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BINARY VECTOR CONSTRUCTION AND *Agrobacterium tumefaciens*-MEDIATED TRANSFORMATION OF LYSOZYME GENE IN SEAWEED *Kappaphycus alvarezii*

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ABSTRACT

Ice-ice disease is the biggest problem in the cultivation of seaweed *Kappaphycus alvarezii*. The disease is caused by bacterial infection and induced by drastic changes of water quality. Lysozyme has the ability to break down bacterial cell wall. The purpose of this research was to construct of a binary vector pMSH1-Lys carrying chicken Lysozyme (Lys) gene and to introduce pMSH1-Lys on *K. alvarezii*. The binary vector expression was transformed into *Agrobacterium tumefaciens* LBA4404 by triparental mating. Thallus was inoculated with *A. tumefaciens* carrying pMSH1-Lys and then the transformed thallus was selected by adding 20 mg/L hygromycin to the culture medium. Polymerase Chain Reaction (PCR) analysis showed that the construction of the binary plasmid pMSH1-Lys was established. Percentage of pMSH1-Lys transformation on *K. alvarezii* was 23.56%, while the efficiency of regeneration was 11.32%. PCR analysis showed that three of the regenerated thallus contained Lysozyme gene. Thus, transgenic *K. alvarezii* was successfully produced. These findings can be useful for studying the mechanisms of seaweed defense against bacterial infection.

Keywords: *Agrobacterium tumefaciens*, genetic transformation, *Kappaphycus alvarezii*, Lysozyme

INTRODUCTION

Kappaphycus alvarezii (Doty) is economically important red tropical seaweed highly demanded for its cell wall polysaccharides, being the most important source of kappa-

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carrageenan in the world (Bixler 1996). Carrageenan is used in industries including food, textile, cosmetic, pharmaceutical and photography (Yu *et al.* 2002). Kappa-carrageenan is used as stabilizers, thickeners and emulsifiers (Bixler 1996).

Seaweed production can be increased by extensification and improvement of cultivation method. However, ice-ice disease is the biggest problem in seaweed cultivation. Ice-ice infection can spread in broad area of cultivation for a week, and cause thallus damage about 60-80% within 1-2 months (Sulistijo, Personal communication, 2002). Carrageenan content in the ice-ice infected seaweed decreases (Amiluddin 2007), and this leads to loss for farmers (Yulianto & Mira 2009).

Bacteria are suspected as causative agents of ice-ice disease, including *Pseudomonas nigricaciens*, *P. fluorescens, Vibrio granii, Bacillus cereus* and *V. agarliquefaciens*. *Vibrio agarliquefaciens* shows the highest pathogenicity (Nasution 2005). Largo (2002) also found *Vibrio* sp. on ice-ice infected thalli. Furthermore, Aris (2011) reported five bacteria on ice-ice infected *K. alvarezii* thalli, namely *Flavobacterium meningosepticum, V. alginoliticus, Pseudomonas cepacia, P. diminuta* and *Plesiomonas shigelloides*.

Genetic engineering techniques can be used to make seaweed resistant to bacterial infections. As the first step, Takahashi *et al.* (2010) had transformed glucuronidase gene into *Porphyra yezoensis* thallus by particle bombardment. Huddy *et al.* (2012) had also successfully introduced the LacZ gene into the *Gracilaria gracilis* thallus by particle bombardment. In plant transgenic production, a foreign gene is generally transferred using *Agrobacterium tumefaciens*. The method has advantages such as having relatively low cost, small copy number of gene and reproducibility (Hiei *et al.* 1997). *A. tumefaciens* contains binary expression vector. The first vector is part of the virulent *A. tumefaciens,* but without helper T-DNA, while the second vector is smaller and contains a T-DNA gene to be inserted (Loeidin 1994).

Lysozyme has been used to generate transgenic fishes that are resistant to bacterial infections, such as zebrafish (Yazawa *et al.* 2006), shrimp (*Litopenaeus vannamei*) (Burge *et al.* 2007) and salmon (*Salmo salar* L.) (Fletcher *et al.* 2011). Lysozyme is small ubiquitous antibacterial enzyme that hydrolyzes β-1, 4-linked glycoside bonds of peptidoglycan, a major cell wall component of gram-positive bacteria (Li *et al.* 2008). In addition, lysozyme is also able to kill gram-negative bacteria infecting bivalves and shrimp (Burge *et al.* 2007). Yazawa *et al.* (2006) established a transgenic zebrafish strain expressing chicken lysozyme gene. In the challenge experiment, 65% of the F2 transgenic zebrafish survived when infected by *Flavobacterium columnare* and 60% fish survived when infected by *Edwardsiella tarda*, whereas all non-transgenic fish were died. The lysozyme lytic activity of F2 in transgenic salmon was 40% greater than that in non-transgenic siblings (Fletcher *et al.* 2011). Chicken lysozyme possessed lytic activities against *Micrococcus lysodeikticus, Flavobacterium columnare, Aeromonas hydrophilla* and *Vibrio anguillarum* (Yazawa *et al.* 2006). Thus, chicken lysozyme is an important component of immune defense against diverse bacterial infections that can be exploited to increase seaweed resistance against pathogens. The aims of this study were to construct a binary plasmid carrying chicken lysozyme gene and to transfer it into *K. alvarezii* thallus by using *A. tumefaciens*.

MATERIALS AND METHODS

Kappaphycus alvarezii and Sterilization

Kappaphycus alvarezii thalli with green color were obtained from Lampung, Sumatera Island, Indonesia. Thalli were maintained in fiber tanks with a flow-through water system, aerated and under a 12/12 hours (light/dark) photoperiod at room temperature. *Kappaphycus alvarezii* thalli were cut about 3 cm in length and then sterilized by 1% iodine solution and detergent. The thalli were maintained in Prevasoli (PES) medium liquid until they were ready to be transformed.

Construction of Lysozyme-expressing Plasmid

Binary vector pMSH1 (Nara Institute of Science and Technology, Japan; Fig. 1) was used for the construction of Lysozyme (Lys) expression vector. Lys gene was amplified from pJfKer-Lys (Yazawa *et al.* 2005; Fig. 2) using specific PCR primers Lys-F: 5'-GCA CTA GTG GCA ACA TGA GGT CTT TGC-3' and Lys-R: 5'-TTG CGG CCG CTC CTC ACA GCC GGC AGC-3'. PCR was performed with 2 minutes initial denaturation at 94 °C and then 35 cycles were run at: 30 seconds denaturation at 94 °C, 30 seconds annealing at 64 °C (at 59 °C for Nos terminator) and 1 minute of extension at 72 °C. The amplified products were electrophoresed on a 2% agarose gel with 1x TAE buffer. The DNA of pMSH1 and PCR product of Lys gen were cut by restriction enzymes *Not*I and *Spe*I. Lys gene was ligated with *Not*I and *Spe*I-digested pMSH1. This recombinant binary vector was designated as pMSH1-Lys. Plasmid pMSH1-Lys was then transformed into *Escherichia coli* DH5α by heat shock (Suharsono *et al.* 2002). Transformant *E.coli* DH5α was identified by PCR method using Lys specific primer, and restricted using *Not*I and *Spe*I enzymes.

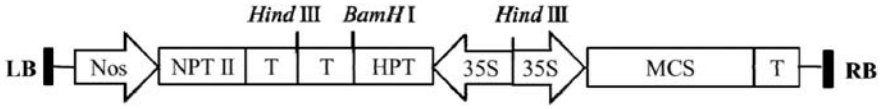

Figure 1. Map of the T-DNA plasmid pMSH1 (NAIST, Japan). NPT II: neomycin phosphotransferase II, a selection marker gene. HPT: hygromycin phosphotransferase, a selection marker gene. MCS: multi cloning site, regions of target genes that is controlled by the cauliflower mosaic virus 35S promoter (CaMV 35S) and terminator (T) nopaline synthase (Nos), containing *Xba*I, *Xho*I, *Sac*I, *Sma*I, *Kpn*I, *Spe*I, *Not*I, *BamH*I

Transformation of pMSH1-Lys into *Agrobacterium tumefaciens*

Plasmid pMSH1-Lys was transformed into *A. tumefaciens* by tri-parental mating (Liberty *et al.* 2008). Tri-parental mating was carried out using *E. coli* DH5α carrying pMSH1-Lys (Kan^R, Hyg^R) as the donor strain, *E. coli* DH1 carrying pRK2013 (Kan^R) as

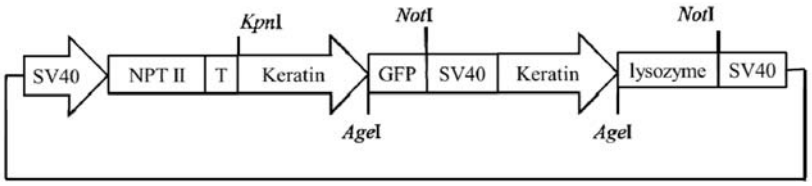

Figure 2. Map of pJfKer-Lys (Yazawa *et al.* 2005). Chicken lysozyme gene is controlled by the promoter of keratin (keratin) Japanese flounder (*Paralichthys olivaceus*). SV40: simian virus 40 terminator. NPT II: neomycin phosphotransferase, GFP: green flourescense protein

the helper strain, and *A. tumefaciens* LBA4404 (Strep^{R}) as the recipient. *A. tumefaciens* was grown at room temperature on LB medium with 50 μg/L streptomycin. *E. coli* DH5α containing pMSH1-Lys was grown at 37 °C on LB medium with 50 mg/L kanamycin and 50 mg/L hygromycin. *E. coli* DH1 bearing helper plasmid pRK2013 was grown at 37 °C on LB medium with 50 μg/L kanamycin.

About 20 μL bacteria consisted of *E. coli* DH5α, *E. coli* DH1 and *A. tumefaciens* were cultured together (conjugation="konjugasi") for 36 hours at room temperature. *A. tumefaciens* conjugated product was identified by antibiotic selection (kanamycin 50 mg/L, hygromycin 50 mg/L and streptomycin 50 mg/L), and PCR method using specific primers: for Lys gene was Lys-F: 5'-GCA CTA GTG GCA ACA TGA GGT CTT TGC-3' and Lys-R: 5'-TTG CGG CCG CTC CTC ACA GCC GGC AGC-3'; for 35S CaMV promoter was 35SCaMV-F: 35S-F: 5'-ATG GCT GGA GTA TTA GCT GGG-3' and Lys-R: 5'-TTG CGG CCG CTC CTC ACA GCC GGC AGC -3; for Nos terminator was Lys-F: 5'-GCA CTA GTG GCA ACA TGA GGT CTT TGC-3' and Nos-R: 5'-CTC ATA AAT AAC GTC ATG CAT TAC A-3'. PCR was performed with 2 minutes initial denaturation at 94 °C and then 35 cycles were run at: 30 seconds denaturation at 94 °C, 30 seconds annealing at 64 °C (at 59 °C for Nos terminator) and 1 minute extension at 72 °C. The amplified PCR products were electrophoresed on 2% agarose gel with 1x TAE buffer.

Preparation of *Agrobacterium* Suspension for Co-cultivation

A single colony of *A. tumefaciens* containing pMSH1-Lys was incubated in 5 mL of LB medium and grown for 36 hours on a 200 rpm shaker at room temperature. The bacterial culture was refreshed in 10 mL of LB medium and grown for 18 hours on a 200 rpm shaker at room temperature to an OD600 of 0.5-1.0. The bacterial culture was centrifuged at 5,000 rpm and the pellet was resuspended in 25 mL of liquid suspension medium containing PES and 100 μM acetosyringone. The suspension was used for thalli transfection.

Production of Transgenic *Kappaphycus alvarezii*

Transgenic *K. alvarezii* were produced as previously reported (Cheney *et al.* 2001). Seaweed thalli (1-2 cm in length) were wounded by sterile syringe and infected by *A. tumefaciens* carrying pMSH1-Lys for 30 minutes in suspension medium containing PES and 100 μM acetosyringone. The infected thalli were transferred to co-cultivation medium (PES medium containing 100 μM acetosyringone) for 3 days and then transferred to recovery medium (PES medium without acetosyringone) for 7 days. Finally, transgenic thalli were selected in PES medium containing hygromycin 20 mg/L for 14 days.

Detection of Lysozyme Gene in Transgenic *K. alvarezii*

Genomic DNA was extracted from putative bud of transgenic *K. alvarezii* using CTAB reagent (Doyle & Doyle 1987). DNA sample (1 μL) was used in a 10 μL PCR mixture. The PCR primers for Lys detection were Lys-F and Lys-R; 35S CaMV-F and Lys-R; and Lys-F and NosT-R. PCR was performed with 2 minutes initial denaturation at 94 °C and then 35 cycles were run at: 30 seconds denaturation at 94 °C, 30 seconds annealing at 64 °C (at 59 °C for Nos terminator) and 1 minute extension at 72 °C. The amplified PCR products were electrophoresed on a 2% agarose gel with 1x TAE buffer.

RESULTS AND DISCUSSION

Construction of Lysozyme Expressing Plasmid

Construction of binary plasmid was done by ligating chicken Lys gene (460 bp) and pMSH1 (12,986 bp). Result of the ligation was 13,449 bp (Fig.3A Lane 1). Verification of *E. coli* DH5α containing pMSH-Lys was performed by digesting pMSH1-Lys with *Not*I and *Spe*I restriction enzymes. The restriction products were two fragments in size of 12,986 bp and 460 bp (Fig.3A Lane 2). The 12,986 bp fragment was the size of pMSH1, and 460 bp was Lys gene fragment. *E. coli* DH5α carrying pMSH1-Lys was also identified using PCR with primers Lys-F and Lys-R; 35S-F and Lys-R, and Lys-F and Nos-R. The results of PCR analysis using those primers were 460 bp, 670 bp and 580 bp, respectively (Fig. 3B). Based on the results, it was concluded that the binary vector pMSH1-Lys was established.

PCR verification and enzyme restriction performed above also confirmed that transformation of pMSH1-Lys into *E. coli* DH5α had been successful. Plasmid pMSH1-Lys contained genes encoding protein resistant to antibiotics. Thus, bacterial colony survived and grew in selective medium containing kanamycin and hygromycin was a pMSH1-Lys transformed *E. coli* DH5α. The pMSH1-Lys could then be used for transformation into *A. tumefaciens*.

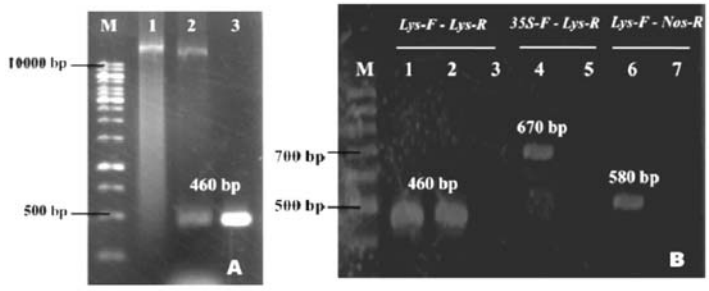

Figure 3. A. Pattern restriction of pMSH1-Lys using *Not*I and *Spe*I enzymes. M: 1 kb DNA ladder marker (Fermentas). Lane 1: plasmid of pMSH1-Lys, Lane 2: *Not*I and *Spe*I digested pMSH1-Lys and Lane 3: PCR product of Lysozyme gene (Lys). B. Identification of *Escherichia coli* DH5α containing Lysozyme gene using PCR with primers Lys-F and Lys-R (Lanes 1, 2 and 3), 35S-F and Lys-R (Lanes 4 and 5) and Lys-F and Nos-R (Lanes 6 and 7). M: 100 bp DNA ladder marker (Fermentas), Lanes 1, 4 and 6 are DH5α containing pMSH1-Lys. Lane 2: positive control, pJfKer-Lys. Lanes 3, 5 and 7 are the negative control (non-transformant Dh5α)

Transformation of pMSH1-Lys into *Agrobacterium tumefaciens*

Transformation of pMSH1-Lys into *A. tumefaciens* was performed by tri-parental mating (Fig.4). Plasmid pMSH1-Lys in *E. coli* DH5α (as a donor) was transferred into *A. tumefaciens* (as a recipient) through the conjugation process by pRK2013 in *E. coli* DH1 as a helper (Fig. 4A labelled as "konjugasi"=conjugation). *E. coli* DH5α containing pMSH1-Lys was resistant to kanamycin and hygromycin, but susceptible to streptomycin. *A. tumefaciens* was resistant to streptomycin, but susceptible to kanamycin and hygromycin. Thus, bacteria grew on the selective medium was *A. tumefaciens* containing pMSH1-Lys derived from tri-parental mating (Fig.4B). *A. tumefaciens* non-transformant was not grown on the selective medium (Fig. 4C).

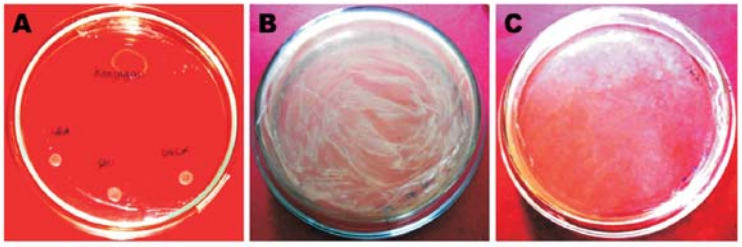

Figure 4. Triparental Mating. A. Results of tri-parental mating grown on LA medium without antibiotics. B. *Agrobacterium tumefaciens* LBA 4404 transformants on selective medium containing 50 mg/L hygromycin, 50 mg/L kanamycin and 50 mg/L streptomycin. C. *A. tumefaciens* LBA 4404 non-transformants did not grow on selective medium

Identification of *A. tumefaciens* transformants was performed by PCR method. As shown in Figure 5, PCR amplification products using Lys-F/Lys-R primer was 460 bp (Lanes 1 and 2), using primers 35S-F/Lys-R was 670 bp (Lanes 4 and 5), and using Lys-F/Nos-R primer was 580 bp (Lanes 7 and 8). This result showed that *A. tumefaciens* contained pMSH1-Lys, and it could then be used for transformation into *K. alvarezii*.

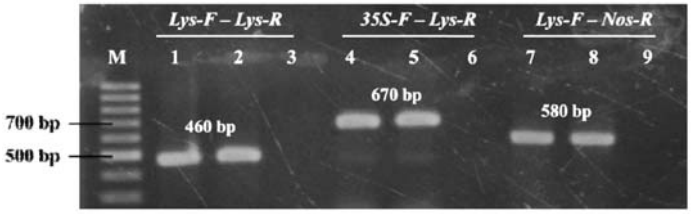

Figure 5. Identification of *Agrobacterium tumefaciens* containing pMSH1-Lys by PCR method. Lanes 1, 2 and 3: PCR product using primers Lys-F and Lys-R. Lanes 4, 5 and 6: PCR product using primers 35S-F and Lys-R. Lanes 7, 8 and 9: PCR product using primers Lys-F and Nos-R. M: 100 bp DNA ladder marker (Fermentas). Lanes 1, 4 and 7 are *A. tumefaciens* LBA4404 from tri-parental mating. Lanes 2, 5 and 8 are a positive control (DH5α containing pMSH1-Lys). Lanes 3, 6 and 9 are a negative control (non-transformant *A. tumefaciens* LBA4404)

Transformation of pMSH1-Lys into *Kappaphycus alvarezii*

K. alvarezii thalli were adapted on the PES culture medium (liquid and solid), and then were transformed with pMSH1-Lys by *A. tumefaciens* (Fig.6). The result showed that after 30 minutes infection with *A. tumefaciens* at OD600 of 0.5 to 0.8, the transformed thalli could grow in PES medium containing 100 μM acetosyringone (Fig.6). The transformed thalli could grow on selective medium containing 20 mg/L hygromycin (Fig. 6A-C). Non-transformant thalli was gradually dead on selective medium containing 20 mg/L hygromycin (Fig. 6D-F). Wild type thalli could grow on PES medium (Fig. 6G-I).

In total, 53 out of 225 transformed thalli (23.56%) grew on hygromycin selective medium (Table 1). This number was lower compared to percentage of gene LacZ transformation on *Gracilaria changii* using particle bombardment methods (80-94%). The result found in this study was thought to be affected by the difference of transformation method. In the next study, genetic transformation protocols of *K. alvarezii* need to be improved for increasing the transformation percentage. There were 3 (11.32%) thalli succeeded to germinate. Efficiency of putative sprouting ratio was determined by the number of putative sprouting thalli grew on hygromycin selective medium. The result showed that the efficiency of putative germination transformed thalli was lower than those of non-transformed ones (22%). It might be caused by *Agrobacterium* infection and antibiotic selective medium. This finding was in accordance with Suma *et al.* (2008) that the addition of antibiotics in selective medium

can cause thallus growth and development decrease. Optimum infection duration and different treatment methods on selective medium in future research is expected to result in higher germination efficiency. Transformation percentage in this study was 23.56% (Table 1).

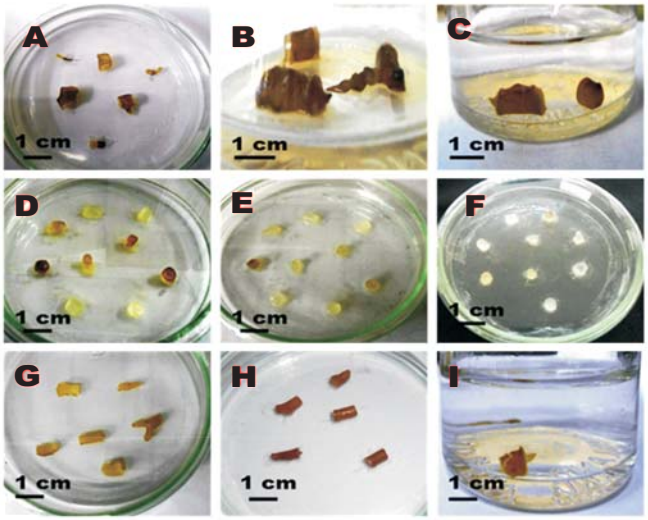

Figure 6. Development of *Agrobacterium tumefaciens* transformed *Kappaphycus alvarezii* thalli at 4 (A), 8 (B) and 12 (C) weeks culture in selective medium containing 20 mg/L hygromycin. Non-transformant thalli at 4 (D), 8 (E) and 12 (F) weeks culture in selective medium containing 20 mg/L hygromycin. Non-transformant thalli in the medium without hygromycin at 4 (G), 8 (H) and 12 (I) weeks culture. In D-F, green color indicates survived thalli, while the white color indicates dead thalli

Table 1. Transformation percentage and putative bud of *Kappaphycus alvarezii* containing lysozyme gene

Treatment	Number of thalli	Number of hygromycin resistant thalli	Percentage of transformation[a)]	Number of putative bud	Number of positive by PCR	Efficiency of putative bud[b)]
Transformation	225	53	23.56%	6	3	11.32%
Control -[1)]	50	0	0	0	0	0
Control +[2)]	50	-	0	11	0	22%

Notes:[a)] The percentage of the number of hygromycin-resistant thalli versus the total number of thalli transformed

[b)] The percentage of the number of thalli that sprouts putative versus number of hygromycin-resistant thalli

[1)] Non-transgenic thalli in hygromycin selective medium

[2)] Non-transgenic thalli in non-hygromycin medium

Presence of Lys gene in *K. alvarerzii* was confirmed by PCR method using three primers set. As shown in Figure 7, transgenic thalli (Lanes 1, 4 and 7) possessed PCR amplicon in the same size with the positive control of pMSH1-Lys plasmid (Lanes 2, 5 and 8), while the non-transgenic thalli showed no amplicon. This indicated that the putative bud derived from *A. tumefaciens* transformation was transgenic carrying Lys gene.

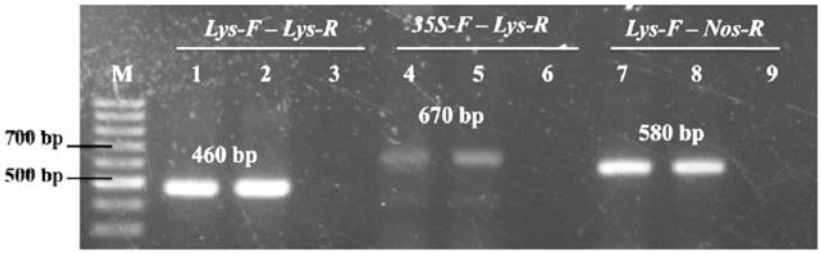

Figure 7. Lysozyme gene detection in *Agrobacterium tumefaciens* transformed *Kappaphycus alvarezii* thalli by PCR method. Lanes 1, 2 and 3: PCR products using Lys-F and Lys-R primers; Lanes 4, 5 and 6 using 35S-F and Lys-R primers; Lanes 7, 8 and 9 using primers Lys-F and Nos-R primers. M: 100 bp DNA ladder marker (Fermentas). Lanes 1, 4 and 7 are transgenic thalli; Lanes 2, 5 and 8 are positive control (plasmid of pMSH1-Lys); Lanes 3, 6 and 9 are negative control (wild type of seaweed).

This study is the first transgenic *K. alvarezii* production using *A. tumefaciens* infection method and Lys gene as the transgene. Analysis of Lys gene activity in transgenic *K. alvarezii* againts ice-ice bacterial agent is in progress. This transgenic *K. alvarezii* can also be useful for studying the mechanisms of seaweed defense against bacterial infection. In addition, transgenic seaweed resistance to ice-ice disease can be useful to ensure seaweed production in the season when ice-ice disease frequently infects. Transformation method developed in this study is beneficial to produce other transgenic seaweed expressing protein that regulates important traits in aquaculture. Examples of interesting genes that can be used to generate transgenic seaweed are cooper/zinc superoxide dismutase (CuZn-SOD) and omega-3 highly unsaturated fatty acids (n-3 HUFA) metabolic enzymes. Those enzymes had been used in plant transgenic production. CuZn-SOD had successfully been transformed in *Nicotiana tabacum* to increase its resistant to environmental stress (Hannum 2012). Transgenic *Arabidopsis* expressing n-3 HUFA metabolic enzymes had also been generated (Robert *et al*. 2005).

CONCLUSIONS

Construction of the binary plasmid pMSH1-Lys was established with size of 13,449 bp. *Agrobacterium tumefaciens* mediated transformation of lysozyme gene could produce transgenic *Kappaphycus alvarezii*. The efficiency of putative bud was 11.32 %, while percentages of positive transformants were approximately 23.56%.

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