phalaenopsis. *Agriculture Science Developments*, 3(10), pp.317–322.

Steeves, T.A. & Sussex, I.M., 1989. *Patterns in Plant Development* 2nd ed., New York: Cambridge University Press. doi: 10.2307/2484650.

Sulasiah, A., Tumilisar, C. & Lestari, T., 2015. Pengaruh Pemberian Jenis Dan Konsentrasi Auksin Terhadap Induksi Perakaran Pada Tunas *Dendrobium Sp* Secara in Vitro The Effect of Types and Concentrations of Auxin on Rooting Induction on Dendrobium sp Bud in In Vitro. *Bioma*, 11(1), pp.59–66.

Sutikno, 2016. *Bahan Ajar Mikroteknik Tumbuhan*, Yogyakarta: Fakultas Biologi Universitas Gadjah Mada.

Waki, T. et al., 2011. The arabidopsis RWP-RK protein RKD4 triggers gene expression and pattern formation in early embryogenesis. *Current Biology*, 21(15), pp.1277–1281. doi: 10.1016/j.cub.2011.07.001.

Yang, X. & Zhang, X., 2010. Regulation of somatic embryogenesis in higher plants. *Critical Reviews in Plant Sciences*, 29(1), pp.36–57. doi: 10.1080/07352680903436291.