Journal of Tropical Biodiversity and Biotechnology
Volume 06, Issue 02 (2021): jtbb61783
DOI: 10.22146/jtbb.61783

Research Article

Detection of *AtRKD4* Protein During Induction of Somatic Embryogenesis in *Dendrobium lineale* Rolfe Transgenic Orchids Carrying *35S::GR::AtRKD4*

Gde Cahyadi Wirajagat[1], Ni Luh Putu Kayika Febryanti[1], Fitriana Puspitasari[1], Dika Sundari[1], Jose Gutierrez-Marcos[2], Endang Semiarti[1]*
1) Faculty of Biology, Universitas Gadjah Mada , Yogyakarta, Indonesia, 55281
2) School of Life Science, University of Warwick, England, CV4 7AL
* Corresponding author, email: endsemi@ugm.ac.id

Submitted: 30 November 2020; **Accepted:** 29 April 2021; **Published online:** 04 June 2021

ABSTRACT
Dendrobium lineale is an Indonesian native orchid from the Spatulata section in *Orchidaceae* Family. This orchid is important because it is usually used as a parental plant in orchid breeding and is predicted to have a potential phytochemistry compound. In addition, in their natural habitat, this orchid is threatened due to forest exploitation and natural disaster. Therefore the precision mass propagation techniques for this orchid need to be conducted. Biotechnological approaches through inserting embryo gene such as *AtRKD4* from *Arabidopsis thaliana* has already been successfully conducted. This study aims to check the integration stability of T-DNA harboring 35S::GR::*AtRKD4* from ten selection transformants and to detect the existence of AtRKD4 protein after induction by Dexamethasone and/ Thidiazuron. The result showed that T-DNA were stably integrated into the genome of *D. lineale* transformants and the AtRKD4 protein with a molecular weight of 28.53 kDa was detected in *D. lineale* transformant plants after being induced by 15 µM DEX and 3 mgL-1 TDZ for 5 days.

Keywords: *AtRKD4* gene, *At*RKD4 protein, *Dendrobium lineale,* dexamethasone, somatic embryo, thidiazuron

INTRODUCTION
Dendrobium lineale Rolfe is an endemic orchid of Papua that is threatened in its habitat due to over-exploitation. *D. lineale* is favored because it shows tall stems and produces many beautiful purplish-white flowers (Rolfe 1889; Pridgeon 1992). This orchid is named *D. lineale* because has lined sepals and petals (Pridgeon 1992). In addition, *D. lineale* can be used for medical purposes such as anti-cancer. It contains types of phytochemicals such as flavonoids, polysaccharides, bibenzyl, phenanthrene, alkaloids, sesquiterpenoids, and steroids. A common problem with *D. lineale* is that it is not easy to breed and grow naturally, so the population of this orchid is less abundant (Semiarti et al. 2020). Several types of orchids are increasingly rare to be found in nature due to overexploitation, domestication, and usage as parental orchids in cultivation (Ivakdalam & Pugesehan 2016). *In vitro* propagation is expected to be the right solution for *ex situ* conservation

because it's possible to produce a mass number of plants with similar characteristics to its parental (Setiari et al. 2018). Propagation through *in vitro* culture can increase the quantity and the number of tillers obtained in a relatively short time (Hartati et al. 2016). Optimization of *in vitro* propagation can be done by using genetic engineering, by inserting foreign genes through *Agrobacterium tumefaciens*. An embryo gene of *Arabidopsis thaliana*, *AtRKD4* gene in this study was inserted into the genome of *D. lineale* protocorms through *A. tumefaciens* strain EHA105 that harbor pTA7002 plasmid, therefore it can induce large numbers of somatic embryos in a relatively short time. The gene construction used an inducible promoter equipped with *Glucocorticoid Response Element* (GRE), requiring dexamethasone (DEX) which is a glucocorticoid compound as an inducer to induce transcription or activation of transgenes (Mursyanti et al. 2015). The insertion of the *AtRKD4* gene into the genome of orchids has been carried out and has successfully demonstrated the formation of somatic embryos in transformant orchid leaves after being induced with DEX on leaves of *Dendrobium phalaenopsis* (Zulwanis et al. 2020).

The formation of somatic embryos in orchid plants can be induced by the addition of growing regulatory substances in the medium of *in vitro* culture. Auxin and cytokinin are commonly used for the induction of somatic embryogenesis (Moradi et al. 2017). Thidiazuron (N-phenyl-N'-1,2,3-thiadiazol-5-ylurea) is a cytokinin class growth regulator known to have potential activity in shoot regeneration and proliferation was effective in inducing somatic embryo formation (Ghosh et al. 2018). TDZ with 3 mgL^{-1} concentration is the best concentration used to induce the formation of somatic embryos in the roots, stems, leaves explants, and protocorm of *P. amabilis* orchid (Mose et al. 2017).

According to Semiarti et al. (2007 & 2011), plant genetic transformation is the right method to improve the quality of orchid plants because it can break barriers between species and insert beneficial traits from superior genes from other plant species. The success of the genetic transformation is shown by the detection and integration of *AtRKD4* transgenes in the orchid genome, and also the activation of these genes in orchid transformants (Setiari et al. 2018). Somatic embryogenesis (SE) is a promising technique used in the proliferation of plants. It is also a way to support *ex situ* conservation (Maruyama & Hosoi 2019). Protein detection of AtRKD4 needs to be done because the function of genes is biochemically carried out by proteins which is the result of gene expression and it determines the character of plants at each stage of their development (Utami et al. 2007). RKD4 Protein (*RWP-RK motif-containing 4 putative transcription factors domain*) is one of two sub-family of RWP-RK protein motifs (amino acid sequences consists of arginine (R), tryptophan (W), prolin (P), arginine (R), and lysine (K) proteins located inside the nucleus which are transcription factors based on similarities of form with basic leucine zipper protein and basic helix-loop-helix. The function of the RKD4 protein is to trigger the expression of genes needed for the initiation of the process of forming a divisional pattern in the zygote and the process of the early development of the embryo (Chardin et al. 2014)

The *AtRKD4* gene has been successfully inserted into the *D. lineale* orchid genome, but the stability of the *AtRKD4* gene integration and the expression of the gene in the orchid genome are not yet known. This research aims to analyze the stability level of *AtRKD4* gene integration in the genome of *D. lineale* transformant and to analyze the expression of the gene which can produce AtRKD4 protein in *D. lineale* transformants.

MATERIALS AND METHODS

Plant Materials and T-DNA Construct

Eighty plantlets of 1 year and 10 months-old *D. lineale* that consisted of 46 non-transformants and 34 transformants carrying T-DNA with the construct of 35S::GR::AtRKD4 were used as plant materials for this work. For protein analysis, 2 non-transformant plantlets and 10 candidates of transformant plantlets of *D. lineale* that carrying the 35S::GR::AtRKD4 construct were used. All plantlets were planted on New Phalaenopsis (NP) basal medium (Islam et al. 1998). The T-DNA contains an inducible system of GVG, which contains a synthetic protein fusion for the GAL4 DNA binding domain, the transcriptional activator VP16, and a portion of the rat Glucocorticoid Receptor (GR) (Figure 1). The expression of the GVG gene was driven by a strong promoter 35S promoter from the *cauliflower mosaic virus* (CAMV). To facilitate transformant selection, T-DNA is also filled with *Hygromycin phosphotransferase* (*HPT*) gene as a selection marker, which confers resistance to hygromycin on the selection medium (Figure 1).

Detection of *AtRKD4* integration in the genome of orchid transformant

DNA isolation of non-transformant and candidates of *D. lineale* transformant

Samples used for genome DNA isolation were 2 samples of non-transformant and 10 samples of transformant plantlets. Leaves of non-transformant plantlets and candidates of transformant plantlets weighing 20 –30 mg, were put in the mortar added 250 µl CTAB 3%, and crushed until smooth in the form of powder using a pestle and then added more 250 µl of CTAB solution. The mixture was homogenized and incubated in a water bath at 60°C for 30 min. The mixture was then added with 500 µL of chloroform solution, and mixed with shaking the tube 6 times inversely, following by incubation with a shaker for 30 min at room temperature. The sample was then centrifuged at 14.000 rpm for 10 min, the supernatant was moved into a new tube. An equal volume of isopropanol was added to the supernatant, mixed well, then incubated at room temperature for 10 min. The mixture was then subsequently centrifuged at 14.000 rpm for 10 min. The pellet of DNA and RNA appeared at the base of the tube, discarded the supernatant, pellets added 500 µl EtOH 70% into the tube, then centrifuged at 10.000 rpm for 5 min to wash the pellet from cell debris. The supernatant was then discarded, and dry up the DNA pellets by using an incubator at a temperature of 37°C for 30 min. The dried pellet of DNA was diluted with 30 µl TE, incubated in the water bath at 60°C for 5 min. Isolated genomic DNA was stored at a temperature of -20°C for subsequent analyses. An aliquot volume of genome DNA suspension was taken to check the concentration by using 0.7% agarose gel electrophoresis.

Detection of T-DNA that carrying *35S::GR::AtRKD4* integration in the genome of orchid transformants plants

Detection of transgene *AtRKD4* and *HPT* in the genome of *D. lineale* were performed by Polymerase Chain Reaction (PCR) method. DNA genome of

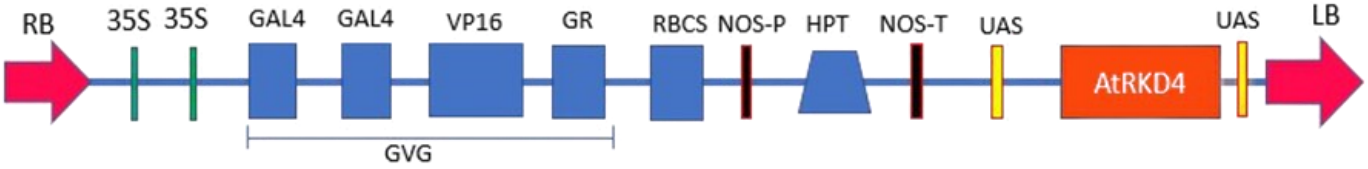

Figure 1. The structure of T-DNA carrying *35S*::GR/UAS::*AtRKD4* in plasmid pTA7002/EHA 105 (Mursyanti et al. 2015).

non-transformant and transformant plant candidates carrying *35S::GR:: AtRKD4* analyzed with PCR using a specific primer for *AtRKD4* and *HPT* genes. Specific primers for the *AtRKD4* gene are AtRKD4F1 (5'GTTCATT TCATTTGGAGAGGACG-3') and AtRKD4 R1 (5'CTTCCATATCTAGG AGAGAATCAAG-3') which produced a 382 bp band of DNA.

Specific primers for HPT genes are HygF (5'-TCGGACGATTGCGT CGCATC-3') and HygR (5'AGGCTATGGATGCGATCGCTG-3') that produced a 545 bp band of PCR product. The PCR process was carried out under the conditions according to the protocols of the Bioline MyTaq™ HS Red Mix 2x. The steps are pre-denaturation stage at 95°C temperature for 1 min, denaturation at 95°C temperature for 15 sec, the annealing stage at 58°C for *AtRKD4* and 61°C for the *HPT* for 15 sec, the extension stage at a temperature of 72°C for 10 sec, the final stage was cooling at 4°C temperature with 35 PCR cycles. Interspace fragment of chloroplast DNA trnL-F (1200 bp) as an internal control of the PCR process. The primers for trnL-F were trnL-F F1 (5' CGAAATCGGTAGACGCTACG-3') and trnL-F R1(5' ATTTGAACTGGTGACACGAG-3'). The results of the PCR were run in electrophoresis gel using 1% agarose gel in TAE 1X buffer with 100 volts for 20 min and observed with UV transilluminator (Extragene).

The PCR products were checked by 1% agarose gel electrophoresis in 1X TAE solution with 3µl of EtBr. The sample was running using electrophoresis with 100 volts for 20 minutes and the bands were observed by UV transilluminator.

AtRKD4 protein analysis on *D. lineale* transformant plantc

Protein analysis of transformant plant was carried out using the Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE) method. The total protein isolation was carried out using transformant plant leaves which had been induced in DEX media 15 µM and TDZ 3 mgL-1 for 5 days. The isolation was carried out by crushing 200 mg leaves by mortar and pestle in 300 µl *Phosphate Buffer Saline* (PBS) with pH 7 as an extraction buffer until it becomes powder and homogenous. The sample was put in a 1.5 ml eppendorf tube. After that, the sample was centrifuged at a speed of 10,000 rpm for 10 minutes with a temperature of 4°C. The acquired supernatant was then transferred into a new tube. A volume of 200 µl supernatant was added with 50 µl 5X sample buffer (3.9 ml of aquabidest; 1.0 ml 0.5 M Tris pH 6.8; 0.8 ml glycerol; 1.6 ml SDS 10%; 0.4 ml 2-mercaptoethanol; 0.4 ml 1% Bromophenol Blue). The mixture was then heated in a water bath at 90°C temperature for 5 minutes to degrade the protein. Samples were stored at -20°C. Eight µl of supernatant was used to measure the total concentration of proteins by using spectrophotometry.

Determination of protein concentration was carried out by using 8 µl supernatant added with 200µl Bradford solution. The protein concentration was measured by spectrophotometry at a wavelength of 595 nm. Bovine Serum Albumin (BSA) proteins were used as a standard to calculate the protein concentrations. The steps of electrophoresis with SDS-PAGE have put a plate glass arranged with a frame from Bio-Rad and printed 12% lower gel. Then the 12% running gel mixed homogeneous (30% acrylamide 4.0 ml; 4X LGB (Lower Gel Buffer) 2.50 ml; distilled water 3.45 ml; TEMED 5 µl, APS 10% 50 µl) the mixture then inserted into a plate glass through its chamber up to one cm from the upper limit of the plate. The upper gel is made like a lower gel. The 5% upper gel mixed homogeneous (30% acrylamide 0.67 ml; 4X UGB (Upper Gel Buffer) 1.25 ml; distilled water 3.05 ml; TEMED 5 µl, APS 10% 50 µl) then the mixture inserted on top of the lower gel that has hardened to full. Electrophoresis was run in 50 volts for 50 minutes and 100 Volts for 60 minutes. The staining process was carried out

using 40% methanol, 10% glacial acetic acid, 50% distilled water, and 0.1% Coomasie Blue for 24 hours. The next process was the destaining stage using 40% methanol, 10% glacial acetic acid, and 50% distilled water.

The profile of the protein was formed, a weight analysis of protein band molecules was performed using the software of microsoft excel. The Linear equations were obtained by molecular weight log measurements and a migration band size of y = -0.1537X + 2.235 with R of 0.9581. The results of the analysis of protein molecular weight can be arranged in a Table.

RESULTS AND DISCUSSION

Integration stability of putative transformants

Semiarti et al. (2018), reported in transgenic *Phalaenopsis "Sogo vivien"* plant carrying T-DNA *35S::Gal4::AtRKD4::GR* is stably integrated with genome after one year of transformation. Zulwanis et al. (2020), reported that transgenic *Dendrobium phalaenopsis* carrying *35S::GR::AtRKD4* so far is stably integrated inside the genome. Despite various percentages.

The detection of *35S::GR/UAS:AtRKD4* integration stability in *D. lineale* was carried out using ten transgenic plantlets and two wild-type plantlets (Figure 2). In general phenotype of non-transfromant and transformant plantlets carrying T-DNA with construct 35S::GR::AtRKD4 there is no significant difference as seen in Figure 2. This is in accordance with the expected, unchanged phenotypes of plants from somatic embryos, the phenotype of transformant plants remains the same as plants derived from zygotic embryos.

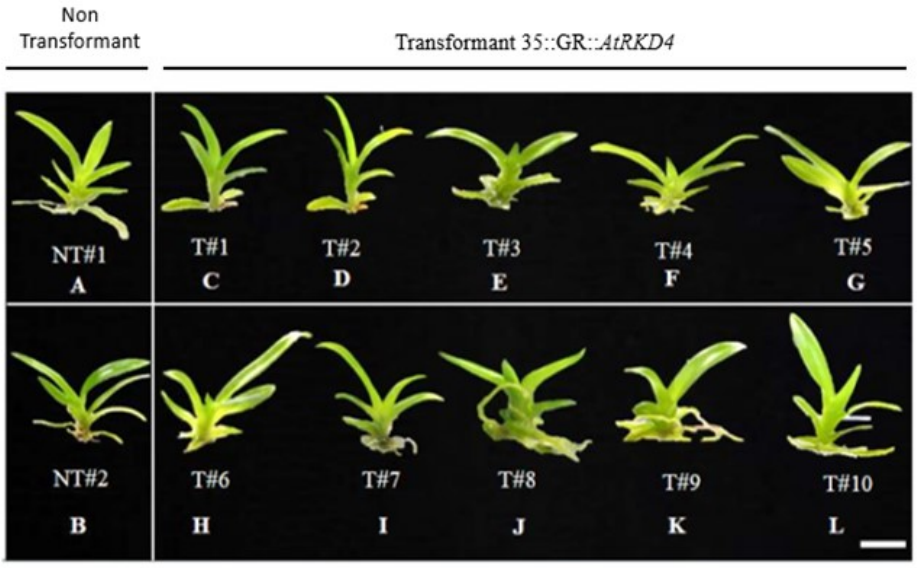

Figure 2. Phenotype of *D.lineale* Putative Transformant carrying T-DNA with 35S::GR::AtRKD4 and non-transformant. . A-B: Non-transformant plants (NT#1, NT#2) and C-L: 10 putative transformant (T#1-T#10), plant age 1 year 10 months. Bar = 1 cm.

Figure 3 shows detection of the stable integration of T-DNA carrying the gene *AtRKD4* in the *D. lineale* transformant orchid indicates that the *AtRKD4* gene could be amplified from all transformant plants, resulted in a DNA fragment of 382 bp in length. *AtRKD4* genes could not be detected in NT plants, indicated that all non-transformant plants did not contain the *AtRKD4* gene in their genomes. The presence of the *AtRKD4* genes in genome DNA of transformant plants indicates there were stable integration of the *35S::GR/UAS:AtRKD4* transgene in the *D. lineale* genomes and that

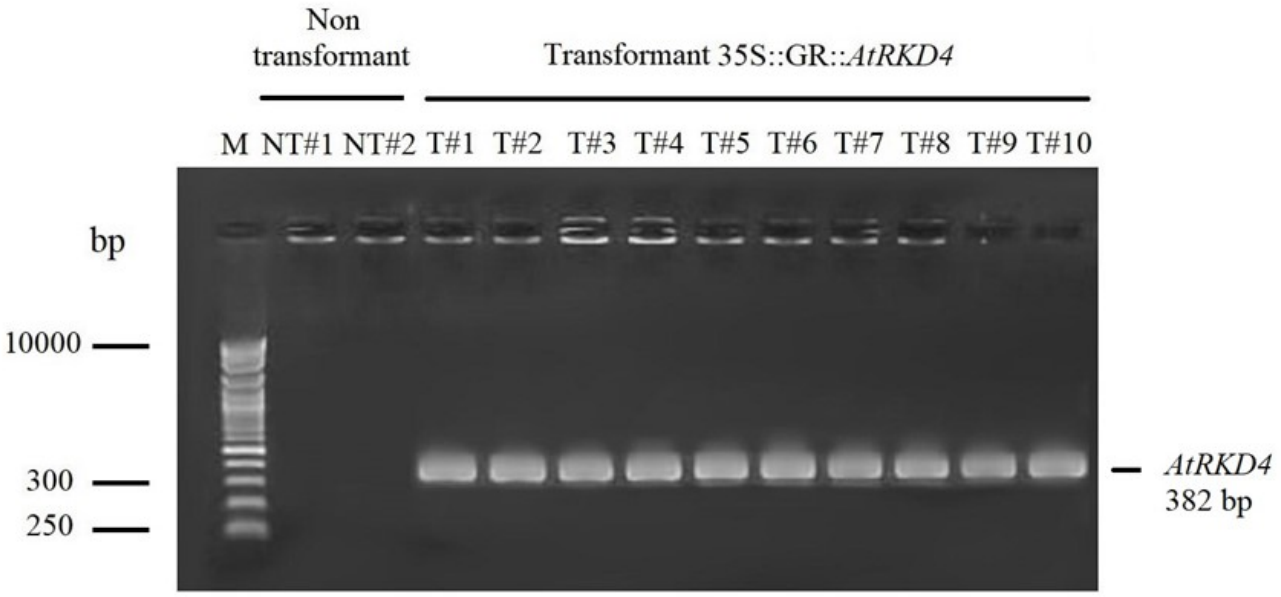

Figure 3. Detection of *AtRKD4* integration in the genome of *D.lineale* transformants carrying T-DNA with 35S::GR::AtRKD4. M: DNA marker. Lanes 2-3: non-transformant (NT); 4-13: transformant.

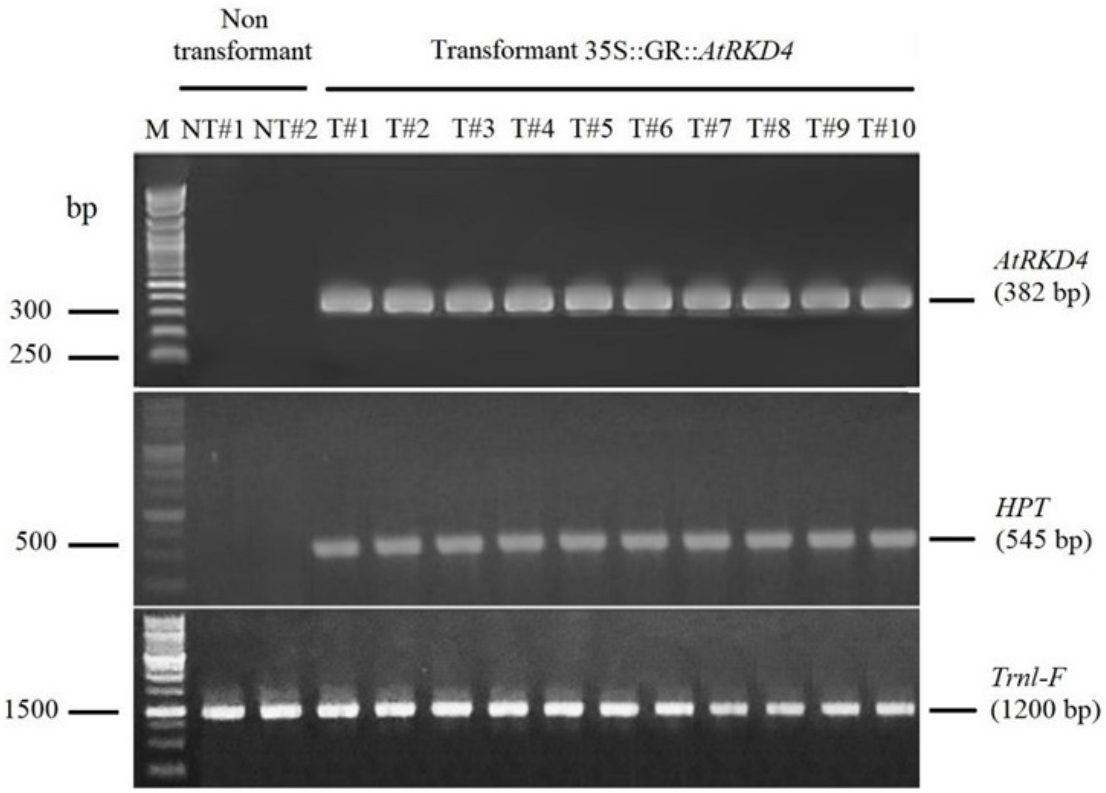

Figure 4. Detection integration of T-DNA harboring 35S::GR::AtRKD4 in the transformant genome of *D. lineale*. A. PCR product of *At*RKD4 gene (382 bp); B. *HPT* gene (545 bp), b.; and C. Interspace fragment of chloroplast DNA *trnL -F* (1200 bp) as an internal control of the PCR process. Lanes 2-3 Non-transformant plants; lanes 4-13: candidates of transformant plants.

during growth and development of transformant the transgene was somatically maintained.

Detection of *D. lineale* transformant lines was carried out by using *HPT* primers that amplified a 545 bp DNA fragment (Figure 4). HPT is an enzyme produced by the *Streptomyces hygroscopicus* bacteria that is resistant to hygromycin. HPT was used as a selection marker found in T-DNA of *A. tumefaciens* induce into the genome of orchids that were used to prove positive

transformant plants. Detection of the *HPT* gene in the plant genomes proved that transformant plants were resistant to hygromycin antibiotics and plantlets can grow well on a medium containing hygromycin (Setiari et al. 2018).

Molecular markers role as control is an important part of plant biotechnology development related to gene regions both in DNA and genomes (Yeşiltaş et al. 2019). The interspace fragment trnL-F of chloroplast genome was used as an internal control of PCR analysis. Chloroplasts have their genome which contains conserved genes and encodes many specific components. It is usually passed down maternally in most angiosperms (Filiz et al. 2018)

Evaluation of T-DNA integration stability was examined by PCR method, which used two inserted genes: HPT (545 bp), and *AtRKD4*(382bp) as indicators to confirm the integration stability of *AtRKD4* transgene into *D. lineale genome* (Figure 4). It is proven that T-DNA transgene carrying 35S::GR::*AtRKD4* is stably integrated into the genome of *D. lineale*. This is reinforced by the amplification of the interspace fragments of trnL-F chloroplast DNA (1200 bp), which indicates that the plant DNA genome has good quality and the PCR reactions have been well underway, then the results can be accounted for the truth.

Induction and Detection of AtRKD4 protein in orchid transformant plants after being Induced by Using DEX and TDZ

Detection of *AtRKD4* gene expression carried out after leaves of transformant plants induced on NP media containing 15 µM DEX or 3 mgL^{-1} TDZ for 1, 3, 5, and 7 days. T-DNA in the pTA7002 plasmid construct used to insert the *AtRKD4* gene contains a GRE activated by steroid hormone such as DEX in the orchid genome. In this study, TDZ played a role as an alternative to activate somatic embryos. TDZ is a powerful synthetic growth regulation exhibiting both auxin-cytokinin-like effects in plants, the indirect effect of TDZ is considered to be its ability to inhibit the enzyme cytokinin oxidase/dehydrogenase which degrades cytokinin (Nisler 2018). TDZ uses in inducing SE were reported by Gow et. al (2018) to induce SE in *Phalaenopsis aphrodite*. Mose et al. (2020) reported that direct somatic embryogenesis in *P. amabilis* orchid can be effectively induced from various explants by using a combination of auxin and cytokinin, with the best combination being 3.0 mg L-1 TDZ and 1.0 mg L-1 NAA in dark condition. Zulwanis (2020) found that SE could be induced by TDZ in leaf explant of transformant plant of *Dendrobium phalaenopsis* orchid that carrying AtRKD4 transgene, therefore it assumed that the existence of TDZ could trigger plant endogenous steroid hormones to drive the expression of *AtRKD4* transgene. The most optimal expression of the *AtRKD4* gene was detected after 5 days with DEX or TDZ. Based on Zulwanis (2020), DNA bands that amplified from the explants were induced using DEX and TDZ during 1 to 9th day, the highest transgene expression was found .in day 5th of induction as the highest DNA band density. These results correspond to the results of gene expression analysed by Febryanti et al. (2020) showing that AtRKD4 mRNA appeared in *D.lineale* transformant samples induced with medium NP +15 µM DEX or NP + 3 mgL-1 TDZ. This proves that the translation process goes well judging by the formation of *AtRKD4* gene expression products in the form of proteins. One of the growth regulators widely used in *in vitro* propagation systems is TDZ (Mahendran & Narmatha Bai 2016). The formation of somatic embryos in *Phalaenopsis* 'Sogo Vivien' could successfully be induced by using 10 mg/L TDZ supplemented into NP medium (Kasi & Semiarti 2016). However, the direct mechanism of TDZ for activating gene expression in SE is still unclear (Modi & Kumar 2018).

DEX works across plasma membrane diffusion and bonded with *glucocorticoid receptors* (GR), which are in the unprotected state, as a cytoplasmic complex with 90 kDa *Heat Shock Protein* (HSP90). The separation of HSP90 was due to the absence of ligands that localized the transcription factor into the nucleus. Inside the nucleus, GR binds to specific DNA sequences and activated the expression of the *AtRKD4* gene (Schena et al. 1991).

AtRKD4 transgene expression at the translation level was known in the appearance of protein formation in the *D. lineale* carried *35S::GR/UAS::AtRKD4* after treated by DEX or TDZ. Fellers et al. (1997) and Tchorbadjieva (2005) identified two proteins with molecular weights of 43 and 27 kDa which were used as embryogenic cell markers in wheat callus. Analysis of protein molecules can be carried out using the SDS-PAGE method. This method was used to detect predicted AtRKD4 protein bands with a molecular weight of ~28.53 kDa by electrophoresis separation (Heda et al. 2016). The isolated total protein from leaves of transformant plant carrying the construct of *35S::GR/UAS::AtRKD4* after 5 days treated by 15 µM DEX, and 3 mgL^{-1} TDZ showed 7 protein bands with various molecular weight. One of the protein band was 28.53 kDa in molecular weight, that can be predicted as AtRKD4 protein (Figure 5). This is supported by data on the absence of protein bands with a size of 28.3 kDa in non-transformant plants.

It is also supported by data showing that in transformant plants that planted on the NP basic medium without additional DEX or TDZ the protein band formation with a size of 28.3 kDa were not detected. Thus, it can be assumed that transgene *AtRKD4* supposed to be well expressed in *D. lineale* transformant plants that carrying 35S::GR::AtRKD4 after being induced by DEX and/TDZ.

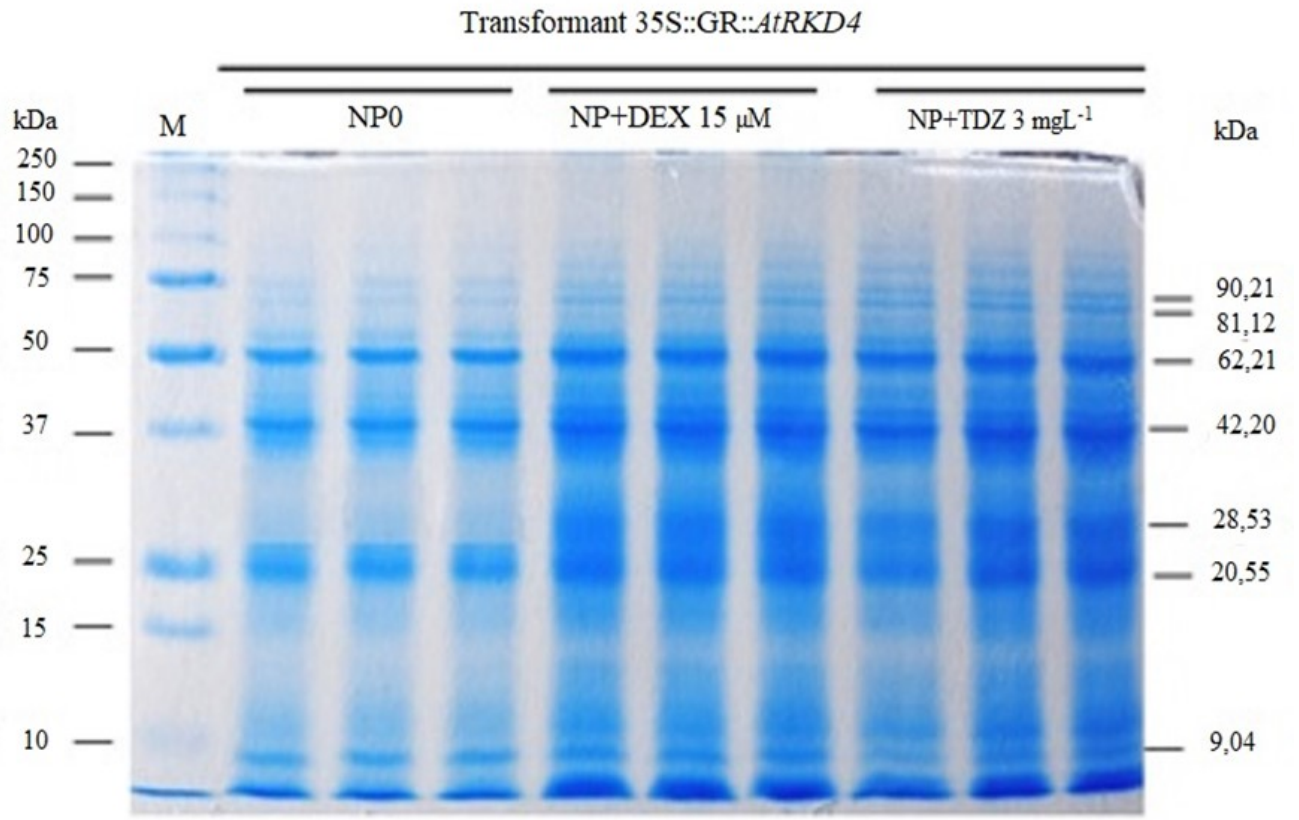

Figure 5. Protein profile of transformant plant *D. lineale* carrying T-DNA with *35S::GR/UAS::AtRKD4*. Lane 1: protein marker 250 kDa (Biorad); Lane2-4: SE induction on NP Basic Medium. Lanes 5-7: SE induction with 15 µM DEX, Lanes 8 -10: induction with 3 mgL^{-1}TDZ.

The molecular size of *D.lineale* proteins induced by DEX and TDZ is determined based on linear equations of molecular weight log measurement and migration of protein band size in SDS-PAGE presented in Table 1.

Table 1. Protein products in the transformant plant *D. lineale* that carry the *AtRKD4* gene after being induced with DEX and TDZ for somatic embryogenesis.

Number of Bands	Protein products of *D. lineale* transformant carrying 35S::GR::AtRKD4 planted on various mediums		
	NP0 (kDa)	NP+DEX 15 µM (kDa)	NP+TDZ 3mgL^{-1} (kDa)
1	90.21	90.21	90.21
2	81.12	81.12	81.12
3	62.21	62.21	62.21
4	43.20	43.20	43.20
5	-	28.53	28.53
6	20.55	20.55	20.55
7	9.04	9.04	9.04

Table 1 showed that there were 7 kinds of protein products that could be detected after 5 days treated by both DEX and TDZ, and only 6 proteins can be detected from in NP0 treatment. Protein with 90,21 kDa was the largest protein detected in all treatments, and protein with a size of 9.04 kDa was the smallest one. AtRKD4 protein with 28.3 kDa can be detected as the fifth band in *D. lineale* transformant that planted on NP medium supplemented by both 15 µM DEX, or 3 mgL^{-1}TDZ. A thick protein band weighing 20.55 kDa was found, thought to be the cycline protein responsible for cell division. This protein is present in all developing cells and plays a role in regulating the transition of phase G1 to phase S and phase G2 to phase M. Cycline proteins have a molecular weight range of 16-25 kDa. While the protein band measuring 9.04 kDa is thought to be a protein that encodes the SAUR (Small Auxin-Up RNA) gene that encodes proteins measuring 9-10 kDa. The SAUR gene is known to be responsible for cell division and lengthening and serves as a promoter for activating genes in protein syntheses such as cycline and expansion proteins (Li et al. 1994).

In orchids, Utami et al. (2007) found a protein measuring 44 kDa during the induction of somatic embryogenesis of the orchid *P. amabilis in vitro* culture. The successful formation of the AtRKD4 protein from the *Arabidopsis thaliana* model plant in the *D.lineale* orchid transformant plant suggests that the embryonic gene can work well in orchids, even though the gene is derived from dicot plants. This will open up opportunities in the future that the *AtRKD4* gene can be used for a wide variety of plants both dicots and monocots for plant micropropagation through somatic embryogenesis.

CONCLUSION

The *AtRKD4* genes are stably integrated into *D. lineale* transformant orchid genome, expressed and function for inducing somatic embryogenesis. All ten transformant plants stably carrying the T-DNA with 35S::GR::AtRKD4 constructs. The AtRKD4 protein was detected in all ten transformants after being induced for 5 days by both15 µM DEX or 3 mgL^{-1} TDZ, which showed the predicted AtRKD protein band in 28.53 kDa molecular weight. The direct role of TDZ in the induction of somatic embryos in this study is

still unclear. Therefore, more research is needed on the function of TDZ in embryo formation in plants, mainly in orchids.

AUTHORS CONTRIBUTION

G.C.W.: data collection, analysis and interpretation data, preparation and writing of the article. N.L.P.K.F.: idea of the experiment, data collection, preparation, and writing of the article. F.P.: data collection, analysis and interpretation data, preparation and writing of the article. D.S.: data collection, analysis and interpretation data, preparation and writing of the article. J.G.M.: founder of T-DNA construct. E.S.: adviser of work, data collection, and analysis, interpretation, critical review.

ACKNOWLEDGMENTS

This research was supported by a grant from the Ministry of Research and Technology Higher Education RI on the Scheme of Rekognisi Tugas Akhir (RTA) 2020. We thank Steffanie Nurliana, M.S for providing transformant plants and non-transformant of *D. lineale*.

CONFLICT OF INTEREST

The authors declare there is no competing interest.

REFERENCES

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.

Febryanti, N.L.P.K. et al., 2020. The expression analysis of AtRKD4 transgene in Dendrobium lineale Rolfe transgenic orchid carrying 35S::GR::AtRKD4 for micropropagation. AIP Proceeding of The 6th International Conference on Biological Science ICBS 2019: "Biodiversity as a Cornerstone for Embracing Future Humanity," 2260 (September), 060021.

Fellers, J.P., Guenzi, A.C. & Porter, D.R., 1997. Marker proteins associated with somatic embryogenesis of wheat callus cultures. *The Journal of Plant Physiology*, 151, pp.201–208

Filiz, E. et al. 2018. Genetic Diversity and Phylogenetic Analyses of Turkish Rice Varieties Revealed by Issr Markers and Chloroplast Trnl-F Region. *Fresenius Environ*, 27(12), pp. 8351–8358.

Ghosh, A., Igamberdiev, A.U. & Debnath, S.C., 2018. Thidiazuron-induced somatic embryogenesis and changes of antioxidant properties in tissue cultures of half-high blueberry plants. *Scientific Reports*, 8(1), pp.1–11.

Gow, W.P. et al., 2018, 'Factors Affecting Thidiazuron-Induced Direct Somatic Embryogenesis of *Phalaenopsis aphrodite*', in Ahmad N., Faisal M. (eds.), *Thidiazuron: From Urea Derivative to Plant Growth Regulator*, pp.317-327, Springer, Singapore.

Hartati, S., Budiyono, A. & Cahyono, O., 2016. Pengaruh NAA dan BAP Terhadap Pertumbuhan Subkultur Anggrek Hasil Persilangan *Dendrobium biggibum* X *Dendrobium liniale*. *Caraka Tani: Journal of Sustainable Agriculture*, 31(1), p.33.

Heda, G.D. et al., 2016. Effects of reusing gel electrophoresis and electrotransfer buffers on western blotting. *Journal of Biomolecular Techniques*, 27(3), pp.113–118.

Islam, M.O., Ichihasi S., & Matsui S, 1998. Control of growth and development of protocorm like body derived from callus by carbon sources in Phalaenopsis. *Plant Biotechnol*, 15, pp.183-187.

Ivakdalam, L.M. & Pugesehan, D.J., 2016. Keragaman Jenis Tanaman Anggrek (*Orchidaceae*) Di Cagar Alam Angwarmase, Kabupaten Maluku Tenggara Barat. *Jurnal Agroforestri*, 11(3), pp.161-168.

Kasi, P.D. & Semiarti, E., 2016. Pengaruh Thidiazuron Dan *Naphthalene Acetic Acid* Untuk Induksi Embryogenesis Somatik Dari Daun Anggrek *Phalaenopsis "*Sogo Vivien*". Jurnal Dinamika*, 7, pp.31-40.

Li, Y. et al., 1994. An Auxine Inducible Element in Soybean SAUR Promoters. *Plant Physiol*, 106, pp.37-43.

Mahendran, G. & Narmatha Bai, V., 2016. Direct somatic embryogenesis of *Malaxis densiflora* (A. Rich.) Kuntze. *Journal of Genetic Engineering and Biotechnology*, 14(1), pp.77–81.

Maruyama, T.E. & Hosoi, Y., 2019. Progress in somatic embryogenesis of Japanese pines. *Frontiers in Plant Science*, 10(January), pp.1–15.

Modi, A., & Kumar, N, 2018, 'TDZ-induced regeneration in *Stevia rebaudiana* Bertoni: an important natural sweetener', in Ahmad N., Faisal M. (eds.), *Thidiazuron: From Urea Derivative to Plant Growth Regulator*, pp.351-358, Springer, Singapore.

Moradi, S. et al., 2017. Direct somatic embryogenesis in *Epipactis veratrifolia*, a temperate terrestrial orchid. *Journal of Horticultural Science and Biotechnology*, 92(1), pp.88–97.

Mose, W. et al., 2017. The Influence of Thidiazuron on Direct Somatic Embryo Formation from Various Types of Explant in *Phalaenopsis amabilis* (L.) Blume Orchid. *HAYATI Journal of Biosciences*, 24(4), pp.201–205.

Mose, W. et al., 2020. Direct Somatic Embryogenesis and Regeneration of an Indonesian orchid *Phalaenopsis amabilis* (L.) Blume under a Variety of Plant Growth Regulators, Light Regime, and Organic Substances. Jordan Journal of Biological Sciences, 13(4), pp.509–518.

Mursyanti, E. et al., 2015. Induction of Somatic Embryogenesis through Overexpression of ATRKD4 Genes in *Phalaenopsis* "Sogo Vivien." *Indonesian Journal of Biotechnology*, 20(1), p.42.

Nisler, J., 2018, 'TDZ: Mode of Action, Use and Potential in Agriculture', in Ahmad N., Faisal M. (eds.), *Thidiazuron: From Urea Derivative to Plant Growth Regulator*, p.48, Springer, Singapore.

Pridgeon, A., 1992, *The Illustrated Encyclopedia of Orchids*, Timber Press, Inc., Oregon.

Rolfe, R. A, 1889. *Dendrobium lineale* Rolfe. *Gardeners' Chronicle*, 2, p.38.

Schena, M., Lloyd, A.M. & Davis, R.W., 1991. A steroid-inducible gene expression system for plant cells. *Proceedings of the National Academy of Sciences of the United States of America*, 88(23), pp.10421–10425.

Semiarti, E. et. al. 2007. Agrobacterium Mediated Transformation of The Wild Orchid Species *Phalaenopsis amabilis*. *Plant Biotechnology*, 24, pp.265-272.

Semiarti, E. et al., 2011. Agrobacterium-Mediated Transformation of Indonesian Orchids for Micropropagation in: Alvarez, M., *Genetic Transformation.* Croatia: Intech, pp.215-240.

Semiarti, E. et al., 2018. Stability of T-DNA Integration in *Phalaenopsis* "Sogo vivien" Transgenic Orchid Carrying 35S::Gal4::AtRKD4::GR. *Biology Medicine & Natural Product Chemistry*, 7(1), pp.5-13.

Semiarti, E., Purwantoro, A., Puspita, S.I., 2020, 'Biotechnology Approaches on Characterization, Mass Propagation, and Breeding of Indonesian Orchids *Dendrobium lineale* (Rolfe.) and *Vanda tricolor* (Lindl.) with Its Phytochemistry', in Merillon JM., Kodja H. (eds.), *Orchids Phytochemistry, Biology and Horticulture*. Reference Series in Phytochemistry. Springer, Cham.

Setiari, N. et al., 2018. Micropropagation of Dendrobium *phalaenopsis* orchid through overexpression of embryo gene AtRKD4. *Agrivita*, 40(2), pp.284–294.

Tchorbadjieva, M.I., 2005. Protein markers for somatic embryogenesis. Plant Cell Monographs, pp.215–233.

Utami, E.S.W. et al., 2007. Embriogenesis Somatik Anggrek Bulan *Phalaenopsis amabilis* (L.) Bl: Struktur dan Pola Perkembangan. *Berkala Penelitian Hayati*, 13(1), pp.33–38.

Yeşiltaş, B.N., & Kolören, O., 2019. Molecular Characterization of Sicyos Species in Ordu And Giresun Provinces. *Turk. J. Weed sci*, 22(1), pp.37–44.

Zulwanis et al., 2020. The expression of AtRKD4 transgene during induction of somatic embryogenesis in transgenic dendrobium phalaenopsis Orchid Carrying 35S::GR::AtRKD4. *AIP Conference Proceedings*, 2260.