CHAPTER III

MATERIALS AND METHODS

3.1 Equipments and reagents

3.1.1 Equipments

100ml bottles (Kaewthai Industries, Thailand)

Augusta safety cabinet (Lio Lab Co. Ltd, Thailand)

Autoclave LS-2D (Rexell Industries Co. Ltd., Taiwan)

Gel electrophoresis system (Mupid, Japan)

Improved Neubauer haemacytometer (Boeco, Germany)

No.1 filter paper (Whatman, England)

Spectrophotometer (Beckman, USA)

Gene Cycler (Bio-Rad Laboratories, USA)

3.1.2 Chemicals

Agarose (FMC Bioproducts, USA)

Calcium chloride (Merck, Germany)

Chloroform (Merck, Germany)

100 mM dATP, dCTP, dGTP and dTTP (Promega, USA)

Magnisium chloride (Merck, Germany)

Magnesium sulfate (Merck, Germany)

Bacto-yeast extract (Himedia Laboratories, India)

Bacto-tryptone (Bio Basic.INC, USA)

Ethidium bromide (Merck, Germany)

Isoamyl alcohol (Merck, Germany)

PCR core reagents (Promega, USA)

Phenol crystal (Merck, Germany)

Polyethylene glycol (Bio Basic.INC,USA)

Sodium acetate (Merck, Germany)

Sodium chloride (Merck, Germany)

Sodium chloride (Prungtip, Thailand)

Sodium hydroxide (Merck, Germany)

16-16-16 fertilizer (Bioplus, France)

Unilate (Bioplus, France)

3.1.3 Antibiotics

Ampicillin (Sigma Chemical Co., USA)

Kanamycin (Sigma Chemical Co., USA)

3.1.4 Kits

RNA Qaiamp blood minikit (Qaigen, Germany)

3.1.5 Enzymes

*Eco*RI (New England BioLabs, USA)

RNaseA (Sigma Chemical, USA)

Stu I (New England BioLabs, USA)

Superscript II (Invitrogen, USA)

T4 DNA ligase (Promega, USA)

Taq DNA polymerase (Promega, USA)

3.1.6 Algae

Dunaliella salina isolated from Samutsakorn province

3.2 Methods

3.2.1 Production of *D. salina* using simple media for the substitution of original J/1 medium

3.2.1.1 Preparation of JR (revised J) medium

JR medium was prepared according to appendix A in 20X stock solution. The solution was filtered through 90mm-diameter Whatman filter paper and kept at 4° C

3.2.1.2 Variation of JR concentrations

The stock solution of JR medium was diluted to 4 levels of concentrations; 0.25x, 0.5, 1x and 2.5x solutions adjusted pH to 7 and were autoclaved before use. *D. salina* was inoculated into each media using $1X10^4$ cell/media solution. After culture number of cells was counted against day of culture by Improved Neubauer hemacytometer under a light microscope.

3.2.1.3 Variation of pH

In oder to investigate the effect of pH on *D. salina* growth, the stock solution of JR medium was diluted into 1X solutions and each adjusted to pH6, pH6.5, pH7, pH7.5, pH8, pH8.5 to pH9. *D. salina* was inoculated into each media as described earlier. After culture, number of cells was counted against day of culture by Improved Neubauer hemacytometer under a light microscope.

3.2.2 Selection condition for herbicide resistance

Bialaphos, sulfonyl urea, was used as selective reagent in this experiment. JR media which contained 5, 6, 7, 8, 9, 10, 11 and 12 ppm of bialaphos were prepared and used for screening study. *D. salina* was inoculated into each media as described and survival rates through living cell count were measured.

3.2.3 Method for gene transformation in *D. salina*

Five hundred microlitres of *D. salina* cells approximately $1x10^5$/ml in concentration was transferred into a microcentrifuge tube. Thirty micrograms of plasmid DNA was added. PEG stock solution (1% w/v) was added into each tube to make condition of: 0%, 0.02%, 0.04%, 0.06%, 0.08% and 0.1% w/v PEG, respectivly and JR medium was added into each tube up to 1 ml. The tubes were then mixed by inversion and incubated for 1 hour. Then, these buffer was all replaced after centrifugation with fresh JR medium containing 8 ppm of bialaphos. Every 40 µl of each tube was sampled and counted by hemacytometer.

The existence of *bar* was confirmed by PCR. The PCR was performed by 10X PCR buffer, 3mM $MgCl_2$, 0.2mM dNTP, 0.2μM of bar forward primer 5'-ggt ctg cac cat cgt ctt c-3', 0.2 μM of bar reverse primer 5'-gaa gtc cag ctg cca gat tc-3' and 0.5U *Taq* Polymerase (Promega, USA). PCR condition was 40 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes. The PCR product was compared with the original product from pBicBar.

3.2.4 Construction of transforming vector, containing GBA gene

RNA extraction was performed by RNA blood minikit (Qaigen, Germany). The first strand cDNA was synthesized by Superscrip II (Invitrogen, USA). The *GBA* cDNA was amplified by PCR in 2 overlapping fragments. The reaction component consisted of 10x PCR buffer, 3mM $MgCl_2$, 0.2mM dNTP, 0.2μM of each primer in table 3.1 and 0.5U Taq Polymerase (Promega, USA). PCR condition was performed using 40 cycles of 93°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, then extended at 72°C for 10 minutes. The PCR product was visualized after electrophoresis.

Table 3.1 2 pairs of primers for amplifying 2 overlapping fragments of *GBA*

Fragment	Primer	Fragment size (nt)
GBA5' portion	Forward: 5'- atg gag ttt tca agt cct tcc aga-3' Reverse: 5'-ccc gaa ttc tgc gga tgg aga agt cac-3'	570
GBA3' portion	Forward: 5'-atg ggg ccc atc cag gct aat cac -3' Reverse: 5'-ccc gaa ttc tca ctg gcg acg cca cag -3'	1338

35S promoter was amplified from pBicBar with the same condition as GBA. After 35S was rendered, it was ligated with GBA5' portion and reamplified to obtain the 35S-GBA5' fragment accordingly (Kuribara et al., 2002) with GBA3' portion to generate a full length fragment containing, 35S promoter, GBA gene. The whole fragment was ligated with pBICBar pre-digested with *Bam*HI / *Eco*RI using T4 DNA ligation and transform in to *E. coli* DH5α as a cloning system.

DH5α competent cells were prepared according to Sambrook *et al.* (1989) and after placing on ice and thawed, cells were mixed by gently flicking. A 50 µl of cells was transfer red to the tube containing ligation reaction or having target plasmid. The tube was placed on ice for 20 minutes for DNA binding, 42°C water bath for 45 seconds for heat-shocked transformation and immediately dipped on ice for 2 minutes. Then a 950 µl of SOC medium was added. The tube was incubated at 37 °C for 1.5 hours with shaking at 120 rpm. 100 µl of transformation culture was plated on a selective LM medium containing 50µg/ml kanamycin. The plate was incubated overnight at 37°C.

Plasmid DNA was isolated using small-scale plasmid extraction method (Sambrook *et al.*, 1989). A colony was inoculated into a sterile tube containing 3 ml LB broth supplemented with 50 µg/ml of Kananmycin and incubated overnight at 37°C. The suspension was transferred to new tube and centrifuged at 3,000 rpm for 3 minutes. The supernatant was carefully discarded. One hundred microlitres of lysozyme buffer was added to the cell pellet and vortexed. Two hundred microlitres of alkaline solution (0.2N NaOH and 1% SDS) was added vortexed and incubated on ice for 5 minutes. Then, one hundred and fifty microlitres of 3M sodium acetate was added. Total reaction was further vortexed and incubated on ice for further 5 minutes. The tube was centrifuged for 5 minutes at 12,000 rpm to pellet cell debris. The supernatant was transferred to a new microcentrifuge tube, equal volume of phenol-chloroform was added and mixed by inversion of the tube. The tube was centrifuged for 5 minutes at 12,000 rpm. The supernatant was transferred to a new tube and equal volume of isopropanol was added and incubated at room temperature for 15 minutes. After that, the tube was centrifuged at 12,000 rpm for 15 minutes. The supernatant was discarded, the pellet was completely dried and resuspended in 30 µl of TE buffer. Two hundred microgram per milliliter of RNase A was added to digest contaminating RNA. The reaction was incubated at 37°C for 30 minutes. Plasmid could store at -20 for several months.

3.2.5 Transformation of GBA gene in *D. salina*

D. salina was transformed according to the best condition obtained in 3.4) with the plasmid containing 35S promoter and full-length GBA obtained in 3.5). In order to detect the existence of target DNA, transformants were performed phenol extraction (Sambrook *et al.*, 1989) to extract for genomic DNA and amplified with GBA5' portion's primer to confirm the integration of DNA. Expression of RNA was detected after RNA extraction and confirmed for mRNA expression by RT-PCR.