

Evaluation of different promoters driving the GFP reporter gene in seaweed *Kappaphycus alvarezii*

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ABSTRACT

Promoter regulates expression level of foreign gene in transgenic organism. This study was performed to select a suitable promoter as the first step towards production of valuable trait-enhanced seaweed by transgenic technology. Green fluorescent protein (GFP) gene was used as a reporter to determine the activity of promoter in seaweed *Kappaphycus alvarezii*. GFP gene constructs driven by cytomegalovirus (pCMV-GFP), cauliflower mosaic virus (pCaMV-GFP), medaka β -actin (pmBA-GFP) and Japanese flounder keratin (pJfKer-GFP) promoters were introduced by electroporation method. Electroporation was performed using a gene pulser (BIORAD) with voltage of 300 V, pulse length of 0.5 ms, pulse numbers of 4, and pulse interval of 0.1 s. Promoter activity was determined by analyzing GFP gene expression level using a fluorescent microscope. The results showed that CMV regulated highest number of filament callus (34.10%±1.49) expressing GFP at medium to strong fluorescence levels. CaMV promoter had relatively similar activity with CMV, but lower number of filament callus expressing GFP (10.48%±0.25). mBA promoter drove GFP expression at medium level and similar number of filament callus (8.85%±2.31) expressing GFP with CaMV, while JfKer promoter had lowest activity by means in number of filament callus expressing GFP (4.79%±0.26) and GFP expression level. PCR analysis for transgenic confirmation showed a DNA band of PCR product from pCMV-GFP and pCaMV-GFP expressing filament callus in the same size (about 0.6 kb) with positive control of plasmid. Thus, CMV and CaMV promoters was an appropriate promoter and foreign gene could be transferred to filament callus by electroporation method. Combining this achievement with developing a culture method of filament callus to be thallus, stable transgenic breeding in *K. alvarezii* can be feasible.

Keywords: *transgenic, promoter, GFP, electroporation, filament callus, Kappaphycus alvarezii*

Introduction

Kappaphycus alvarezii is one of the major sources of kappa-carrageenan for food, chemical and pharmaceutical industries (McHugh, 2003). This seaweed has been prioritized by the Ministry of

Marine Affairs and Fisheries as one of aquaculture commodities targeted to increase on 2014. Increased production of seaweed farming can be achieved through extension and use of high quality seeds in terms of fast growth, resistant to disease and fluctuation of environmental conditions. Seaweed farming extensification program conducted by the Directorate of Aquaculture successfully increased production of seaweed. However, various problems faced in achieving the production target such as the growth rate being decrease due to disease infection and

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environmental degradation (Vairappan, 2006; Largo *et al.*, 1997). In addition, disease and environmental factors are also affecting the quality and quantity of carrageenan (Vairappan *et al.*, 2008). On the other hand, the increase in carrageenan consumption worldwide requires more production and high level of bioactive compound in farmed seaweed. Genetic engineering techniques have therefore been expected to improve seed stocks of seaweeds (Hallman, 2007). In fact, transgenic research have been conducted on *Laminaria japonica* using β -glucuronidase (Li *et al.*, 2009), *Kappaphycus alvarezii* using *LacZ* gene (Wang *et al.*, 2010) and *Gracilaria changii* using *LacZ* gene as reporter (Huddy *et al.*, 2012; Gan *et al.*, 2003). In Wang *et al.* study, *Kappaphycus alvarezii* transgenic was produced by micro-particle bombardment method (Wang *et al.*, 2010).

Application of gene transfer (transgenesis) technology in order to increase growth (Devlin *et al.*, 1994, Nam *et al.*, 2001; Kobayashi *et al.*, 2007), resistant to disease (Yazawa *et al.*, 2006; Parenrengi, 2010) and adaptation ability to the extreme environmental condition (Wang *et al.*, 1995) have extensively been reported in various finfish and shellfish species. In seaweed, however, research is still limited to development of transgenic method and analysis of promoter activity in *Laminaria japonica* Aresch and *Undaria pinnatifida* Suringer (Qin *et al.*, 2005).

The ability of promoter used to control the expression of foreign gene, in this study green fluorescent protein (GFP) gene was used as reporter, is one of determinant factors affecting the success of transgenesis. Various promoters have been analyzed for their activities in fish, such as cytomegalovirus/CMV (Volckaert, 1994; Arenal *et al.*, 2008; Traxler *et al.*, 1999), β -actin (Yoshizaki, 2001; Alimuddin *et al.*, 2005), heat-shock protein, keratin (Gong *et al.*, 2002; Yazawa *et al.*, 2005), myosin light chains (Alimuddin, 2003) and elongation factor-1 α (Yasawa *et al.*, 2005; Alimuddin *et al.*, 2007). Promoter

activity level is determined by the match between transcription factors (*cis* regulators) of the promoter with *trans* regulators in the host (Iyengar *et al.*, 1996; Alimuddin, 2003). Generally, promoters activity originating from the same species or family with the fish will be made transgenic are higher than those of originating from different species. In addition, number of promoter which has been isolated is still limited. Thus, it is necessary to evaluate the activity of existing promoters in order to produce seaweed transgenic.

In this study, as an initial step to produce high quality (such as high level of carrageenan content and disease resistant) seed of seaweed using transgenesis technology, four different promoters that drives GFP gene was introduced to obtain a suitable promoter for seaweed. The results of study showed that the two virus derived promoters (CMV and CaMV) and medaka β -actin promoter can regulate high foreign gene expression level in seaweed.

Materials and Methods

Source of *K. alvarezii* filament callus

K. alvarezii used in this study was derived from the collection of Brackishwater Aquaculture Research Center, Maros, South Sulawesi. Thalli segment, callus and filament callus were cultured in the laboratory condition according to method described by Reddy *et al.* (2003) and Rajamuddin (2010). Thalli segment was culture to obtain callus using solid agar medium containing growth regulators, while callus was culture in semi-solid medium to produce filament callus. Filament callus were then regenerated in a liquid medium.

GFP construction vectors

Four GFP expression vectors driven by different promoters, i.e. cytomegalovirus (pCMV-GFP, Clontech), cauliflower mosaic virus (pCaMV-GFP, BIOS Cambia Labs.), Japanese medaka β -actin (pmBA-GFP, Takagi *et al.*, 1994) and Japanese flounder

keratin promoters (pJfKer-GFP, Yazawa *et al.*, 2005) were examined. The first two promoters was virus origin, while the last two was from the fish.

Transformation protocol

Each GFP expression vector in concentration of 100 ng/ μ L was mixed with *K. alvarezii* callus mass as filament form. Transformation was performed by electroporation method using *Gene Pulser II* (Biorad, USA) in voltage of 300 V, pulse length 0.5 ms, pulse number 4, and pulse interval 0.1 s at 2 mm cuvette. Those electroporation parameters were obtained from preliminary study that produced high transformation efficiency and cell viability.

GFP gene expression analysis

GFP expression was analyzed using a fluorescent microscope (Olympus BH2-RFCA) equipped with reflected light fluorescent attachment (BH2-RFC2). Observation was conducted every hour started at 1 h after electroporation until GFP expression level being stable. Number of cells expressing GFP and their expression level was observed after GFP was stably expressed. GFP expression levels were qualitatively classified as faint, medium and strong fluorescence.

Transformant regeneration and detection

Transformant carrying GFP was screened and regenerated by culturing the electroporated filament callus in the semi-solid medium containing kanamisin 100 ppm for 1 month. Detection of transformant was conducted using fluorescent microscope and PCR method. Genomic DNA was extracted from the highest and lowest GFP expressing transformant using the Puregene DNA Isolation Kit (Gentra, USA). PCR amplification was carried out using a set primer of GFP-F (5'-GGTCGAGCTGGACGG-3') and GFP-R (5'-ACGAACTCCAGCAGG-3'). Amplification of GFP fragment was conducted for 35 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 1

min. PCR product was electrophoretically separated using 1% agarose gel.

Results and Discussion

GFP gene expression

The electroporation parameters used in this study was obtained in our preliminary experiment (unpublished data). As shown in Figures 1A-D, electroporated filament callus mass showed green fluorescence in some of embryos, while no GFP expression in non-electroporated control was observed (Fig. 4E). This indicated that GFP gene construct was successfully transferred to filament callus of seaweed. Further, percentage of filament callus expressing GFP gene was different between gene construct used (Table 1, Fig. 4). Highest percentage of filament callus expressing GFP was obtained by using CMV promoter, followed by CaMV, mBA and JfKer (Table 1). GFP gene expression levels driven by CMV, CaMV and mBA were relatively similar while JfKer was lower compared to that of the first three promoters (Fig. 4). The first two promoters is virus origin, while the last two is fish origin. Foreign gene expression levels driven by virus origin promoter were similar with mBA promoter of Japanese medaka fish origin, but it was lower compared to that of JfKer promoter of Japanese flounder fish origin. Furthermore, initial time to express GFP after electroporation was fastest in filament callus carrying pCMV-GFP and pCaMV-GFP constructs compared with pmBA-GFP, and the later was pJfKer-GFP. Thus, differences in the level and initial time of foreign gene to express are possibly related to difference in recognition of *trans*-acting protein of the host with *cis*-acting element of promoter (Iyengar *et al.*, 1996; Alimuddin, 2003).

Hae is hour after electroporation. GFP-expressing cell percentage are given as means \pm S.D. Different superscript letters in GFP-expressing cell percentage indicate significant differences ($p < 0.05$) as determined by one-way ANOVA followed by Duncan's test.

Table 1. Initial time, level of expression and percentage of cell expressing GFP in *Kappaphycus alvarezii* filament callus after electroporation using different gene constructs

GFP constructs	Initial time to express GFP (hae)	GFP expression levels	GFP-expressing cell percentage (%)
pCMV-GFP	1	Mid to strong	34.10±1.49 ^a
pCaMV-GFP	2	Mid to strong	10.48±0.25 ^b
pmBA-GFP	2	Mid to strong	8.85±2.31 ^b
pJfKer-GFP	3	Weak	4.79±0.26 ^c

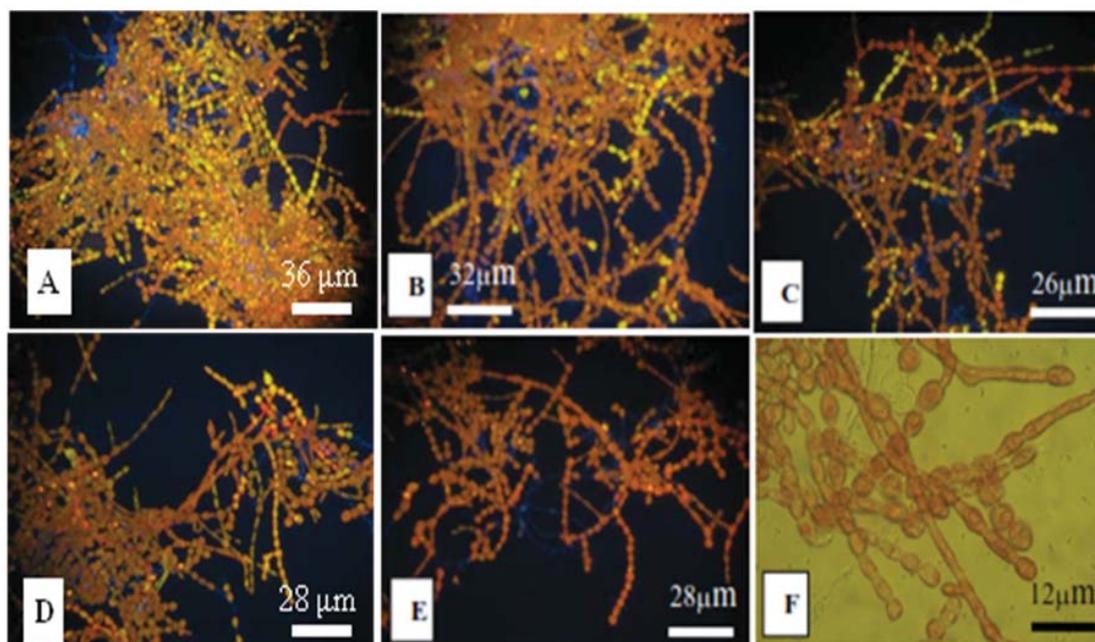


Figure 4. GFP gene expression at the electroporated filament callus of *K. alvarezii* using pCMV-GFP (A), pCaMV-GFP (B), pmBA-GFP (C), pKeratin-GFP (D) constructs, and non-electroporated control observed by fluorescent microscope (E) and by light microscope (F).

Transformant regeneration

All gene constructs used in this study is contained kanamycin resistance gene so that the transformant can live in the medium containing kanamycin. By adding 100 ppm of kanamycin into the culture medium, the electroporated filament callus survived and grew (Fig. 5A-D), while non-electroporated control died after 1 month incubation (Fig. 5E). This indicated that the electroporated filament callus carried foreign gene construct. Furthermore, number of filament callus expressing GFP after incubation for 1 month (Fig. 5A-D) was higher compared to that of initial condition (Fig. 4), indicating the mitotic division in transgenic cells. Number of

GFP-expressing filament callus using pCMV-GFP and pCaMV-GFP constructs was relatively similar and higher than that of pmBA-GFP and pJfKer-GFP. This might be correlated to initial number of cells expressing foreign gene before they regenerated. In addition, GFP-expressing cells derived from mitotic division may become a transgenic stable, though this needs a proof by Southern blot analysis.

PCR analysis for transgenic confirmation

PCR analysis was performed to re-confirm that the green fluorescence in the survived filament callus of *K. alvarezii* in the kanamycin-contained medium is from GFP

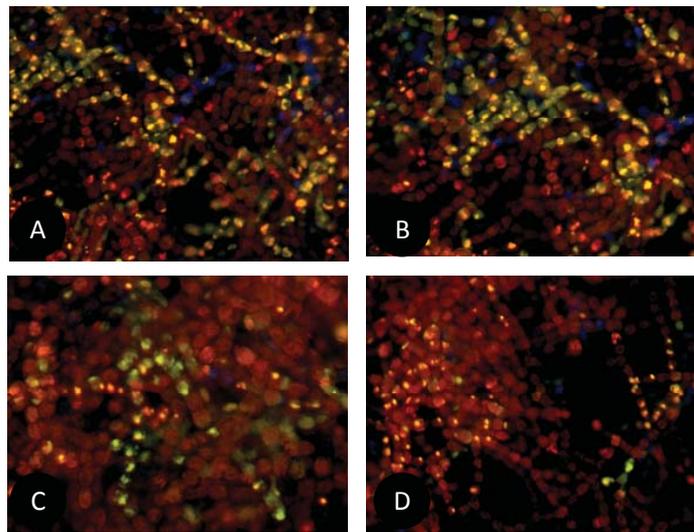


Figure 5. A constitutive GFP gene expression in filament callus of *Kappaphycus alvarezii* after culturing in the medium containing kanamycin 100 ppm for 1 month. (A) pCMV-GFP, (B) pCaMV-GFP, (C) pmBA -GFP, (D) pJfKer-GFP.

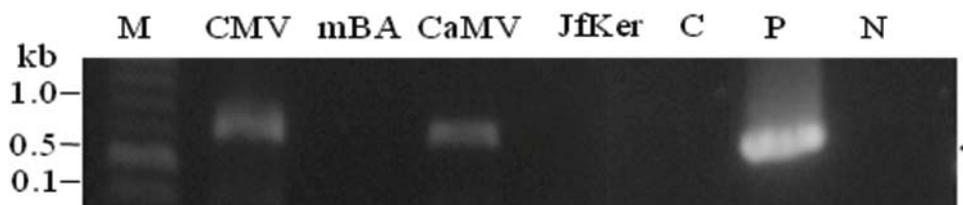


Figure 6. Result of transgene detection by PCR analysis with DNA template extracted from filament callus of *Kappaphycus alvarezii*. Lane CMV, mBA, CaMV and JfKer are PCR product (marked by arrow head) amplified using genomic DNA from pCMV-GFP, pmBA-GFP, CaMV-GFP and pJfKer-GFP expressing filament callus, respectively; C= a sample product with genomic DNA from non-transgenic filament callus; lane P and N= a sample PCR product with pCMV-GFP plasmid and without DNA template, respectively. M= is 2-log ladder DNA marker (BioLabs, Inc., New England). The amplified fragment is about 0.6 kb in size.

transgene expression. As shown in Fig. 6, a DNA band of PCR product from pCMV-GFP (CMV) and pCaMV-GFP (CaMV) expressing filament callus in the same size (about 0.6 kb) with the positive control of pCMV-GFP plasmid (P) was obtained, but no PCR product in the non-electroporated control (C). This indicated that the somatic embryos showing green fluorescence were transgenic carrying pCMV-GFP and pCaMV-GFP gene constructs. PCR product from pmBA-GFP showed negative of DNA band, although number of GFP expressing filament callus using pCaMV-GFP construct was relatively similar.

Conclusion

Electroporation method could be used to transfer GFP gene into filament callus of *Kappaphycus alvarezii*. Cytomegalovirus (CMV) and cauliflower mosaic virus (CaMV) promoters are more suitable for *K. alvarezii* in controlling GFP gene expression compared with medaka β -actin and Japanese flounder keratin promoters.

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