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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 food industries. 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 a 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 the 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*. The callus cells of *K. alvarezii* were transformed using *Agrobacterium tumefaciens* strains LBA4404 and EHA105 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). The regeneration of transformed callus cells into transgenic plantlets was successfully performed using the somatic embryogenesis technique. The results showed that the highest percentage of putative transgenic micropropagule formation occurred at the 20-40 mg/L concentration of acetosyringone. Polymerase chain reaction (PCR) analysis on the twenty transgenic plantlets indicated that the *Ga* gene was successfully introduced into the genomic DNA of all of them. The highest transformation efficiency was in the co-cultivation treatment of 20-40 mg/L acetosyringone (22-28%). The transformation efficiency produced by *Agrobacterium tumefaciens* EHA105 (23%) was not significantly different from that produced by the LBA4404 (15%).

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 the substance carrageenan which is known for its high economic values, particularly in the pharmaceuticals, foods and cosmetic industries as a stabilizer, thickener, gelling agent, and emulsifier. However, its cultivation is constrained by the ice-ice disease which causes whitening of the thallus (bleaching) that resulted in a decrease in the carrageenan content (Mendosa *et al.* 2002). The 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 makes the seaweed susceptible to pathogenic microorganisms such as the *Vibrio* marine bacteria and the *Cytophaga-Flavobacterium* group (Largo *et al.* 1999).

A possible approach to this problem is through genetic engineering that produces a transgenic seaweed that can tolerate extreme changes in seawater temperature and salinity 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.*

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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 al.* 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, the transgenic thallus buds grown from thallus explants were small and difficult to regenerate into plantlets that could be cultivated in coastal areas (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 Superoxide dismutase (SOD) gene, but the growth rates of the transgenic thallus exposed to environmental stresses had not been reported, so the effectiveness of the gene in increasing tolerance to environmental stress in transgenic thallus was still unclear. The authors then decided 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 co-cultivation of callus 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, the authors 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 EHA105 strains carrying the binary vector expression 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 were induced from the 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 the 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, the calli were 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 before this study began in 2015. Therefore, it was necessary to test the sensitivity to determine the concentration of hygromycin to be used in the 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 the thallus color were also recorded by taking photographs. 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 was then used in culture medium to select the hygromycin-resistant callus cells.

Preparation of *A. tumefaciens* Strains

This study used two strains of *A. tumefaciens* (LBA4404 and EHA105) as carriers of the pGWB502 binary vector containing hygromycin-resistant gene and *Ga* gene under the control of a constitutive 35S CaMV promotor (Fajri 2015; Fig. 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 was then centrifuged at 6000 rpm and 4˚C for 10 min and the pellets resuspended in PES medium (20 ppt salinity) containing acetosyringone.

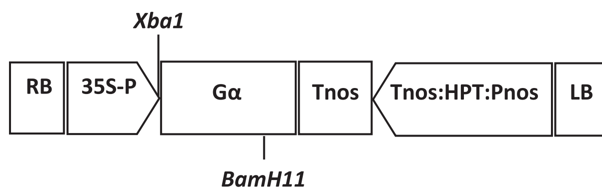

Figure 1 T-DNA regions of the pGWB502-*Ga* binary vector (Fajri 2015)

Co-cultivation and Selection of Hygromycin-resistant Callus

A. tumefaciens LBA4404 or EHA105 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, the 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. The 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 calli were subcultured in P0 medium for 2 months then again in liquid P0 medium (without Bacto agar; 1 callus/40 mL P0 medium) until the micropropagules were 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 EHA105, as well as the 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 for 12 weeks (Sulistiani & Yani 2014). During the micropropagule culture, the medium was continuously aerated using an air blower (aerated culture; Fig. 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 weights of the putative transgenic micropropagules were measured every week, and the 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. The 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'. 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 (Thermo Fisher Scientific).

RESULTS AND DISCUSSION

Effect of Acetosyringone Concentration on the Callus Growth following Co-cultivation

After co-cultivation, some calli died in P1 medium as they bleached and could not grow. The concentration of acetosyringone in the culture medium significantly affected the percentage of callus growth 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%; Fig. 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 EHA105 resulted in a higher degree of callus growth (48%) than did LBA4404 (25%; Fig. 3). This showed that, in addition to the concentration of acetosyringone, *A. tumefaciens* strain also affected the callus growth of *K. alvarezii*, with the LBA4404 strain being more detrimental than the EHA105 strain.

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 (Fig. 4A) and the average weight of micropropagules increased by 15.6 mg (Table 1). In P1 medium containing hygromycin at 20-40 mg/L, the 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 the micropropagule thallus cells died and were desiccated (Table 1). The death of the micropropagules was also indicated by a color change of the entire thalli of the micropropagules, which were previously brown but later turned white or underwent 100% bleaching (Fig. 4B).

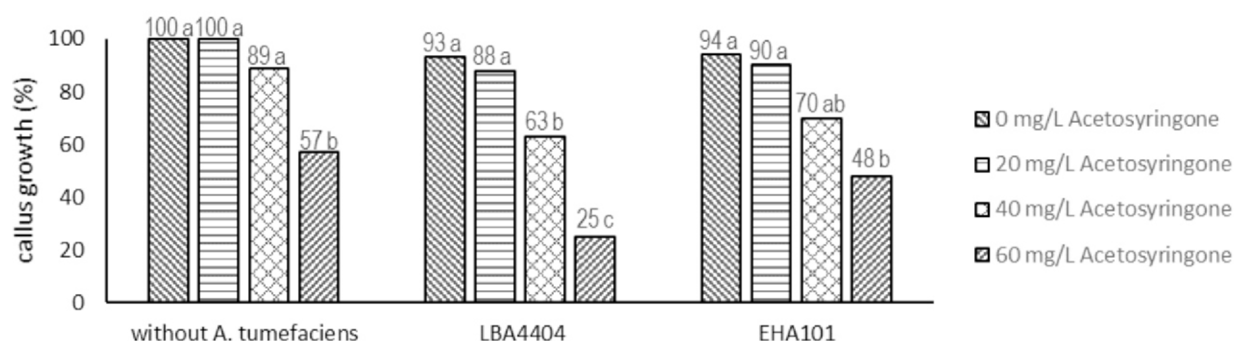

Figure 3 Percentage of callus growth in the absence or presence of *A. tumefaciens* LBA4404 or EHA105 at 0-60 mg/L acetosyringone (The means followed by the same letter are not significantly different based on Duncan's multiple-range test at p <0.01, n = 10)

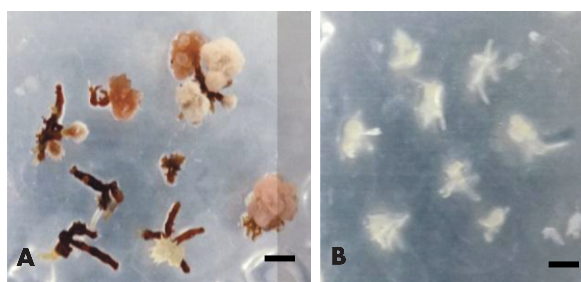

Figure 4 Growth of *K. alvarezii* micropropagules and calli after one month cultured in P1 medium: (A) without hygromycin; (B) containing 50 mg/L hygromycin (scale bars = 0.5 cm)

Table 1 The weight gains 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 means followed by the same letter are not significantly different based on Duncan's multiple-range test at p <0.01 (n = 7)

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.

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

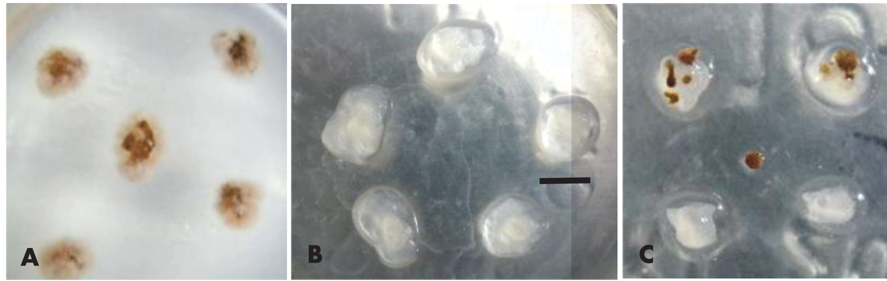

Figure 5 Selection of hygromycin-resistant callus: (A) co-cultivated callus before selection (P1 medium); (B) after 2 weeks selection in P1 medium containing 50 mg/L hygromycin; (C) growth of hygromycin-resistant callus after selection (brown callus) (scales bars = 5 mm)

Regeneration of Hygromycin-resistant Callus into Micropropagules

The 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 (Fig. 6A), which then each grew one thallus bud (Fig. 6B). Subsequently, more thallus buds grew on each of the globular shaped micropropagule to a length of 5-10 mm in 2-3 months (Fig. 6C). 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 was also crucial in the regeneration of micropropagules from *K. alvarezii* callus because the composition of this medium has complete trace metals and vitamins for the optimal growth of macroalgae (Harrison & Berges 2005). Moreover, liquid PES medium has been widely reported to be very effective in morphogenesis of seaweed callus (Kumar *et al*. 2007; Baweja *et al*. 2009).

The 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).

Transformation Efficiency and the Growth of Putative Transgenic Micropropagules

Determination of transformation efficiency (%) was carried out by calculating the percentage of co-cultivated calli which regenerated into putative transgenic micropropules. 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 factors. However, acetosyringone concentration as a single factor significantly affected the transformation efficiency. Co-cultivation at 20-40 mg/L resulted in the highest transformation efficiency (22-28%) and was significantly different than that at 60 mg/L (7%; Table 2). Since co-cultivation in 60 mg/L acetosyringone resulted in a lower callus growth rate (Fig. 3), the possibility of obtaining hygromycin-resistant calli or micropropagules was lower at this concentration than with co-cultivation at 20-40 mg/L.

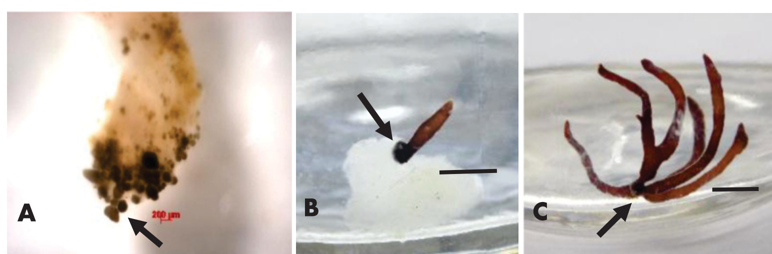

Figure 6 Regeneration of hygromycin-resistant callus into micropropagule: (A) growth of globular-shaped micropropagule (arrow) on embryogenic callus (observation under trinocular microscope); (B) growth of one and (C) several thallus buds on a globular-shaped micropropagule (arrow) after 3 months in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

Table 2 Transformation efficiency of *Kappaphycus alvarezii* in different acetosyringon concentration and *Agrobacterium tumefaciens* strains

Acetosyringone (mg/L)	Transformation efficiency (%)		Mean (%)
	LBA44044	EHA105	
20	22	22	22 *a*
40	24	32	28 *a*
60	0	14	7 *b*
Mean	15	23	

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

The results of this study indicated that the EHA105 strain produced higher transformation efficiency (23%) than transformation efficiency using the LBA4404 strain (15%) (Table 2), but based on analysis of variance these were not significantly different. This study was the first use of *A. tumefaciens* EHA105 on genetic transformation of macro algae. Previous genetic transformation studies on *K. alvarezii* used *A. tumefaciens* LBA4404 (Handayani *et al.* 2014; Triana *et al.* 2016), as well as seaweed *Porphyra yezoensis* (Cheney *et al.* 2001). In this study, the EHA105 strain resulted in quite high transformation efficiency (23%). This showed that besides LBA4404 strain, *A. tumefaciens* EHA105 was very potential to be used for genetic transformation of macro algae.

Co-cultivation with LBA4404 resulted in the largest number (45 micropropagules) of putative transgenic micropropagules at 20 mg/L acetosyringone (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 the co-cultivation with EHA105, the highest number of putative transgenic micropropagules were produced at 40 mg/L acetosyringone (25 micropropagules). The putative transgenic micropropagules from co-cultivation with EHA105 were mostly normal (Table 3). The percentage of normal micropropagules following co-cultivation with EHA105 (95-100%) was higher than that from co-cultivation with LBA4404 (47-67%). Therefore, EHA105 was superior to LBA4404 for gene transformation of *K. alvarezii* callus.

Table 3 The number of putative transgenic micropropagules from co-cultivation with *Agrobacterium tumefaciens* LBA4404 and EHA105 at several acetosyringone concentrations

A. tumefaciens	Acetosyringone concentration (mg/L)	Number of micropropagule	Number of normal micropropagule	Percentage of Normal micropropagule (%)
LBA4404	20	45	21	47
	40	18	12	67
	60	0	0	0
EHA105	20	18	18	100
	40	25	24	96
	60	19	18	95

The normal thallus of micropropagule exhibited a large degree of branching with thallus lengths of 0.5-1 cm after 3 months in liquid P0 medium (Fig. 7A). Abnormal micropropagules had shorter and smaller thalli (Fig. 7B) and some did not even form a thallus branch (Fig. 7C, 7D). In gene transformation using *A. tumefaciens*, the transgene is randomly inserted into the plant genome (Hooykaas & Schilperoort 1992; Qu & Qin 2014; Hwang *et al.* 2017). The emergence of an abnormal morphology in transgenic micropropagules is probably due to inserting of *Ga* genes into the genomic DNA at the site of genes that encode important factors for the normal morphological development of seaweed so that the genes can not be expressed (Qu & Qin 2014).

Growth of Putative Transgenic Plantlets

The micropropagules grew into propagules or plantlets with 1-3 cm thallus lengths after 12 weeks in aerated culture. Based on the analysis of variance, the *A. tumefaciens* strains used to transfer the gene had influenced the daily growth rate (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 EHA105 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 EHA105 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; Fig. 8).

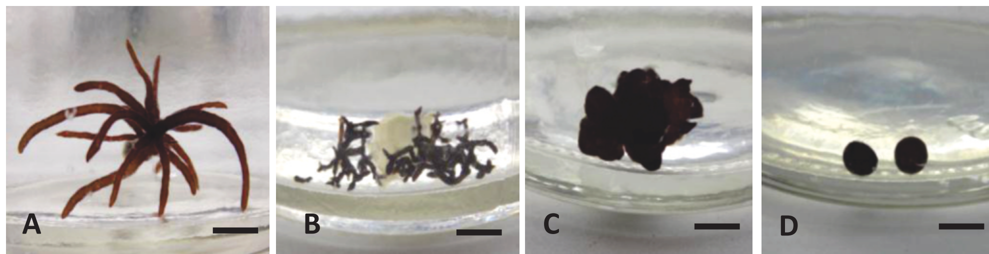

Figure 7 Morphology of putative transgenic micropropagules with: (A) normal thallus; (B-D) abnormal thallus (scale bars = 5 mm)

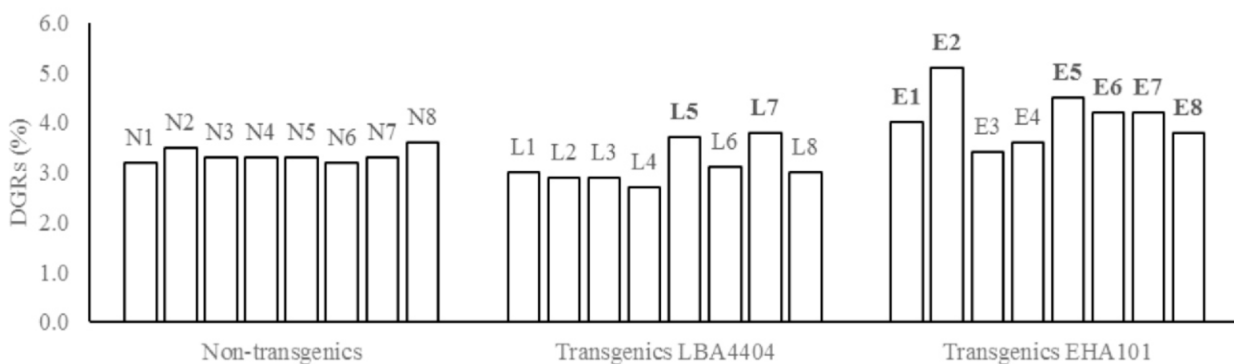

Figure 8 The daily growth rate (DGR) of non-transgenic plantlets (N1-8) and putative transgenics derived from co-cultivation with LBA4404 (L1-8) or EHA105 (E1-8) after 12 weeks in aerated cultures

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 (Fig. 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 (Fig. 9B). This was probably due to the influence of the *Ga* genes that had been introduced into the transgenic plantlets, since *Ga* protein had been shown to play a critical role in regulating cell proliferation and promoting growth in *Arabidopsis* (Ullah 2001; Chen *et al.* 2006) and Rice (Izawa *et al.* 2010).

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

Integration of *Gα* Gene in the Genome of Transgenic Plantlets

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, including 12 clones with DGRs which were already observed in the aerated culture (L5-L8, E1-E8) (Fig. 10). DNA of pGWB502-*Ga* plasmid was used as a positive control and DNA of non-transgenic plantlets was used as a negative control. The results 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 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) (Fig. 10). The fast-growing transgenics possibly possess a higher expression level of *Ga* genes than those transgenics with nomal growth rates. Gene transformation on *K. alvarezii* callus using *A. tumefaciens* EHA105 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).

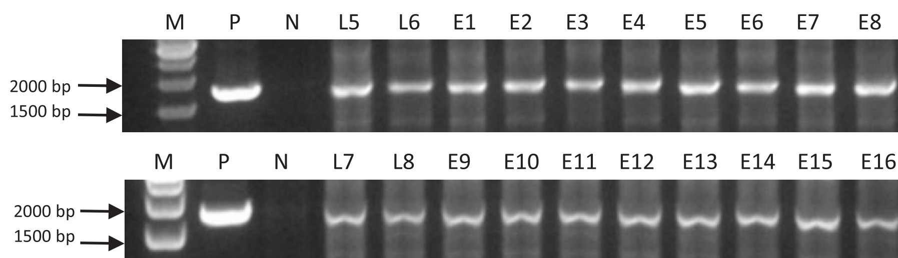

Figure 10 PCR amplification of genomic DNA of *K. alvarezii* transgenic plantlets with 35S-F and tNOS-R primer. M: DNA marker ladder, P: pGWB502-*Ga* plasmid, N: non-transgenic plantlet, L5-L8: transgenics derived from LBA4404 co-cultivation, E1-E16: transgenics derived from EHA105 cultivation.

CONCLUSION

Transgenic plantlets of *K. alvarezii* carrying the *Ga* gene derived from soybean (*Glycine max* var Slamet) were successfully produced. The highest transformation efficiency was found in 20-40 mg/L acetosyringone treatment and significantly different from that at 60 mg/L acetosyringone treatment. The transformation efficiency from co-cultivation with *A. tumefaciens* EHA105 (23%) was higher than that from co-cultivation with the LBA4404 (15%), but not significantly different.

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