Agrobacterium-MEDIATED GENETIC TRANSFORMATION OF SEAWEED *Kappaphycus alvarezii* USING *Gα* GENE AND CALLUS CULTURES

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ABSTRACT

Cottonii seaweed (*Kappaphycus alvarezii* Doty) is one of the most important commercial sources of carrageenans which are widely used in the pharmaceuticals and the food industry. A problem in the cultivation of this seaweed is the ice-ice disease, which is caused by extreme changes in environmental conditions such as temperature and seawater salinity. Gene transformation to produce Cottonii seaweed transgenics that are tolerant to environmental stress is potential solution to this problem. *Ga* gene encodes for the heterotrimeric G protein α subunit is a gene that plays a role in tolerance to biotic and abiotic environmental stresses. This study aimed to: (a) introduce the *Ga* gene into callus cells of *K. alvarezii* and regenerate transformed callus cells to transgenic plantlets; (b) determine the appropriate concentration of acetosyringone and *Agrobacterium tumefaciens* strain for gene transfer into the callus of *K. alvarezii*. Callus cells of *K. alvarezii* were transformed using *Agrobacterium tumefaciens* strains LBA4404 and EHA101 carrying the expression vector pGWB502-*Ga* with a CaMV-35S promoter. The calli and *A. tumefaciens* were co-cultivated in several concentrations of acetosyringone (20, 40, 60 mg/L). Regeneration of transformed callus cells into transgenic plantlets was successfully performed using the somatic embryogenesis technique. The result showed the highest percentage of putative transgenic micropropagule formation occurred with 20-40 mg/L acetosyringone. Polymerase chain reaction (PCR) analysis on twenty transgenic plantlets indicated that the *Ga* gene was successfully introduced into the genomic DNA of all of them. The transformation using EHA101 produced more transgenic plantlets that were morphologically normal and grew faster in aerated culture than using LBA4404. This study concluded that the appropriate method of gene transfer into *K. alvarezii* callus was co-cultivation with *A. tumefaciens* EHA101 in 20-40 mg/L concentration of acetosyringone.

Keywords: *Agrobacterium tumefaciens*, *Ga* gene, *Kappaphycus alvarezii*, transgenic plantlets

INTRODUCTION

Cottonii seaweed (*Kappaphycus alvarezii* Doty) is a red algae species (Gigartinales, Rhodophyta). It is one of the leading commodities for Indonesian fishery and marine industries because it produces carrageenan which have high economic values. Carrageenan has many uses in the pharmaceuticals, foods and cosmetic industries as a stabilizer, thickener, gelling agent, and emulsifier. A constraints in the cultivation of Cottonii seaweed is the ice-ice disease, which causes the thallus to change color to white (bleaching) and resulted in a decrease in the carrageenan content (Mendosa *et al.* 2002). Ice-ice disease is caused by extreme changes in environmental factors that are unsuitable for optimal growth, such as high temperatures and low seawater salinity (Parenrengi *et al.* 2011). These conditions can lead to physiological stress, which causes the seaweed to become susceptible to pathogenic microorganisms such as *Vibrio* marine bacteria and *Cytophaga-Flavobacterium* group (Largo *et al.* 1999).

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A possible solution to this problem is to genetic engineer transgenic seaweed that are tolerant to environmental changes, such as temperature and salinity of sewater that often occurs in coastal waters. One of the genes that plays a role in plant resistance to biotic and abiotic environmental stresses is the heterotrimeric G protein (Chakraborty *et al.* 2015). The G protein consists of 3 subunits (α, β and γ), the *Ga* gene encodes for the α subunit of this protein, a component that plays a universal role in cellular signal transduction. When this protein is active, it passes on the signals received by extracellular receptors to enzymes, ion channels, and other intracellular effectors on the inside of the cell membrane. In plants, the *Ga* protein affects growth, cell proliferation, defense against disease, stomata movement, channel regulation, sugar sensing and some hormonal responses (Urano *et al.* 2013).

Gene transformation of *K. alvarezii* using *Agrobacterium tumefaciens* had been previously achieved using thallus explants (Daud 2013; Fajriah 2014; Handayani *et at.* 2014). However, these work showed that the efficiency of gene transformation was low, i.e., 7.5% with the *PaCS* gene (Daud 2013), 3.1% with the *MaMt2* gene (Fajriah 2014) and 23.56% with *Lys* gene (Handayani *et al.* 2014). In addition, transgenic thallus buds grown from thallus explants were small and difficult to regenerate into plantlets that could be cultivated in coastal area (Handayani *et al.* 2014; Jeihandanu 2015). The genetic transformation to obtain *K. alvarezii* that is tolerant to environmental stress was done by Triana *et al.* (2016) using SOD (Superoxide dismutase) gene, but the growth rate of transgenic thallus in environmental stresses had not been reported, so the effectiveness of the gene in increasing tolerance to environmental stress in transgenic thallus was still unclear. We sought to advance the work by investigating *A. tumefaciens*-mediated genetic transformation of *K. alvarezii* using callus explants since the procedure to induce and regenerate embryogenic callus of *K. alvarezii* into plantlets through somatic embryogenesis technique is available (Sulistiani *et al.* 2012; Sulistiani & Yani 2014).

The use of callus for co-cultivation with *A. tumefaciens* has been widely used in plants, including coffee (*Coffea arabica*), where the efficiency of gene transformation reaches 90-93% (Ribas *et al.* 2011). In addition, the regeneration of a plant from a single gene-transformed cell through the somatic embryogenesis techniques can produce transgenic plants that are not chimeras, i.e., plants that are not mixture of two or more genetically different cells. Taking the facts together, we hoped to efficiently produce transgenic seaweed plantlets that are resistant to environmental stress.

Therefore, this study aimed to: (a) introduce the *Ga* gene from soybean (*Glycine max* var Slamet) into the callus of *K. alvarezii* using the *A. tumefaciens* LBA4404 and EHA101 strains carrying the expression binary vector pGWB502-*Ga* with a CaMV-35S promoter; (b) regenerate genetically transformed callus cells into transgenic plantlets; (c) determine the optimal concentration of acetosyringone for callus co-cultivation; and (d) identify an appropriate *A. tumefaciens* strain for gene transfer into the callus of *K. alvarezii*.

MATERIALS AND METHODS

Callus Preparation

The calli in this study were induced from apical thalli of *K. alvarezii* collected in Takalar, South Sulawesi, Indonesia. Callus induction was carried out by planting pieces of thallus in P1 medium that is Provasoli enriched seawater/PES medium (Provasoli 1968) which was solidified with 8 g/L Bacto agar and contained 1 mg/L 6-Benzylaminopurine (BAP) and 2.5 mg/L indole-3-acetic acid (IAA) (Sulistiani *et al.* 2012). Before co-cultivation, callus was cultured in P0 medium that is PES medium containing 4 g/L Bacto agar without plant growth regulators for 4 months.

Sensitivity test of *K. alvarezii* cells to Hygromycin.

The sensitivity of micropropagules of *K. alvarezii* to hygromycin was unknown when we began this study in 2015. Therefore, it was necessary to test the sensitivity to determine the concentration of hygromycin to use in culture medium to select genetically transformed callus cells after co-cultivation. Micropropagules were planted on P1 medium containing hygromycin

at concentration 0, 20, 30, 40 and 50 mg/L. The micropropagules were weighed prior to planting and after 4 weeks of treatment to determine their weight gain. Changes in thallus color were also recorded by photographing. Micropropagules were considered dead if they did not increase in weight and thallus color had turned entirely white. The hygromycin concentration that caused micropropagule death would be used in culture medium to select hygromycin-resistant callus cells.

Preparation of *A. tumefaciens* strains

This study used two strains of *A. tumefaciens* (LBA4404 and EHA101) as carriers of the pGWB502 binary vector containing hygromycin-resistance gene and *Ga* gene under the control of a constitutive 35S CaMV promotor (Fajri 2015; Figure 1). Thirty µl of bacteria from a glycerol stock were added to 3 ml LB (Luria Broth) medium containing 50 mg/L hygromycin, 100 mg/L streptomycin and 50 mg/L spectinomycin. The culture was shaken at 150 rpm at 28°C in dark room for 48 hours. Then 1 ml of the culture was added into 25 ml of fresh LB/antibiotic medium and cultured until OD_{600} = 0.9-1 (~ 24 hours). Two ml of this culture was diluted into 25 ml of fresh LB/antibiotic medium and shaken in the dark at 250 rpm and 28°C until OD_{600} = 0.5 (~2 hours). The culture then centrifuged at 6000 rpm and 4°C for 10 min and pellets resuspended in PES medium (20 ppt salinity) containing acetosyringone.

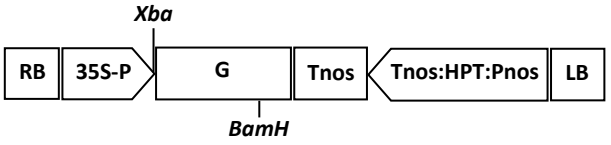

Figure 1 T-DNA regions of the pGWB502-*Ga* binary vector (Fajri 2015)

Co-cultivation and selection of hygromycin-resistant callus

A. tumefaciens LBA4404 or EHA101 was suspended (OD_{600} = 0.5) in liquid PES medium containing different concentration of acetosyringone (0, 20, 40, 60 mg/L). The same solutions were also produced without *A. tumefaciens*. Calli (about 5 mg/callus) were immersed in solution containing or lacking *A. tumefaciens* for 10 minutes. Then, calli were removed and co-cultivated in solid PES medium containing 4 g/L Bacto agar with the same concentration of acetosyringone for 3 days in the dark. Calli were then immersed in sterile seawater containing 200 mg/L cefotaxime for 10 minutes and then grown in P1 medium containing 100 mg/L cefotaxime for 4 weeks. The calli were then subcultured in P1 medium to induce further growth. After 4 weeks, the calli were subcultured in P1 medium containing 50 mg/L hygromycin to select callus cells that were resistant to hygromycin. After 2 weeks, the calli were then subcultured in P1 medium to induce further growth of hygromycin-resistant callus cells.

Regeneration of hygromycin-resistant callus into putative transgenic plantlets

Hygromycin-resistant callus were subcultured in P0 medium for 2 months then again in liquid P0 medium (without Bacto agar; 1 callus/40 mL P0 medium) until micropropagules formed, i.e., 2-4 months (Sulistiani & Yani 2014). The callus cultures were continuously agitated by a rotary shaker at 120 rpm and 22-24°C under cool white fluorescent tube lighting at 1500 lux with a 12 hours light: 12 hours dark cycle. The calli were subcultured to fresh P0 medium every month. After 4 months in P0 medium, the percentage of micropropagule formation (%) in each co-cultivation treatment was calculated.

The putative transgenic micropropagules derived from the co-cultivation of LBA4404 and EHA101, as well as non-transgenic micropropagules (8 clones of each) were transferred to autoclaved bottles containing 500 ml of liquid P0 medium (1 clone /bottle) and grown into putative transgenic plantlets (12 weeks; Sulistiani & Yani 2014). During micropropagule culture, the medium was continuously aerated using an air blower (aerated culture; Figure 2) and the medium was replenished weekly. The Aerated culture was conducted at 22-24°C and 1500 lux with a 12 hours light : 12 hours dark cycle. The weight of putative transgenic micropropagules was measured every week, and daily growth rates (DGRs) were calculated using the formula of Dawes *et al.* (1994).

Figure 2 Regeneration of micropropagules into putative transgenic plantlets in the aerated culture

PCR analysis of putative transgenic plantlets

Isolation of genomic DNA was carried out on twenty putative transgenic plantlets (after 4 months in aerated culture). Thallus pieces from plantlets (60 mg) were quickly frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. Subsequently, DNA extractions were conducted using a PowerPlant® Pro DNA Isolation Kit (MO BIO Laboratories, Inc) and by following the manufacture's instruction. PCR analysis was performed using a 35S-F primer: 5'-ATG GCT GGA TTA GCT GGG-3 'and tNos-R primer: 5'-CTC ATA AAC GTC ATG CAT TAC A-3' (Hannum 2012). PCR was carried out in a 25 µL reaction volume containing 100 ng genomic DNA, 5 pmol 35S-F primer, 5 pmol tNOS-R primer, 0.5 U KOD FX Neo DNA polymerase (TOYOBO), 12.5 µL 2x PCR buffer, 5 µL 2 mM dNTPs, and double-distilled water. Amplification was performed as follows: pre-denaturation at 95ºC for 5 min, followed by 35 cycles of denaturation at 94ºC for 30 seconds, annealing at 55ºC for 30 seconds, and extension at 72ºC for 90 seconds. Final extension was at 72ºC for 5 min followed by cooling at 20ºC for 10 min. PCR products were electrophoresed on 1.2% (w/v) agarose gels in 1x TAE buffer and visualized by staining with SYBR Safe™ Safe DNA Gel Stain (ThermoFisher Scientific).

RESULTS AND DISCUSSION

Effect of acetosyringone concentration on callus growth following co-cultivation

After co-cultivation, some calli died in P1 medium, they were bleached and could not grow. The concentration of acetosyringone in culture medium significantly affected to the percentage of callus grow after co-cultivation. In the absence of *A. tumefaciens*, 0-40 mg/L acetosyringone had the highest callus growth rate (100-89%), which were significantly higher than that at 60 mg/L acetosyringone (57%; Figure 3). Acetosyringone is a phenolic compound required for *A. tumefaciens*-mediated transformation because it activates *vir* genes on Ti plasmid whose function is to excise the plasmid T-DNA region and promote its transfer and integration into the plant genome (Stachel *et al.* 1985). This study indicated that acetosyringone as a phenolic compound could be toxic to *K. alvarezii* callus at 60 mg/L concentration. High accumulation of phenolic compound in culture medium could become a toxic compound that ultimately damage or kill plant cells and tissues (Jones & Saxena 2013). Furthermore, co-cultivation in 60 mg/L acetosyringone with EHA101 resulted in a higher degree of callus grow (48 %) than did LBA4404 (25%; Figure 3). This showed that, in addition to the concentration of acetosyringone, *A. tumefaciens* strain used also affected callus growth, with the LBA4404 strain being more detrimental than EHA101 strain to calli of *K. alvarezii*.

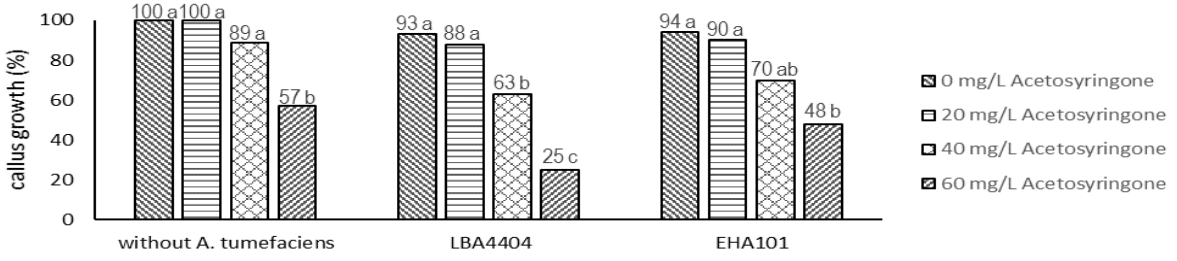

Figure 3 Percentage of callus growth in the absence or presence of *A. tumefaciens* LBA4404 or EHA101 at 0-60 mg/L acetosyringone (The numbers followed by the same letter are not significantly different based on Duncan's multiple-range test at $p < 0.01$, $n = 10$)

Sensitivity of *K. alvarezii* cells to hygromycin and selection of hygromycin-resistant cells

Callus induction (P1) medium was used for the sensitivity test. Micropropagules grown for one month in P1 medium without hygromycin formed calli at the tips of thalli (Figure 4A) and the average weight of micropropagules increased by 15.6 mg (Table 1). In P1 media containing hygromycin at 20-40 mg/L, callus growth decreased and so that the micropropagule weight gain also decreased (Table 1). The weight of micropropagules in 50 mg/L hygromycin actually decreased (-0.2 mg) after 1 month in P1 medium because micropropagule thallus cells died and were desiccated (Table 1). The death of micropropagule thallus cells was also indicated by a color change of the entire thalli of the micropropagules, which were previously brown, to white (100% bleaching; Figure 4B).

The sensitivity of algal cells to hygromycin has a large range. A lethal effect of hygromycin on gametophytes of the red algae *Porphyra yezoensis* was observed at 2 g/L (Takahashi *et al.* 2011). In contrast, the red algae *Griffithsia japonica* is sensitive at 5 mg/L hygromycin (Lee *et al.* 2000) while the green algae *Chlamydomonas reinhardtii* exhibits toxicity at 1 mg/L (Berthold *et al.* 2002). Therefore, it was necessary to test the sensitivity of *K. alvarezii* cells to hygromycin in order to determine an appropriate concentration of hygromycin for the selection of genetically transformed cells of *K. alvarezii*. The results of this study showed that *K. alvarezii* cells were sensitive to hygromycin at 50 mg/L and this concentration was chosen to select hygromycin-resistant callus cells of *K. alvarezii* after co-cultivation.

Co-cultivated calli were cultured in P1 medium for 2 months to induce additional callus growth before selection in 50 mg/L hygromycin (Figure 5A). Subsequently, the calli were subcultured in P1 medium containing 50 mg/L hygromycin. After 2 weeks, all calli were being bleached (Figure 5B), resistant callus cells were not visible at the time. All calli were subcultured to P1 medium lacking hygromycin to induce growth of resistance cells. After 3 months, the growth of hygromycin-resistant callus began to appear from bleached callus (Figure 5C).

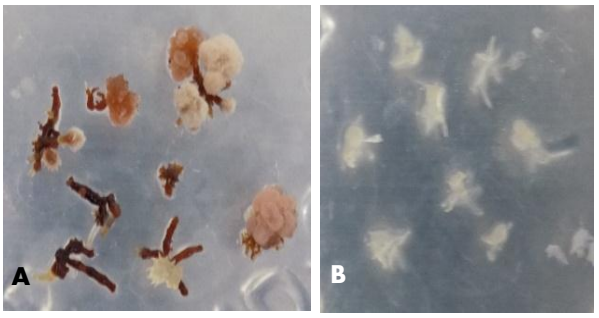

Figure 4 Growth of *K. alvarezii* micropropagules and calli after one month cultured in P1 medium: (A) without hygromycin; (B) containing 50 mg/L hygromycin

Table 1 The weight gain and percentage of micropropagule bleaching after one month in P1 medium containing hygromycin

Hygromycin (mg/L)	Mean initial weight (mg)	Mean final weight (mg)	Mean weight gain (mg)	Mean micropropagule bleaching (%)
0	6.3	21.9	15.6 *a*	0 *a*
20	6.4	7.1	0.7 *b*	73 *b*
30	6.1	6.2	0.1 *b*	71 *b*
40	6.3	6.5	0.2 *b*	96 *bc*
50	6.6	6.4	-0.2 *b*	100 *c*

Note: The numbers followed by the same letter are not significantly different based on Duncan's multiple-range test at $p < 0.01$ ($n = 7$).

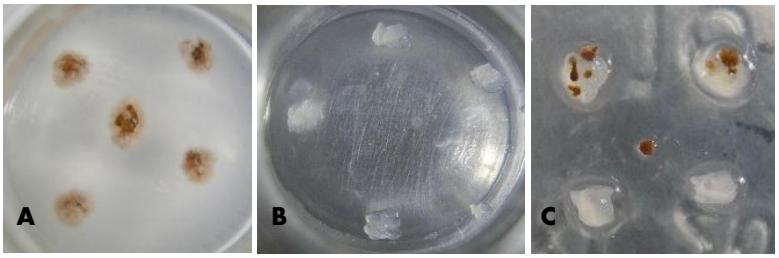

Figure 5 Selection of hygromycin-resistant callus: (A) co-cultivated callus before selection (P1 medium); (B) after 2 weeks selection in P1 medium containing 50 mg/L hygromycin; (C) growth of hygromycin-resistant callus after selection (brown callus).

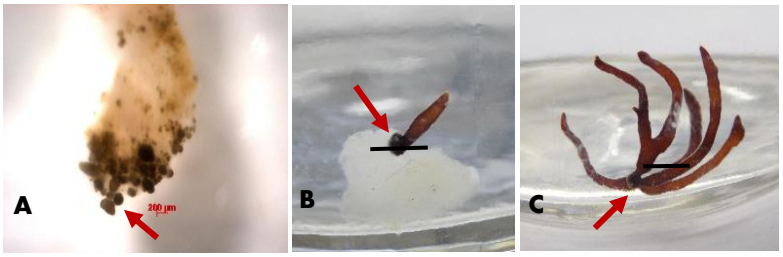

Figure 6 Regeneration of hygromycin-resistant callus into micropropagule: (A) growth of globular-shaped micropropagule (arrow) on embryogenic callus (observation under trinocular microscope); (B) growth of one and (C) several thallus buds on globular-shaped micropropagule (arrow) after 3 months in liquid P0 medium (Scale bars = 5 mm).

Regeneration of hygromycin-resistant callus into micropropagules

Regeneration of hygromycin-resistant callus into transgenic plantlets was conducted through the somatic embryogenesis technique. After one month in liquid P0 medium, the callus cells formed globular-shaped embryo/micropropagule (Figure 6A), which then each grew one thallus bud (Figure 6B). Subsequently, more thallus buds grew on each the globular shaped micropropagule to a length of 5-10 mm (Figure 6C) in the period 2-3 months. This is the first report on the success of obtaining transgenic micropropagules of *K. alvarezii* through somatic embryogenesis techniques. The use of liquid medium plays an important role in the morphogenesis of *K. alvarezii* callus into somatic embryo or micropropagule (Sulistiani & Yani 2014). In addition to the physical form of the medium, the use of PES medium composition is also crucial in the regeneration of micropropagule from *K. alvarezii* callus because the composition of this medium had complete trace metals and vitamins for optimal growth of macroalgae (Harrison & Berges 2005). Liquid PES medium has been widely reported to be very effective in morphogenesis of seaweed callus (Kumar *et al.* 2007; Baweja *et al.* 2009).

Development of somatic embryo in the plant can be divided into three general sequential morphological stages: globular-, heart-, and torpedo-shaped (Zimmerman 1993). Following this, the somatic embryo germinates to form shoot and root. This study showed that the development of the somatic embryo in the seaweed *K. alvarezii* is different than in plant. The embryogenic callus only develops into a globular-shaped embryos/micropropagules and then directly grow a thallus bud, which more resembles the development of the zygotic embryo of *K. alvarezii* i.e., carpospore (2n) and tetraspore (n), which germinated into young thallus (Azanza & Aliaza 1999).

Effect of acetosyringone concentration and *A. tumefaciens* strains on the growth of putative transgenic micropropagule

Based on analysis of variance, the *A. tumefaciens* strain factor did not significantly affect the percentage of calli that formed micropropagules, and neither did the combination of *A. tumefaciens* strain and acetosyringone concentration factor. However, acetosyringone concentration as a single factor significantly affects the percentage of micropropagules formed. Co-cultivation at 20-40 mg/L resulted in the highest degree of

micropropagule formation (22-28%) and was significantly different than at 60 mg/L (7%; Table 2). Since co-cultivation in 60 mg/L acetosyringone resulted in a lower callus growth rate (Figure 3), the possibility of obtaining hygromycin-resistant calli or micropropagules was lower at this concentration than with co-cultivation at 20-40 mg/L. Thus, the highest efficiency of gene transformation on *K. alvarezii* callus was obtained using 20-40 mg/L acetosyringone in co-cultivation medium.

Co-cultivation with LBA4404 resulted in the largest number of putative transgenic micropropagules at 20 mg/L acetosyringone (45 micropropagules; Table 3). This is because, in that co-cultivation, two calli produced 10-13 micropropagules even though the thallus size of these micropropagules was abnormal (shorter and smaller than normal size). In co-cultivation with EHA101, the highest number of micropropagules (25) were produced at 40 mg/L acetosyringone. The putative transgenic micropropagules from co-cultivation with EHA101 were mostly normal (Table 3). The percentage of normal micropropagules following co-cultivation with EHA101 (95-100%) was higher than from co-cultivation with LBA4404 (47-67%). Therefore, EHA101 was superior to LBA4404 for gene transformation of *K. alvarezii* callus.

The normal thallus of micropropagule exhibited a large degree of branching with thallus length 0.5-1 cm after 3 months in liquid P0 medium (Figure 7A). Abnormal micropropagules had shorter and smaller thalli (Figure 7B) and some did not even form a thallus branch (Figure 7C and 7D). In gene transformation using *A. tumefaciens*, the transgene is randomly inserted into the plant genome. The emergence of abnormal morphology in transgenic micropropagules is probably due to *Ga* genes inserting in genomic DNA at the site of genes that encode important factors for the normal morphological development of seaweed so that the genes can not expressed.

Tabel 2 Percentage of micropropagules formation (MF) after co-cultivation

Acetosyringone concentration (mg/L)	MF (%) after co-cultivation with		Mean of MF (%)
	LBA44044	EHA101	
20	22	22	22 *a*
40	24	32	28 *a*
60	0	14	7 *b*
Mean of MF	11.5	17	

Note: The numbers followed by the same letter are not significantly different based on Duncan's multiple-range test at $p < 0.01$ (n = 10).

Table 3 Putative transgenic micropropagule production from co-cultivation with *A. tumefaciens* at different acetosyringone concentrations

A. tumefaciens	Acetosyringone concentration (mg/L)	Number of micropro-pagule	Number of normal micropropagule	Percentage of Normal micropropagule (%)
LBA4404	20	45	21	47
	40	18	12	67
	60	0	0	0
EHA101	20	18	18	100
	40	25	24	96
	60	19	18	95

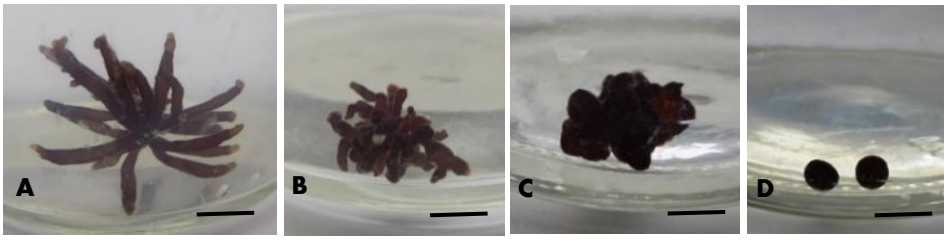

Figure 7 Morphology of putative transgenic micropropagules with: (A) normal thallus; (B-D) abnormal thallus (Scale bars = 5 mm).

Effect of *A. tumefaciens* strains on the growth of putative transgenic plantlets

Micropropagules grew into propagules/plantlets with 1-3 cm thallus lengths after 12 weeks in aerated culture. Based on an analysis of variance, the *A. tumefaciens* strains were used to transfer gene influenced the DGR of putative transgenic plantlets in aerated culture. A Duncan's multiple-range test at $p < 0.01$ ($n = 8$) showed the mean DGR of putative transgenic plantlets produced with LBA4404 co-cultivation (3.1%) was not significantly different from the mean DGR of non-transgenic plantlets (3.3%). However, the mean DGR of putative transgenic plantlets from EHA101 co-cultivation (4.1%) was significantly higher than the DGR of non-transgenic plantlets and putative transgenic plantlets from LBA4404 co-cultivation. This was because 6 of 8 putative transgenics derived from EHA101 co-cultivation had higher DGR than non-transgenics (E1, E2, E5, E6, E7, E8), while this was the case with only 2 putative transgenics derived from LBA4404 (L5 and L7; Figure 8).

Based on plantlet morphology, eight of the transgenic clones with high DGRs generally exhibited highly branched thalli (L5, L7, E1, E2, E5, E6, E7, E8). In aerated cultures, the thallus branches continued growing, and some separated to form new plantlets (Figure 9A). The separation of a thallus branch from the parent thallus resulted in faster growth. Conversely, non-transgenic and transgenic plantlets with DGRs lower than the eight clones (N1-N8, L1-L4, L6, L8, E3, E4) had fewer branches and exhibited no thallus branch separation (Figure 9B). This was probably due to the influence of the *Ga* genes that have been introduced into the transgenic plantlets, since *Ga* protein has been shown plays a role in regulating cell proliferation and promotes growth in *Arabidopsis* (Ullah, 2001; Chen *et al.* 2006) and Rice (Izawa, 2010).

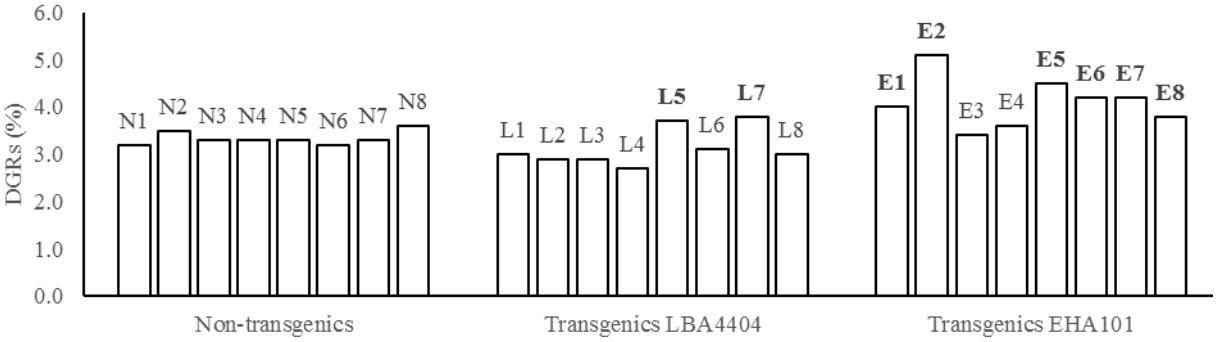

Figure 8 The daily growth rate (DGR) of non-transgenic plantlets (N1-8) and putative transgenics derived from co-cultivation with LBA4404 (L1-8) or EHA101 (E1-8) after 12 weeks in aerated cultures

Figure 9 Morphological comparison of non-transgenic (A) and putative transgenic (B; E5 clone) plantlets after 12 weeks in aerated culture (ruler grid = 1 mm)

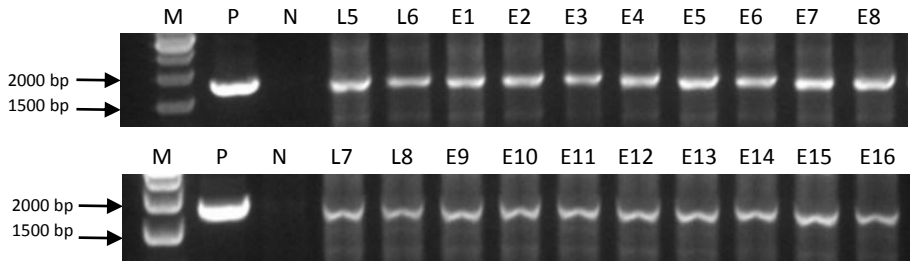

Figure 10 PCR amplification of genomic DNA of *K. alvarezii* transgenic plantlets with 35S-F and tNOS-R primer. M: DNA marker ladder, P: pGWB502-*Ga* plasmid, N: non-transgenic plantlet, L5-L8: transgenics derived from LBA4404 co-cultivation, E1-E16: transgenics derived from EHA101 cultivation.

PCR analysis using 35S-F and tNOS-R primers successfully amplified the 35S-*Ga*-tNos region (~1.9 kb; Fajri 2015) from the genomic DNA of twenty putative transgenic plantlets (Figure 10), including 12 clones which were already observed the DGRs in the aerated culture (L5-L8, E1-E8). DNA of pGWB502-*Ga* plasmid was used as a positive control and DNA of non-transgenic plantlets was used as a negative control. The result of PCR analysis confirmed that the *Ga* gene derived from the Slamet cultivar of soybean under the constitutive control of a CaMV 35S promoter had indeed been successfully introduced into the genomic DNA of all the putative transgenic plantlets examined. It also showed that PES medium containing 50 mg/L hygromycin was very effective in selecting genetically transformed callus cells of *K. alvarezii*.

Based on PCR analysis, *Ga* genes had been introduced into the genomic DNA of all transgenic plantlets, both with high DGRs (L5, L7, E1, E2, E5, E6, E7, E8) or normal DGRs (L6, L8) (Figure 10). It might be possible that the fast-growing transgenics possess a higher expression level of *Ga* genes than transgenics with nomal growth rate. Gene transformation on *K. alvarezii* callus using *A. tumefaciens* EHA101 strain yielded more transgenic with a high level of *Ga* gene expression than the LBA4404 strain. In the transgenic plant, transgene expression level is influenced by many factors such as the site of integration, gene silencing, and transgene copy number (Yang *et al.* 2005; Żmien'ko *et al.* 2014). We are currently determining the *Ga* gene expression levels of the *K. alvarezii* transgenics produced in this study using quantitative real-time PCR.

CONCLUSION

Transgenic plantlets of *K. alvarezii* carrying the *Ga* gene derived from soybean (*Glycine max* var Slamet) were successfully obtained. Gene transfer into *K. alvarezii* was more successful using *A. tumefaciens* strain EHA101 (with 20-40 mg/L acetosyringone) than with strain LBA4404, since it produced more morphologically normal transgenic plantlets that had DGRs that were higher than non-transgenic plantlets.

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