



Micropropagation of *Dendrobium phalaenopsis* Orchid Through Overexpression of Embryo Gene *AtRKD4*

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ABSTRACT

To increase the efficiency of crop production from *Dendrobium phalaenopsis* orchids, mass propagation has been performed by inducing somatic embryogenesis through Agrobacterium-mediated transformation of the Arabidopsis embryo gene *AtRKD4* into orchid protocorm (developing orchid embryo). The three-week-old protocorms of *D. phalaenopsis* were genetically transformed with T-DNA carrying 35S :: GAL4 :: *AtRKD4* :: GR through *A. tumefaciens* strain EHA 105. The cultures were maintained in VW medium with 10 mg L⁻¹ Hygromycin. Due to the existence of glucocorticoid response element (GR) in the T-DNA construct, the transformed protocorms were transferred into VW medium with the addition of 15 µM Dexamethasone in 6 weeks after transformation to activate the transgene. A total of 12% protocorms has been confirmed for Hyg⁺ by using PCR. The expression of embryo gene *AtRKD4* was confirmed by cDNA analysis using *AtRKD4* specific primers and Actin primers as a positive control experiment. The expression level of *AtRKD4* in 2.5-month-old *D. phalaenopsis* transformant shoots was 7 times higher than non-transformant plants, and increased to 86 times higher in 8-months, that much higher than that of non-transformant. These results provide an improved method for genetic transformation of *D. Phalaenopsis* and will (eventually) increase production efficiency in the future.

INTRODUCTION

Dendrobium phalaenopsis (Indonesian: *anggrek larat*) is a native orchid species of Indonesia (Ivakkdalam & Pugesehan, 2016), which is originated from Larat Island, the province of Maluku. *D. phalaenopsis* is one of 12 protected orchid species in Indonesia under the government regulation No. 7 the year of 1999 due to the scarcity of this orchid in nature (Ivakkdalam & Pugesehan, 2016). *D. phalaenopsis* orchids are widely cultivated by orchid collectors and preferred after *Phalaenopsis amabilis* because they have round flowers similar to *Phalaenopsis*, though the number of flowers in one inflorescence is more than *Phalaenopsis*, so it is often used as a parental species for hybrid orchids (Widiastoety, Solvia, & Soedarjo, 2010). Due to a high value of flower for commercial use,

people interest to collect this orchid from their natural habitat in the forest to be cultivated at home or nursery. This is considered as the reason causing the scarcity of *D. phalaenopsis* orchids in their natural habitat, decreasing its population and endangering its existence. Therefore an effort for the conservation of this *D. phalaenopsis* orchid should be conducted.

Orchid germination from seeds is difficult, as the seeds lack of endosperm and contain poorly differentiated embryos, rendering them ineffective for producing large quantities of seedlings (Mondal, Aditya, & Banerjee, 2016; Shekarri, Kafi, Deilamy, & Mirmasoumi, 2014). Tissue culture (*in vitro*) techniques are very useful for orchid cultivation, and facilitate micropropagation and conservation of rare orchids (Semiarti et al., 2010). *In vitro* seed

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germination of rare species *Vanda tricolor* Lindl. var. *suavis* from Bali obtained the highest number of plantlets by using NP (New Phalaenopsis) medium supplemented with 100 g L⁻¹ tomato juice (Dwiyani, Yuswanti, Darmawati, Suada, & Mayadewi, 2015). It is promising that the *in vitro* cultivation (micropropagation) utilizing somatic embryogenesis process can produce plantlets that morphologically and genetically uniform. Meanwhile, Jainol & Gansau (2017) got the highest percentage (96 %) of protocorm like bodies (PLB) from leaf tip explants of Bornean endemic orchid, *Dimorphorchis lowii* is cultured on Murashige and Skoog medium supplemented with Thidiazuron and Naphtalene acetic acid. PLB is special term for somatic embryo in orchids and somatic embryogenesis is the process of embryo formation derived from somatic cells (Lee, Hsu, & Yeung, 2013). PLBs are formed from parts of plants that are not normally involved in embryo formation, such as leaf tissue, roots, and stems. Endosperms and seed coats are not found in somatic embryos. Somatic embryogenesis is an appropriate system for plant propagation both for micropropagation and for producing plant material as a target of genetic transformation to obtain transgenic crops. The process of somatic embryogenesis enables the producing embryos and plants from undifferentiated somatic cells (Elhiti, Stasolla, & Wang, 2013).

In plants, there is genetic regulation of somatic embryogenesis. One of the genes that induce somatic cell changes into embryogenic cells is the *RKD4* gene. *RKD4* gene is able to induce the formation of somatic embryos. This gene encodes a protein that has RWP-RK motif (amino-acid sequence pattern consists of arginine (R), tryptophan (W), proline (P), arginine (R) and Lysine (K) amino acid) in a very early stage of embryo initiation in *Arabidopsis* (Chardin, Girin, Roudier, Meyer, & Krapp, 2014). *RKD4* protein is abnormally changed the pattern of embryo gene expression in seedling. Protein *RKD4* (RWP-RK Domain Containing 4) is an RWP-RK group of plants specific transcription factors. There are similarities in the structure of these transcription factors ranging from unicellular algae to high-level organisms, especially in the form of basic leucine zippers and basic helix proteins involved in DNA binding and transcriptional activity regulation (Waki, Hiki, Watanabe, Hashimoto, & Nakajima, 2011).

In *Chlamydomonas*, *RKD4* plays a role in initiating the first division of zygote to form embryos,

either directly or indirectly, associated with the availability of nitrogen (Chardin, Girin, Roudier, Meyer, & Krapp, 2014). The *RKD4* gene will be expressed in the early embryonic development process for the first division pattern formation on the zygote. If *RKD4* does not work, it will disrupted elongation and division process of the zygote. Specific genes that play a role in early embryonic development will be active after *RKD4* is expressed. Expression of *RKD4* is limited to proper embryos and suspensors. *RKD4* transcription results are not widely detected in postembryonic tissues (Waki, Hiki, Watanabe, Hashimoto, & Nakajima, 2011).

Waki, Hiki, Watanabe, Hashimoto, & Nakajima (2011) inserted the *indRKD4ox* gene into *Arabidopsis* and the overexpression of this gene was able to induce somatic embryo formation in transformant *A. thaliana* roots after the gene was induced with synthetic steroid hormone, Dexamethasone (Dex). Mursyanti, Purwantoro, Moeljopawiro, & Semiarti (2015) successfully inserted the *AtRKD4* gene in protocorm *Phalaenopsis* "Sogo Vivien", which resulted in the leaves of transformant orchids being induced by 15 µM Dex in order to form somatic embryos.

Therefore, in this study, *A. tumefaciens*-mediated genetic transformation has been performed on *D. phalaenopsis* protocorm with insertion of T-DNA 35S :: GAL4 :: AtRKD4 :: GR construct for mass propagation of the orchid. Conventional propagation is less efficient because of the time consuming, and the low rate of reproducibility.

MATERIALS AND METHODS

Plant Materials and Culture Condition

This study was conducted at Laboratory of Biotechnology, Faculty of Biology, Universitas Gadjah Mada (UGM), Yogyakarta from 2012-2017. The plant materials used in this study was protocorms (specific term of growing orchid embryos) of *Dendrobium phalaenopsis*. Protocorms were developed by sowing orchid seeds *in vitro* on Vacin and Went (VW) medium as described by Semiarti et al. (2010) supplemented with 2 g L⁻¹ peptone. Orchid seeds were obtained from 2.5-month-old orchid pods after self-pollination.

Morphological Analysis and Protocorm Anatomy

Protocorm growth and development were observed weekly for eight weeks to determine the embryonic growth phase of seeds based on Semiarti

et al. (2010). The observations were performed using a binocular stereo microscope (Eschenbach Binoculars DM150DB, Germany) and documented using a digital camera (Canon Ixus 132 HD, Japan).

Histological Preparation of Protocorm Samples

For histological preparation, seeds and protocorms (0-8 weeks after planting) were treated using paraffin method following the method of Hoang, Kane, Radcliffe, Zettler, & Richardson (2017). The anatomy slides were observed by a light microscope.

The Plasmid Vector and the Bacterial Strain

The pTA7002 plasmid with the T-DNA construct of 35S::GAL4::*AtRKD4*::GR (obtained from Dr. Jose Gutierrez-Marcos, University of Warwick, UK; Fig.1) in *A. tumefaciens* strain EHA105 was used in this research. The T-DNA is composed of multiple strong promoters 35S, GVG, GAL 4 and VP16 with inducible Glucocorticoid response element (GRE) for selective induction system with glucocorticoid hormone (Dexamethasone). For a selection of transformant, 10 mg L⁻¹ Hygromycin antibiotic was added in VW medium following the method described by Mursyanti, Purwantoro, Moeljopawiro, & Semiarti (2015).

Transformation and Regeneration of Transformants

The transformation method follows the protocol described by Semiarti et al. (2010) with a modification in the kind of medium used in this work was Vacin and Went (VW), and the incorporation of 25 mg L⁻¹ of acetosyringone for cocultivation. Then protocorms were thoroughly washed with sterile aquades, followed by VW liquid medium added with 25 mg L⁻¹ meropenem (Sanbe) two times then shaken at 125 rpm for two nights to eliminate the growth of *A. tumefaciens*.

The medium was renewed every day. On day-3, protocorms were washed with sterile distilled water, then soaked in VW liquid medium + 25 mg L⁻¹ meropenem for 30 minutes in accordance with the

procedure of Mursyanti, Purwantoro, Moeljopawiro, & Semiarti (2015). Finally, protocorms were air dried for 30 minutes, cultured on a solid VW medium supplemented with 25 mg L⁻¹ meropenem for two weeks following the method of Mursyanti, Purwantoro, Moeljopawiro, & Semiarti (2015). Protocorms were then transferred onto new selection VW medium, + 25 mg L⁻¹ meropenem and 10 mg L⁻¹ hygromycin, for six weeks to avoid the growth of *Agrobacterium*. The culture was incubated at 25 °C. Developing shoots were analyzed by PCR with *AtRKD4* and *HPT* primers to confirm the integration of transgenes into the orchid genome and calculated the frequency of transformation.

Polymerase Chain Reaction (PCR) Analysis of Transformants

Detection of *AtRKD4* and *HPT* transgenes in the *D. phalaenopsis* genome was performed by PCR method with specific primers. Five non-transformant and 28 putative transformant DNA samples were analyzed by direct PCR using KAPA3G Plant PCR Kit (KapaBiosystem).

The PCR process used *AtRKD4* and *HPT* genes primers that amplified 382 bp DNA band and 810 bp DNA bands of *AtRKD4* and *HPT*, respectively. Amplification of 1000 bp chloroplast DNA fragment *trnLF* cpDNA was used as an internal control as described by Gardiner, Kocyan, Motes, Roberts, & Emerson (2013). The sequences of primers will be delivered upon request of DNA using primers. The PCR reaction was as follows: an initial denaturation at 95 °C for 5 minutes, denaturation at 95 °C for 30 seconds, annealing at 58 °C (*AtRKD4* and *HPT*) and 51 °C (*trnLF*) for 30 seconds, extension (polymerization) at 72 °C for 30 seconds, final extension phase at 72 °C for 10 minutes and cooling at 4 °C for 10 minutes. The PCR process was performed in 35 cycles. The PCR product was separated on 1.4 % agarose gel in TBE 0.5X buffer at 50 volts for 50 min and visualized under UV transilluminator.

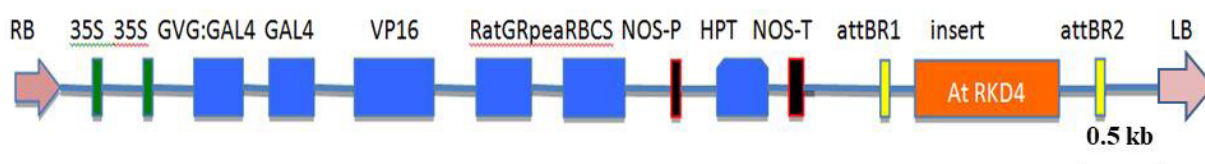
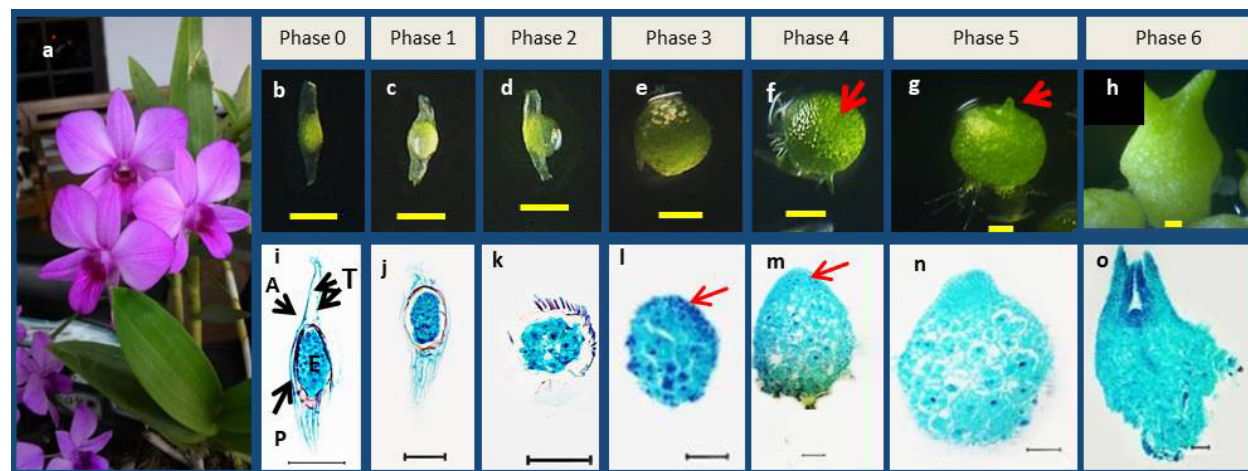


Fig. 1. The structure of T-DNA carrying 35S::GAL4::*AtRKD4*::GR in plasmid pTA7002/EHA 105



Remarks: Arrows point to SAM (Shoot Apical Meristem); LP = Leaf Primordium; T = Testa; E = Embryo (consist of parenchymal cells); A = Anterior; P = Posterior. Bars: 0.1 mm in b; 0.5 mm in c-h; 100 μ m in i-o.

Fig. 2. Flower and the development of embryo of *Dendrobium phalaenopsis* orchid. a. flower morphology, b-h. Morphology of embryo development, i-o. Anatomy of embryo development. b,i. Phase 0; c,j. Phase 1; d,k. Phase 2; e,l. Phase 3; f,m. Phase 4; g,n. Phase 5; h,o. Phase 6.

Induction of Embryo Somatic and Analysis of *AtRKD4* Gene Expression

Protocorms grown on the selection medium were dripped in 15 μ M Dex, then cultured in a VW medium plus 15 μ M Dex for 1 week. The protocorms were then cultured onto hormone-free VW medium. The formation of somatic embryos in protocorms were observed every week with a stereo microscope (Eschenbach Binoculars, Germany) and photographed with a digital camera (Canon Ixus 132 HD, Japan).

Analysis of 35S::GR::*AtRKD4* Gene Expression on *D. phalaenopsis*

AtRKD4 gene expression was analyzed by isolating total RNA from leaves of four non transformant and ten transformant candidates using Total RNA Mini Kit Plant (Geneaid) and cDNA synthesized from mRNA using Thermo ScientificTM RevertAidTM First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., US). The *AtRKD4*, HPT, and Actin primers were used for cDNA amplification with PCR method under amplification reaction the same as described above.

RESULTS AND DISCUSSION

Embryo Development of *Dendrobium phalaenopsis*

Morphological observation of seeds (Fig. 2a-2h) showed that the development of *D. phalaenopsis*

orchid seeds started by embryo's swelling and changing of color from yellow to green during germination (phase 1). In phase 2, the embryo became more swollen and enlarged but it was still inside the testa (seed coat). The enlarged embryo bursted out of the testa and was globular in shape. At this phase, the embryo was called a protocorm (phase 3). Following this, the protocorm formed a bipolar structure. On the top surface, protocorms showed flat shapes and developed shoot apical meristem (phase 4). In phase 5, a shoot primordia on the shoot meristem was developed. In phase 6, the shoot primordia was rising and then growing into shoots.

The process of orchid germination *D. phalaenopsis* consists of 6 stages. This corresponds to the process of seed germination in the *Phalaenopsis amabilis* orchid which also includes six stages of embryonic development. The color of the embryo changes from yellow to green in the second stage. In the third stage, the embryo forms a bipolar structure. In the fourth stage, leaf primordia begins to form and in the fifth stage has formed the first two leaves. In the sixth stage, protocorms have formed three leaves (Semiarti et al., 2010).

Anatomical observation of seed embryos (Fig. 2i-2o) showed that the orchid seed embryos in phase 0 and phase 1 were composed of a cell mass with a strong colored cell in nucleus which is stronger than the color in the other part of the cell and

enclosed by a visible seed coat (Fig. 2i and 2j). The next phase, embryo formed round-shapes, where the testa was broken (phase 2), and the embryonic cells were not yet polarized (Fig. 2k). The cells of the embryo which burst out of the testa began to enlarge and the polarization of the embryo became more apparent (phase 3). The cells at the anterior part of the embryo were smaller in size (15 %) than those at the posterior end (85 %). The anterior cells seemed like meristematic areas, while the posterior cells were parenchymatic areas (Fig. 2l). In phase 4, the anterior cells were more compact at the anterior end of an embryo and formed the dome of the shoot apical meristem. The meristematic dome grew (Fig. 2m) and formed a cone shape (Fig. 2n) in phase 5. It consisted of highly stained meristematic cells with a large nucleus. In phase 6, the meristematic dome elongated rapidly and formed a leaf primordia and then formed the shoot (Fig. 2o).

In addition to morphological observations, the development of *D. phalaenopsis* embryonic anatomy was also observed. The embryo consists of undifferentiated and closed cellular masses by a testa made up of dead cells (without protoplasts). Polarized cells form a meristematic region called the shoot apical meristem (SAM). In SAM, leaf primordia form and grow into shoots. This is in accordance with Yeung (2017) that the oval-shaped orchid seed embryo occupies the center of the seed and consists of undifferentiated cell mass. Embryos are covered with transparent testas consisting of dead cell sheets as a protector. Testa will degenerate at the time of seed germination. At this stage, the anterior-posterior axis is obvious. The anterior end is located near a chalaza called a chalazal, plumular, or apical edge. The posterior tip is located near a micropyle called a micropylar, radicular or basal end. Cells at the posterior end are four times larger (by volume) than cells on the anterior edge. The apical poles rapidly change their shape to cone following several

cell divisions, mostly anticlinal. the development of late-stage embryo apical part quickly turns into a cone shape due to anticycle division in meristem cells. Cells in the meristem region have a large nucleus. Meanwhile, large parenchymal cells are filled with starch. Then the shoot primordia is formed on the meristem ends and form buds.

Transformation Efficiency and Shoot Production from Putative Transformant of *D. phalaenopsis* Protocorms

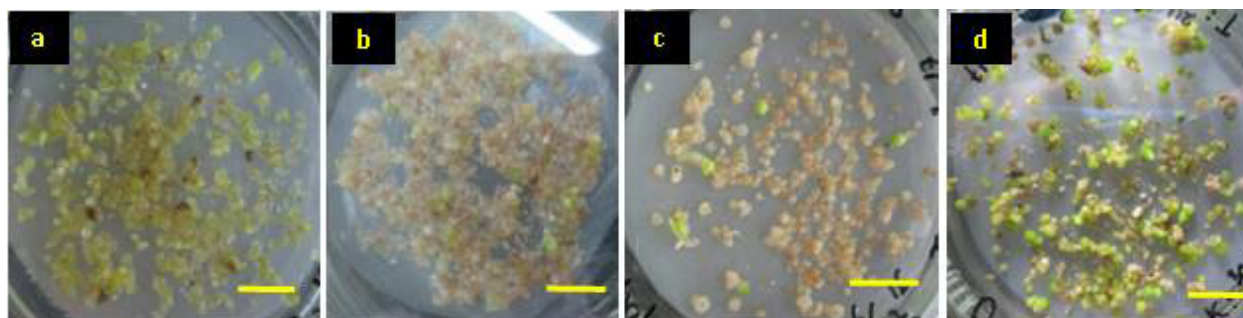
The percentage of *D. phalaenopsis* putative transformant was examined six weeks after transformation, it showed that resistance to the 10 mg L⁻¹ hygromycin antibiotic was 12.10 % (Table 1, Fig. 3). There were 126 protocorms from 1041 examined protocorms that able to grow in the selection medium containing hygromycin antibiotics. The transformant protocorm candidates were expected to contain hygromycin (*HPT*) gene which was located in the 35S::GAL4::AtRKD4::GR T-DNA construct. The percentage of hygromycin resistance of non-transformant protocorms was 0.75 % (Table 1, Fig. 3), whereas in the transformed protocorms with plasmid pTA7002 (vector only), the percentage was 3.16 % (Table 1, Fig. 3).

Molecular Analyses of Transformants

D. phalaenopsis protocorms that survived on selection medium were subsequently subcultured to VW medium for regeneration to obtain the plantlet and to detect the integration of *AtRKD4* and *HPT* genes. Transgene detection in small samples (crude sample) of leaves from non transformant and transformant candidates was done by amplifying a target DNA fragment from *AtRKD4* and *HPT* genes. DNA amplification was conducted from crude samples rather than isolated DNA because the size of plantlets was still very small, and was further observed to determine its growth and development manner.

Table 1. The frequency of transformation of 35S:: GAL4::AtRKD4::GR gene in *D. phalaenopsis* orchid protocorms examined at six weeks after transformation

Genotype	Experiment	No of protocorm examined	Number of positive protocorm on selective medium	Frequency of transformation (%)
NT		133	1	0.75
TV		475	15	3.16
T35S::GAL4::AtRKD4::GR	1	431	54	15.20
	2	404	43	10.64
	3	206	29	14.08
Total		1041	126	12.10 (average)



Remarks: Bars = 1 cm

Fig. 3. The appearance of protocorms on selection medium after transformation. a. Non transformant protocorms grown on hygromycin-free medium, b. Non transformant protocorm grown on hygromycin selection medium; c. pTA7002 putative transformant protocorms showed green color; d. 35S::GAL4::*AtRKD4*::GR putative transformant protocorms showed green color grown on hygromycin selection medium

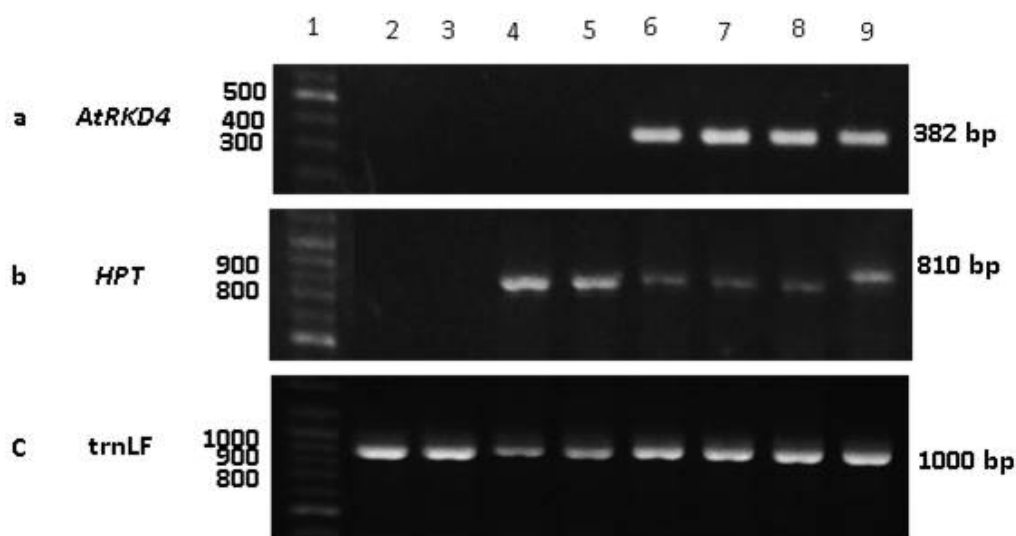


Fig. 4. Detection of *AtRKD4* and *HPT* genes integration in the genome of *D. phalaenopsis* transformants. A. 382 bp DNA fragment *AtRKD4* gene, B. 810 bp DNA fragment of *HPT* gene, C. 1000 bp DNA interspace cpDNA fragment of trnLF, Lane 1. 100 bp DNA ladder as size marker; Lane 2 and 3. Non-transformant plants, Lane 4 and 5. pTA7002 Vector putative transformant; Lane 6-9. Putative transformant

The results showed that the integration of *AtRKD4* (382 bp) and *HPT* (810 bp) genes were detected in the genome of *D. phalaenopsis* transformant candidates (Fig. 4a and 4b). It was confirmed with the amplification of trnL-F (1000 bp) as an internal control for PCR reaction (Fig. 4c). The detected *AtRKD4* gene in the orchid genome has shown that the *AtRKD4* gene was successfully integrated into the orchid genome, and this is proven by the ability of transformants to form somatic embryos after induction with the Dexamethasone glucocorticoid hormone. Detection of the *HPT* gene in the orchid genome suggested that the

plantlets of transformant candidates contained an integrated gene that was resistant to hygromycin antibiotics. Therefore the plantlets can grow well on a hygromycin containing medium. Fragment of trnL-F non coding chloroplast DNA can be amplified in both non transformant and transformant genomic DNAs, an evidence that PCR reaction works well and can be used as a positive control to determine the PCR results. This is consistent with reports from Gardiner, Kocyan, Motes, Roberts, & Emerson (2013) which have stated that trnL-F is very good to be used as molecular marker.

In the non-transformant plant, *AtRKD4* gene was not detected (Fig. 4a and 4b), only trnL-F fragment was detected (Fig. 4c), while in the candidate pTA7002-transformant plants (inserted by vector only without *AtRKD4* transgene), the fragment of *HPT* genes were detected (Fig. 4b), supported by the evidence of the trnL-F fragment in all samples (Fig. 4c).

Protocorms *D. phalaenopsis* was cultivated for three days to optimize the integration of T-DNA into the protocorms genome. According to Semiarti et al. (2010), cocultivation for more than three days will lead to overgrowth of *A. tumefaciens*. After cocultivation and elimination of *A. tumefaciens*, using *HPT* primers the detection of putative transformant was performed by PCR to confirm whether the protocorms contained the *HPT* gene or not (non-transformant that not containing the *HPT* gene).

Hygromycin phosphotransferase is an enzyme produced by *Streptomyces hygrosopicus* bacteria that is resistant to hygromycin, therefore it can be used as selection marker to obtain positive transformant. Bunnag & Pilahome (2012) suggest that selective agents are important for transformant selection process because it can prevent the growth of non-transformant plant. The *HPT* gene is one of the marker genes of choice, and the other is the *NPT* gene (Neo Phosphotransferase). According to Kaur & Singh (2010), the *HPT* or *NPT* gene encodes

a phosphotransferase enzyme that can lead to tolerance to aminoglycoside antibiotics such as hygromycin, kanamycin, and neomycin. Hygromycin is an aminoglycoside antibiotic that can kill plant cells by inhibiting transcription and translation. Wright (2010) explained that one of the mechanisms of resistance is to destroy enzymes. Increasing hygromycin concentration will result in higher toxicity of hygromycin in the target cells, therefore, too high concentration of hygromycin will cause the cell death, even in transgenic plants. This suggests that the appropriate concentration of Hygromycin is necessary for the selection of transformant and transgenic. Because it can not build a resistance system that will inhibit the regeneration of plants and cause death in plants.

Analysis the Expression of *AtRKD4* Transgene in Transformant Plantlet

Protocorms that successfully form somatic embryos or multi-shoot plantlets were analyzed in the level of mRNA to detect the expression of *AtRKD4* transgene in transformant plants. DNA gel electrophoresis (Fig. 5A) showed the detection of an 810 bp DNA fragment corresponds to the *HPT* gene in the sample of putative pTA7002- transformants (vector only, Lane 3) and in the samples of putative transformants with *AtRKD4* T#2 -T# 5 (Fig. 5a, Lanes 5-8), but it was not identified in the non-transformant samples (Lane 2).

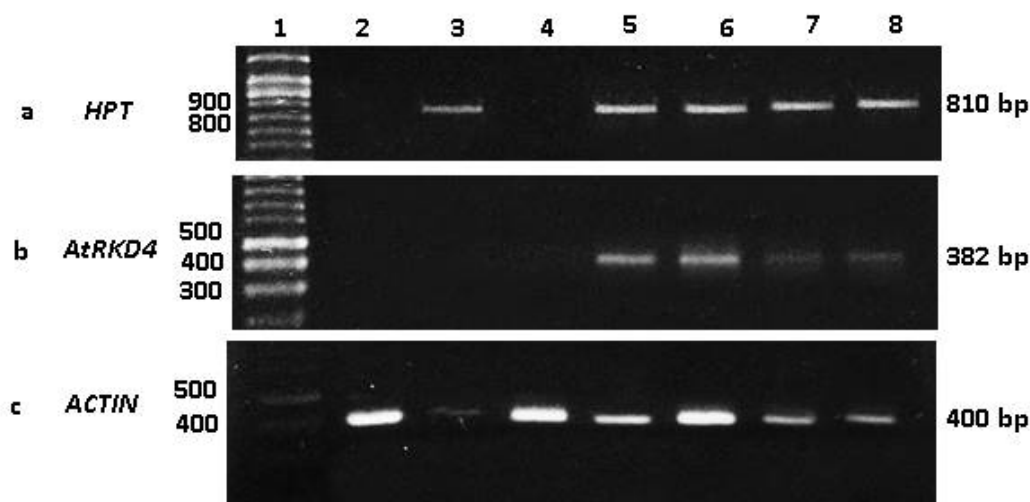


Fig. 5. Expression of the 35S::GAL4::*AtRKD4*::GR gene in putative transgenic *D. phalaenopsis* plants. Reverse transcription-PCR analysis of transcripts of the 35S::GAL4::*AtRKD4*::GR gene using *HPT* primers (A), *AtRKD4* primers (B) or *ACTIN* primers in a wild-type *D. phalaenopsis* plant (2), in putative *D. phalaenopsis* transformants with the pTA7002 vector (3), or transformant plantlets (4-8). The product of PCR control was amplified with a specific primer for *ACTIN* gene transcripts (C)

Table 2. Number of shoots formed in putative transformed protocorms two months after treatment with Dex

	Experiment	Number of protocorms examined	Number of growing protocorms	Number of protocorms producing shoot	Number of shoots produced
T35S::GAL4::AtRKD4::GR	1	669	100	44	264
	2	330	40	29	198
	3	483	70	37	325
	4	441	40	26	167
Total		1923	250	136	954

The expression of *AtRKD4* gene was demonstrated by detection of *AtRKD4* cDNA in transformant plant samples (determined by RT-PCR), and detected as 382 bp band from transformant plant T # 2 -T # 5 (Fig. 5b, lane 5-8), but undetected in transformant plants treated with the pTA7002 vector only without the *AtRKD4* gene (Lane 3) and in the non-transformant plant sample (Lane 2) (Fig. 5b). The sample in Lane 4 was a putative transformant plant sample but does not express the *AtRKD4* or *HPT* genes. A 400 bp band corresponding to *ACTIN* gene transcription ("house-keeping" positive control) appeared on all samples both transformant and non-transformant samples (Fig. 5c). This indicated that the *ACTIN* gene was expressed in all plant samples, both transformants and non-transformants. The *AtRKD4* and *HPT* genes were expressed only in transformant plants, and were not expressed in non-transformant plants.

The *AtRKD4* gene was induced or activated by glucocorticoid hormone (Dexamethasone, Dex). Therefore, to induce somatic embryo formation, transformed protocorms were treated with medium containing Dex. Two months after treated with Dex, only 250 protocorms survived from 1923 examined protocorms (13 %) (Table 2). Out of the 250 protocorms, there were only 136 protocorms produced shoots (54.4 %). Nine hundred fifty four shoots in total were regenerated from 136 protocorms, it indicated that in average, each protocorm produced seven shoots. The DNA analyses confirmed that these transformants shoots positively contained of *AtRKD4* transgene in its genome.

According to Shires, Florez, Lai, & Curtis (2017) dexamethasone is a glucocorticoid hormone that is required to induce somatic embryo formation correspond to the activity of HSP90 and activator binding to a target promoter containing some TF-GR receptors (Transcription Factor-Glucocorticoid Receptor) site in upstream part of short DNA fragments encoding the TATA box. HSP is a small

heat shock protein that also transiently accumulates during somatic embryo maturation and germination in oak and involved in the dedifferentiation of explant cells during somatic embryogenesis (Elhiti, Stasolla, & Wang, 2013). After attaching the activator to the target promoter, the target promoter transcription can be induced (Shires, Florez, Lai, & Curtis, 2017). In this study, the transcription factor designated GVG, also contains yeast transcription factor GAL4, Herpes simplex protein 16 (VP16) virus and Glucocorticoid Receptor domain.

Transformant and Non-transformant Protocorms Development

Non-transformant and putative transformants protocorms had a slight difference on developmental process during shoot formation. The development of non-transformant protocorms follows the following process of protocorms forming the first leaf primordia on higher peripheral parts of SAM (Shoot Apical Meristem; Fig. 6). Next they form the second leaf (Fig. 6d) and then the root (Fig. 6e). Non-transformant protocorms form single shoots (Fig. 6e). Prospective protocorms form somatic embryos, with the formation of two protocorm like bodies (PLBs) (Fig. 6f), then form three PLBs (Fig. 6g), further form five PLBs (Fig. 6h), then form seven PLBs (Fig. 6i). They subsequently form leaf primordia to form buds out of each PLB, and finally form a multishoot (7 buds) (Fig. 6j).

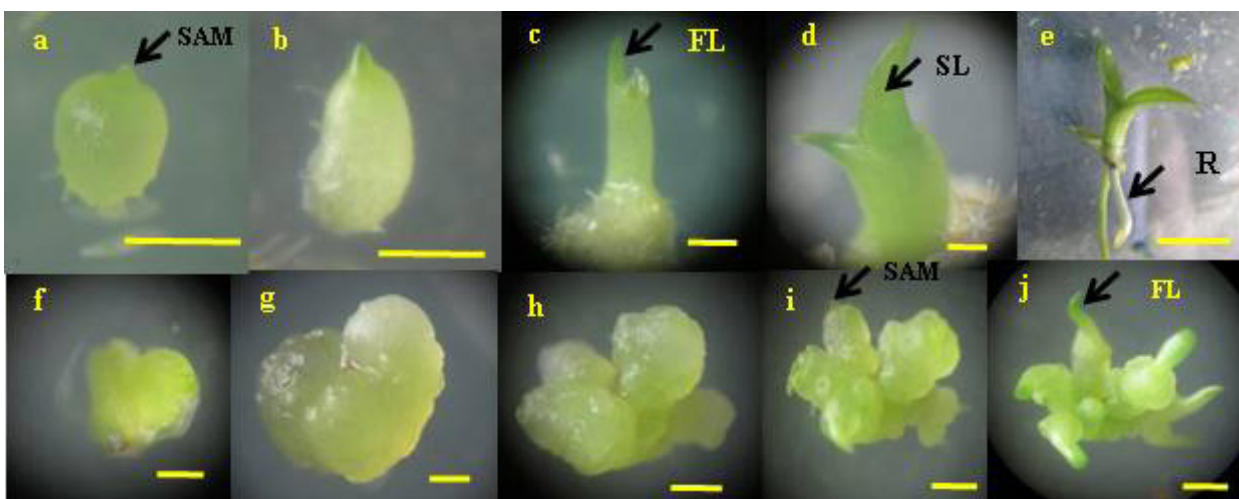
Phenotypic Analysis of 8 Months Regenerated Transformant Plants

Multishoots were successfully formed in transformant plants. Fig. 7 showed that eight months old transformant plants cultured in regeneration medium formed a shoot in an average of 139.17 per plant. In contrast, non-transformant plants produced an average only two shoots per plant. This suggested that transformant plants formed a much higher number of shoots per plant than that of non-transformant plants. This phenomenon is similar to

that shown by Jainol & Gansau (2017), that because of the activity of the transgene *AtRKD4*, every cell in protocorm can undergo a change of self-identity, there is dedifferentiation occurred to form somatic embryos, and each embryo then grows into shoot.

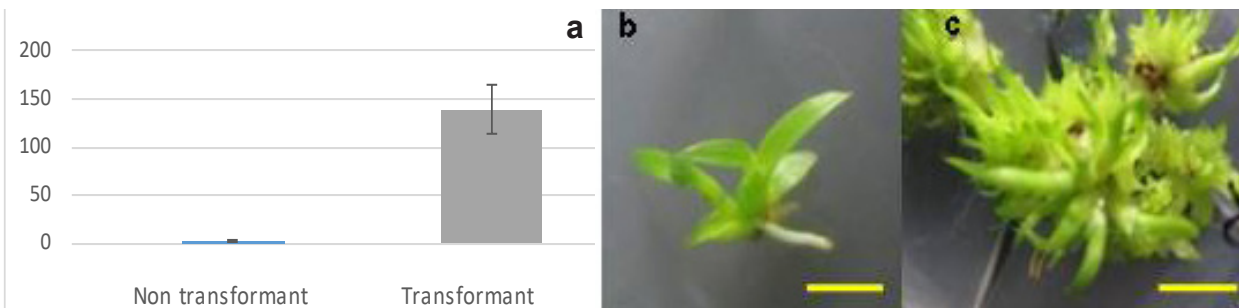
The high production of shoots in orchid transformant was consistent to the result of ectopic expression of *AtRKD4* in *Arabidopsis* (Chardin, Girin, Roudier, Meyer, & Krapp, 2014), that causes some plant growth distortions including abnormal tissue proliferation. This data indicates that the *RKD4* gene in *Arabidopsis* is necessary for embryonic pattern formation. According to Waki, Hiki, Watanabe,

Hashimoto, & Nakajima (2011), the *RKD4* gene encodes proteins as a transcription factor expressed very early in the process of zygotic embryogenesis. Meanwhile, in *Arabidopsis*, *RKD4* is highly expressed in the ovum, especially in the tissues of reproductive organs. *RKD4* overexpression will induce a somatic embryogenesis process, irrespective of the effects of exogenous growth regulators, whereas loss of *RKD4* expression will interfere with the formation of auxin assisted embryo axis and the initiation of organ primordials.



Remarks: Bar = 0.5 cm

Fig. 6. Development of non-transformant (a - e) and putative transformant (f - j) *D. phalaenopsis* orchid protocorms. a. Embryos at protocorm with SAM (arrow) stage, b. Elongated protocorm, c. protocorm forms the first leaf (FL) at shoot stage, d. protocorm at shoot stage forms second leaf (SL), e. Root (R) forms in the single shoot, f - j. Putative transformant protocorm began to form somatic embryos, f. Two embryo somatic, g. Three embryo somatic, h. Five embryo somatic, i. Seven embryo somatic and j. Protocorm form multishoot



Remarks: Bars b and c: 0.5 cm

Fig. 7. The formation of shoot in *D. phalaenopsis* transformant harbor 35S::GAL4::*AtRKD4*::GR. (a) The average number of shoot formed in transformant and non transformant plants. (b) Non-transformant plant and (c) transformant plant on regeneration medium (both plants are eight month old after sowing)

CONCLUSION AND SUGGESTION

Somatic embryos can be induced in the *D.phalaenopsis* orchid protocorm by insertion of the exogenous gene of *AtRKD4* from *A. thaliana* model plant, although both groups are different between Monocots and Dicots. This suggests that *AtRKD4* gene is a very conserve gene in performing its function in very early stages for the initiation of embryogenesis in plants. Thus, the insertion of *AtRKD4* gene into orchid genom is suggested to solve the problem for rapid mass propagation of orchids and other plants in large quantities.

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