CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Rice seeds

Rice (*Oryza sativa* L.) cultivar Khoa Dok Ma Li 105 (KDML105)

Rice (*Oryza sativa* L.) line FL530

2.1.2 Instruments

Autoclave: Labo Autoclave MLS-3020 (Sanyo Electric Co., Ltd.)

Automatic micropipette: pipetman P2, P20, P100, P1000 (Gilson Medical Electronics S.A.)

Balance: Sartorius CP423s (Scientific Promotion Co.)

Biophotometer (Eppendorf)

Film Cassette with intensifying screen: 35x43 cm (Cokamuto)

Centrifuge 5417C (Eppendorf)

-20 °C Freezer (Sharp)

-80 °C Freezer (Revco)

Gel Doc™ (Syngene)

Gel mate 2000 (Toyobo)

GeneAmp PCR System 2400 (Perkin-Elmer)

Gene Pulser: Micropulser™ (Bio-RAD Laboratories)

GS Gene Linker™: UV Chamber (Bio-RAD Laboratories)

Hybridization oven (Hybrid)

Incubator: BM-600 (Memmert Gambh)

Incubator shaker: Innova™ 4000 (New Brunswick Scientific)

Laminar flow: HS-124 (International Scientific Supply Co., Ltd.)

Magnetic stirrer: Fisherbrand (Fisher Scientific)

Magnetic stirrer and heater: cerastir (Clifton)

Microcentrifuge: PMC-880 (Tomy Kogyo Co., Ltd.)

Microwave Oven (Panasonic)

Orbital Shaker: LD-40 (Lacinco)

pH meter: pH900 (Precisa)

PCR workstation Model#P-036 (Scientific Co.)

Power supply: Power PAC 1000 (Bio-RAD Laboratories)

Refrigerated centrifuge 5804R (Eppendorf)

Spectrophotometer: DU®640 (Beckman Coulter)

UV transilluminator: 2001 microvue (San Gabriel California)

Vacuum blotter model 785 (Bio-RAD Laboratories)

Vacuum Pump (Bio-RAD Laboratories)

Vortex mixer: Model K 550-GE, Scientific Inc., USA.

Water bath: Isotemp210 (Fisher Scientific)

2.1.3 Materials

Filter paper: Whatman No.1 (Whatman International Ltd.)

Microcentrifuge tube 0.6 and 1.5 ml (Axygen)

0.22µm Millipore membrane filter (Millipore)

Nipro disposable syringe (Nissho)

KODAK BioMax MS film scientific imaging film: 18x24 cm (Kodax)

PCR: Mastercycler gradient (Eppendorf)

PCR Thin wall microcentrifuge tube 0.2 ml (Eppendorf)

Pipette tips10, 100, 1000 µl (Axygen)

Hybond-N^+ membrane (Amersham)

2.1.4 Chemicals and reagents

Absolute ethanol (BDH)

Abscisic acid (5-[1-hydroxy-2,6,6-trimethyl-4-oxo-cyclohexen-1-yl]-3methyl-{2Z,4E}-pentadienoic acid)

Acetic acid glacial (BDH)

Agarose: Seakem LE Agarose (FMC Bioproducts)

Ammoniun Sulfate (Sigma Chemical Company)

Ammonium persulfate (Sigma Chemical Company)

Ampicillin (Sigma Chemical Company)

Bacto agar (DIFCO)

Bacto tryptone (Scharlau)

Bacto yeast extract (Scharlau)

Bovine Serum Albumin (Sigma Chemical Company)

5-Bromo-4-chloro-3-indole-beta-D-galactopyranoside; X-gal (Sigma Chemical Company)

Bromophenol blue (Merck)

Calcium sulfate (Carlo Erba Reagenti)

Calf thymus DNA (Sigma Chemical Company)

Chloroform (Merck)

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

Dithiothreitol (Sigma Chemical Company)

Diethyl pyrocarbonate: DEPC (Sigma Chemical Company)

Ethidium Bromide (Sigma Chemical Company)

Ethylene diamine tetraacetic acid (EDTA) (Carlo Erba Reagenti)

Ethylene diamine tetraacetic acid (EDTA) disodium salt dehydrate (Carlo Erba Reagenti)

Ferrous sulfate (Carlo Erba Reagenti)

Formamide (Fluka)

Formaldehyde (Sigma Chemical Company)

Ficoll type 400 (Sigma Chemical Company)

Glycerol (BDH)

Glacial acetic acid (Carlo Erba Reagenti)

Hydrochloric acid (Merck)

Isoamylalcohol (Merck)

Isopropanol (Merck)

Iso-1-thio-β-D-thiogalactopyranoside: IPTG (Serva)

Lamda DNA (Promega)

Lithium Chloride (Sigma Chemical Company)

Magnesium sulfate (Sigma Chemical Company)

Methanol (Merck)

Methylene blue (Carlo Erba Reagenti)

Oligo dT (Promega)

Phenol crystal (BDH)

Phenol Solution (Sigma Chemical Company)

Polyvinyl pyrrolidone (Sigma Chemical Company)

Potassium acetate (Merck)

Potassium nitrate (BDH)

Random Hexamers (Promega)

Sodium acetate (Carlo Erba Reagenti)

Sodium Chloride (Carlo Erba Reagenti)

Sodium dodecyl sulfate (Sigma Chemical Company)

Sodium dihydrogen orthophosphate (Carlo Erba Reagenti)

di-Sodium dihydrogen orthophosphate anhydrous (Carlo Erba Reagenti)

Sodium hydroxide (Carlo Erba Reagenti)

Triethanolamine (Sigma Chemical Company)

Triple super phosphate

Tri-Reagent® (Invitrogen Life Technologies)

Tris-(hydroxyl methyl)-aminomethane (Fluka)

Triton X-100 (Merck)

Unilate

Xylene Cyanol FF (Sigma Chemical Company)

2.1.5 Enzymes

DNA polymerase, Large (Klenow) fragment (Biolabs)

Restriction endonucleases: *Hind* III, *Eco*RI, *Msp* I, *Nco* I, *Spe* I (Biolabs)

MMLV reverse transcriptase (Promega)

RNase A (Promega)

RQ1 RNase-free DNase (Promega)

T4 DNA ligase (Promega)

Taq DNA Polymerase (Fermentus)

2.1.6 Microorganism

Escherichia coli strain DH5α with genotype *F'*, *Φ*80d*lac*ZΔM15, Δ(*lac*ZYA-*arg*F) U169 *end*A1, RecA1, *hsd*R17 (r_K-m_{K+}), *deo*R, *thi*-1, *sup*E44, λ⁻ *gyr*A96, *rel*A1

2.1.7 Kits and Plasmid

QIAquick™ Gel Extraction kit (Qiagen)

QIAquick™ Plasmid Extraction kit (Qiagen)

pGEM®-T vector system I (Promega), a vector for cloning (Appendix B)

2.1.8 Radioactive

[α-^{32}P] dCTP (1000-3000 Ci/mmole) (Amersham Biosciences Biotech)

2.2 Bacterial growth medium

Luria-Bertani broth (LB medium) (Maniatis et al., 1982)

The following medium was used LB medium containing 1% peptone, 0.5% NaCl and 0.5% yeast extract was prepared and adjusted pH to 7.2 with NaOH. For agar plate, the medium was supplemented with 1.5% (w/v) agar. Medium was steriled for 20 minutes at 121° C. If needed, selective antibiotic drug was then supplemented.

2.3 Methods

2.3.1 Database searches and analyses of gene structures and chromosomal distribution

To identify members of the *Oryza sativa* L. EF-hand-containing protein family, first, The Institute of Genomic Research (TIGR; http://www.tigr.org/tdb/e2k1/osa1/) was searched for Interpro Database Matches by five different methods including HMMPfam, HMMSmart, BlastProDom, ProfileScan, and superfamily. Proteins shown to contain an EF-hand motif or in the family of Ca^{2+}-binding proteins which included domains PF00036, SM00054, PD000012, PS50222, and protein family SSP47473, respectively by each method were collected. In addition, the Basic Local Alignment Search Tool (BLAST) algorithms (BlastP) using the protein sequences of rice CaM1 [GenBank: NP_912914] and CBL3 [GenBank: NP_643248] as query sequences against the rice genome were conducted. Nucleotide and amino acid sequences as well as information regarding each gene of interest were obtained. Gene annotations at the Rice Annotation Project Database (RAP-DB) were also used to confirm the existence and sequences of

these genes. Gene structure and locations were determined by comparing their full length cDNA with the corresponding genomic DNA sequences obtained from GenBank and searches of the identified loci at TIGR. Information from EST sequences was used when discrepancy was found. Gene duplication was determined according to the analyses of chromosomal segmental duplication of the rice genome by TIGR.

2.3.2 Alignments and tree construction

If necessary, predictions of coding regions were verified using available EST and cDNA sequences. Deduced sequences of proteins identified by InterProScan as containing an EF hand were subjected to phylogenetic analysis. Multiple alignments of amino acid sequences were performed by ClustalX using default settings. Alignments were carried out and protein trees were constructed using the neighbor-joining algorithm by Clustal X (default settings). Bootstrap analysis with 1000 replicates was used to evaluate the significance of the nodes. Comparison of OsCaM proteins with those from other species by multiple sequence alignment was performed by ClustalW. GenBank accession numbers for the sequences used in the alignment are as follows: ACaM2 [GenBank: AAA32763]; HvCaM [GenBank: AAA32938]; T-CaM1 [GenBank: AAC49578]; ZmCaM [GenBank: CAA52602]; SCaM1 [GenBank: AAA34013]; PCM5 [GenBank: AAA85155]; MmCaM [GenBank: NP_033920]; CMD1p [GenBank: AAA34504]. For generating the phylogenetics tree of the full-length Arabidopsis and rice CaMs and CMLs protein sequences, we used Clustal X (default settings) and neighbor-joining method with Bootstrap analysis with 1000 replicates.

2.3.3 Amino acid identity and motif analyses of proteins

Deduced amino acid sequences CaM and CaM-like proteins were aligned with one another by Align (http://www.ebi.uk.ac) and the percentage of amino acid identity was calculated by dividing the number of identical amino acids by the total number of amino acid residues of the aligned sequences. All of the protein sequences were analyzed for EF hands and other domains using InterProScan (http://www.ebu.ac.uk/InterProScan). Positions of the EF hands were located using information from the prediction by InterProScan and by comparing the complete sequences of all proteins with the plant EF-hand consensus sequence. All identified EF hand sequences were aligned with ClustalX and a consensus sequence was generated. To locate sequences for protein modification and targeting, computer programs: Myristoylator (http://www.expasy.ch/tools/myristoylator/) and targetP (http://www.cbs.dtu.dk/services/TargetP/) were used.

2.3.4 Expressed Sequence Tags

ESTs corresponding to *Cam* and *CML* genes were identified by performing BLAST searches of the *Oryza sativa* EST database and by searching UniGene entries corresponding to all genes at GenBank (http://www.ncbi.nlm.nih.gov/). Expression characteristics of all genes were determined based on the types of libraries from which ESTs were derived and from literature reviews.

2.3.5 Preparation of rice seedlings

Seeds of the indica rice cultivar (*Oryza sativa* L.) were obtained from Kasetsart University. Healthy rice seeds (Khoa Dok Ma Li 105 (KDML105) and FL530) were rinsed with deionized water and soaked 20 minutes in 30%, 20% and 10% solution of Clorox with shaking respectively, then extensively washed with deionized water for at least 3 times. Washed seeds were germinated in tray lined with moist paper towel for 7 days in the dark. After 7 days, germinated seeds were transferred to WP No.2 Solution (Vajrabhaya and Vajrabhaya, 1991) (see in Appendix C) and grown for 2 weeks under 16-hr light/8-hr dark photoperiod. To test the effect of abscisic acid (ABA), 3-week old seedlings were sprayed with 100 µM ABA or a buffer (0.5% (v/v) Triton X-100, 2.5% (v/v) ethanol) as control and incubated for 2 hours. The ABA-treated and untreated seedlings were transferred to fresh medium containing 0.5% (w/v) NaCl. Leaves and roots were collected and immersed in liquid nitrogen at 0, 0.5, 1, 2 and 4 hours.

2.3.6 Total RNA preparation

Total RNA was isolated by hot phenol procedure based on that described by Verwoerd et al., 1989. *Oryza sativa* L. tissues were ground in liquid nitrogen using chilled mortars and pestles. The plant material was kept frozen and ground to a fine powder. Then, the ground tissues were added into 500 µl of hot extraction buffer (80 °C) (phenol, 0.1 M LiCl, 100mM Tris-HCl (pH8.0), 10mM EDTA and 1% SDS). After that, the mixtures were homogenized by vortexing for 30 seconds. Consequentially, 250 µl of chloroform:isoamyalcohol (24:1) was added and votex again. The mixture was

centrifuged at 14,000×g for 5 minutes at 4 °C. The upper aqueous phase was transferred to a fresh microcentrifuge tube. RNA was precipitated by the addition of one volume of 4 M LiCl and stored overnight at -20 °C. The mixture was left at room temperature for 5-10 minutes and centrifuged at 14,000×g for 20 minutes at 4 °C. After centrifugation, the pellet was dissolved in 250 µl water. After that 25 µl (0.1 volumes) of 3 M NaOAc was added. RNAs was precipitated with 550 µl (2 volumes) of cold absolute ethanol and place at -20 °C for overnight. Then, the pellet was collected by centrifugation at 14,000×g for 20 minutes at 4 °C. The pellet was washed with 300 µl of 70% ethanol and briefly air-dried. The total RNA was resuspended with 30 µl of diethyl pyrocarbonate (DEPC)-treated water. The concentration was estimated by measuring the optical density at 260 nm, and calculating in µg/ml unit, using the following equation:

$$[\text{RNA}] = A_{260} \times \text{dilution factor} \times 40^*$$

* The absorbance at 260 nm (A_{260}) of 1.0 corresponds to the RNA of approximately 40 µg/ml (Sambrook et al., 2001).

2.3.7 Formaldehyde-agarose gel electrophoresis

Formaldehyde agarose gel electrophoresis was used to analyze RNA. A 1.5% (w/v) formaldehyde agarose gel was prepared. The gel slurry was boiled until complete solubilization, and allowed to cool to 50 °C. The melted agarose gel was mixed with formaldehyde 8.4 ml in 100 ml agarose gel and poured into a chamber set.

Twenty micrograms of the total RNA were mixed with sample buffer and incubated at 65 °C for 10 minutes. The sample was loaded into the 1.5% (w/v)

formaldehyde agarose gel. The RNA Ladder (Fermentas, USA) was used as a standard RNA marker. Electrophoresis was carried out in reservior buffer at 70 volts, until bromphenol blue reached approximately ¾ of the gel length. The total RNA was visualized as fluorescent bands using a UV transilluminator and photographed.

2.3.8 PCR Amplification of mRNA sequences

a) DNase Treatment

Ten micrograms of the total RNA sample were added to the DNase treatment mixture (1x M-MLV Reverse Transcriptase (M-MLVRT) buffer, 40 units of recombinant RNasin® ribonuclease inhibitor, and 1 unit of RQ1 DNase). The reaction was incubated at 37 °C for 1 hour. Consequently, the reaction was incubated in boiling water for 2 minutes and quick-chilled on wet ice to terminate the reaction.

b) Reverse Transcription

One microgram of the DNase-treated RNA was added to the reverse transcription mixture (1x M-MLVRT buffer, 100 ng of oligo$(dT)_{15}$ primer, 100 ng of the dNTP mix, and 200 units of M-MLVRT). The reaction was incubated at room temperature for 10 minutes, then at 42 °C for 60 minutes and immediately placed in a boiling water for 3 minutes and quick-chilled on wet ice to terminate the reaction. The reactions were stored at -20 °C or used to assemble PCR reactions immediately on ice.

c) PCR Amplification

PCR amplification was carried out using forward and reverse oligonucleotide primers that were synthesized by Bioservice Unit (BSU) of The National Science and Technology, Thailand. Their sequences were as follows:

OsCam1-1

OsCam1-1F	5'-GAAGCCAGGCTAAGCCCAGC-3'
OsCam1-1R	5'-GCAAGCCTTAACAGATTCAC-3'

OsCam1-2

OsCam1-2F	5'-CTTCGTTGATCCACTCACCC-3'
OsCam1-2R	5'-ACACAATCTCCTCTGCCTTA-3'

OsCam1-3

OsCam1-3F	5'-CCCCTCGCCGCCTCGCCACC-3'
OsCam1-3R	5'-CCCATAACCAAATGCTGTCA-3'

OsCam2

OsCam2-F	5'-GAGGAGGGTTCCCATTAAAT-3'
OsCam2-R	5'-CGCAAGCTAAGCATCACAAT-3'

OsCam3

OsCam3-F	5'-CCTTCCTCTCTCTCTCGCTC-3'
OsCam3-R	5'-CCCCCTGTGTTGATCCAAAT-3'

OsCML1

OsCML1-F	5'- GCTTTGCTCGCCTTCTCGAA -3'
OsCML1-R	5'- GGATCACGCACTTCTGGCCA -3'

PCR amplification by *Taq* polymerase was performed as follows: pre-denaturation at 94 °C for 5 minutes, denaturation at 94°C for 2 minutes, annealing at 55°C for 1 minute, and extension at 72°C for 2 minutes for *OsCam1-1*; *OsCam1-2*; *OsCam2*; and *OsCam3* and a program of 94°C for 2 minutes, 58°C for 1 minute, and 72°C for 2 minute for *OsCam1-3* and *OsCML1*. The final extension step was performed at 72 °C for 10 minutes. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

2.3.9 Agarose gel electrophoresis

Agarose gel electrophoresis is the standard method used for separation DNA fragments on the basis of their molecular weight and used for rough estimation of DNA on Basis of its direct relationship between amount of DNA and the level of the fluorescence after ethidium bromide staining. The ladder obtained from restriction enzyme digestion was analyzed according to their sizes. The size was estimated from standard curve of molecular weight markers, i.e., fragment of Lamda DNA digested with *Hin*dIII and fragment of pBR322 digested with *Msp*I. The calibration curve was plotted between logarithmic molecular massed and their relative mobilities. Agarose was solubilized by heating in a microwave oven and then allowed to cool to 50-60°C before pouring into a plastic gel former with a present well-forming comb. The concentration of

agarose gel was used varies with the size of the DNA fragment to be separated. Generally 1.0-1.8%gel in Tris-acetate-EDTA (TAE) buffer was used.

The DNA were mixed with 10% (v/v) of DNA gel loading Buffer (0.1M EDTA/NaOH pH 7.5, 50% (v/v) of glycerol, 1% (w/v) of SDS, 0.5 (w/v) of xylene cyanol FF, and 0.5 (w/v) of Bromophenol blue). The mixture was loaded into slots of the gel which was submerged in electrophoretic chamber filled with TAE. Electrophoresis was carried out at constant 100 volts. The duration of the running times was depended on the size of DNA. Generally, the gel was run until bromophenol blue move to 1 cm from the bottom of the gel. After electrophoresis, the gel was stained with ethidium bromide solution (5-10 μg/ml in distilled water) for 3-5 minutes and the destained with an appropriate amount of water with gently shaking for 10 minutes to remove unbound ethidium bromide from agarose gel. The DNA fragment were visualized as fluorescent bands under an UV transilluminator and photographed.

2.3.10 Extraction of DNA fragment from agarose gel

Extraction of DNA fragment from agarose gel was performed using QIAquick gel extraction kit protocol (QIAGEN, Germany). After electrophoresis, the desired DNA fragment was excised as gel slice from an agarose gel using a scalpel and transferred to a microcentrifuge tube. Three volumes of the buffer QG (supplied by manufacturer) were added and incubated for 10 minutes at 50°C or until the gel slice has completely dissolved. The gel mixture was vortexed every 2 to 3 minutes during the incubation period. The gel mixture should be in yellow after the gel is completely dissolved. The mixture was transferred into the QIAquick column and centrifuged at 12,000 rpm for 1

minute. The flow through solution was discarded. Another 500 µl of buffer QG was added and centrifuged at 12,000 rpm for 1 minute. After this step, a 750 µl of buffer PE (supplied by manufacturer) was added to the QIAquick column and centrifuged at 12,000 rpm for 1 minute. The flow through solution was discarded. The QIAquick column was centrifuged to remove a trace element of the washing solution. The QIAquick column was placed into a sterile 1.5 ml microcentrifuge tube. DNA was eluted by an addition 30 µl of steriled water to the center of the QIAquick column and let the column standing for 1 minute, and then centrifuged at 12,000 rpm for 1 minute. The DNA solution was used for cloning in the next experiment.

2.3.11 Cloning of *Cam* and *CML* genes and DNA Sequencing

a) PCR Amplification

PCR amplification was carried out using forward and reverse oligonucleotide primers that were synthesized by Bioservice Unit (BSU) of The National Science and Technology, Thailand. Their sequences are as follows:

The coding region of *OsCam1-1i*

OsCam1-1i-CD-F	5'-GAAGCCAGGCTAAGCCCAGC-3'
OsCam1-1i-CD-R	5'-CATCATCGGCAGGGGAAGTG-3

The 3' untranslated region of *OsCam1-1i*

OsCam1-1i-3'-F	5'-CTGCCGATGATGGCATAGTA-3'
OsCam1-1i-3'-R	5'-GCAAGCCTTAACAGATTCAC-3'

The coding region of *OsCML1i*

OsCML1i-CD-F	5'- GCTTTGCTCGCCTTCTCGAA -3'
OsCML1i-CD-R	5'- GGATCACGCACTTCTGGCCA -3'

The 50 µl of reaction mixture contained 5 U of *Taq* DNA polymerase, 200 µM dNTPs, 1x PCR buffer, 2.5 mM $MgCl_2$, 50-100 pmole of DNA template, and 10 pmole of each primer. PCR amplification was performed as follows: pre-denaturation at 94 °C for 5 minutes, denaturation at 94°C for 2 minutes, annealing at 55°C for 1 minute, and extension at 72°C for 2 minutes for *OsCam1-1* and a program of 94°C for 2 minutes, 58°C for 1 minute, and 72°C for 2 minute for *OsCML1*. The final extension step was performed at 72 °C for 10 minutes. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

b) Ligation

The resulting PCR products were purified by the QIAquick Gel Extraction Kit (see in 2.3.10). The purified PCR products were ligated to the pGEM®-T vector (see in Appendix B). A suitable molecular ratio between vector and inserted DNA in a mixture of cohesive-end ligation is usually 1:3. To calculate the appropriate amount of PCR product (insert) used in ligation reaction, the following equation was used:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert:vector molar ratio} = \text{ng of insert}$$

The 10 µl ligation reaction was composed of 5 µl of 2× T4 DNA ligase buffer, 3 weiss units of T4 DNA ligase, and the appropriate amount of the PCR product. The reaction was incubated at 4 °C overnight.

c) Making competent cell of *E. coli* for electroporation

Competent *E. coli* strain DH5α was prepared according to the method of Sambrook *et al.* (1989). A single colony of *E. coli* strain DH5α was inoculated in 10 ml LB broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl) and incubated at 37 °C with shaking 250 rpm for overnight. The starting culture was inoculated into 1 litre of LB broth and continued culture at 37 °C with shaking 250 rpm for 3-4 hours until the optical density at 600 nm (OD_{600}) of cells reached 0.4-0.6. The cells were chilled on ice for 15-30 minutes and harvested by centrifugation at 8,000 rpm, 4 °C for 15 minutes. The supernatant was removed as much as possible. The cell pellet was washed with 100 ml cold sterile deionized water, resuspended by gently mixing and centrifuged at 8,000 rpm, 4 °C for 15 minutes. The supernatant was discarded. The cells was resuspended and centrifuged further with 50 ml cold sterile deionized water, followed by 20 ml of cold steriled 10% glycerol twice. Finally, the cells were resuspended in 300 µl of cold 10% glycerol. This cell suspension was derived into 40 µl aliquots and stored at -80 °C for later used.

d) Electrotransformation

The cuvettes and sliding cuvettes holder were chilled on ice. The competent cells were gently thawed on ice. Forty microlitres of competent cells were mixed well with one to three microlitres of the ligation reaction, and then placed on ice for 1

minute. The cells were transferred to a cold cuvette and transformed by setting the GENE pulser apparatus (Bio-RAD) as follows: 25 F, 200 Ω of the pulse controller unit, and 2.50 kV. After one pulse was applied, the cells were immediately resuspended with 1 ml of LB broth. The cell suspension was incubated at 37 °C with shaking 250 rpm for 60 minutes. Finally, this cell suspension was spread on the LB agar plates which contained 100 μg/ml ampicillin, 25μl of 25mg/ml Iso-1-thio-β-D-thiogalactopyranoside (IPTG), and 25μl of 25mg/ml 5-Bromo-4-chloro-3-indole-beta-D-galactopyranoside (X-gal) and incubated at 37 °C overnight.

e) Plasmid DNA isolation

The selected clones was grown in one ml of LB broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl) containing 100 μg/ml of ampicillin and incubated overnight at 37 °C with shaking 250 rpm. The cells were spinned in a microcentrifuge at 14,000×g for 1 minute at 4 °C. The cells were resuspended in Lysis buffer (50mM of Tris base, 10mM of $Na_2EDTA.H_2O$ and 100 μg/ml of RNaseA) and mixed by pipetting up and down. The suspension was allowed to stand at room temperature for 5 minutes, then 300 μl of Alkaline-SDS solution (200mM NaOH and 1% SDS) was added. The suspension was inverted several times to mix and allowed to stand on ice of 5 minutes. 300 μl of High salt solution (3M of potassium acetate) was added to the mixture. The suspension was mixed gently and allowed to stand for 10 minutes on ice. The insoluble salt-genomic DNA precipitate was then removed by centrifugation at 14,000 rpm, 4 °C for 15 minutes. The supernatant was transferred to a fresh microcentrifuge tube and the nucleic acid was

precipitated by adding 480 µl (0.6 volumes) of isopropanol. The sample was mixed thoroughly and immediately centrifuged for 30 minutes to collect the precipitated DNA. The pellet was resuspend in 90 µl of sterile water and the suspension was vortexed gently. 10 µl of 3M sodium acetate, pH7 and 300 µl of cold absolute ethanol was added to the mixture. The mixture was mixed and chilled on ice overnight. The DNA was collected by centrifuging at 14,000×g for 20 minutes at 4 °C. The pellet was rinsed with 300 µl of 70% ethanol and allowed to dry for 10-15 minutes. The plasmid DNA was resuspended with 50 µl of sterile water. The concentration was estimated by measuring the absorbance at 260 nm, and calculated in µg/ml unit, using the following equation:

$$[\text{DNA}] = A_{260} \times \text{dilution factor} \times 50^*$$

* The absorbance at 260 nm (A_{260}) of 1.0 corresponds to the DNA of approximately 50 µg/ml (Sambrook et al., 1989).

f) Restriction enzyme digestion

Restriction endonuclease was used to cut DNA based on its specific binding property and cleaving double-stranded DNA at a specific sequence. The condition of digestion was performed as recommended by the enzyme manufacturer. Typically, a reaction contains about 0.5-1 µg of DNA. In a final volume of 10 µl containing 1x enzyme reaction buffer and 2-5 units of restriction enzyme. This digested DNA was analyzed by agarose gel electrophoresis as describe above.

g) Sequencing Analysis

The selected clones carrying the PCR fragment were extracted by QIAquick™ Plasmid Extraction kit and nucleoide sequences of the inserts were determined. DNA sequencing using M13 reverse primer was carried out at Macrogen, Korea.

2.3.12 Probe preparation

a) PCR Amplification

PCR amplification was carried out using forward and reverse oligonucleotide primers that were synthesized by Bioservice Unit (BSU) of The National Science and Technology, Thailand. Their sequences were as follows:

The 3' untranslated region of *OsCam1-1*

OsCam1-1-3'UTR-F	5'-CTGCCGATGATGGCATAGTA-3'
OsCam1-1-3'UTR-R	5'-GCAAGCCTTAACAGATTCAC-3'

The 3' untranslated region of *OsCam1-2*

OsCam1-2-3'UTR-F	5'-GGCAGAGGAGATTGTGTGTT-3'
OsCam1-2-3'UTR-R	5'-GGACATACCAAGGACAAGAA-3'

The 3' untranslated region of *OsCam1-3*

OsCam1-3-3'UTR-F	5'-TGCCATCTAGTTACTCTCCT-3'
OsCam1-3-3'UTR-R	5'-CACCATGCTCTACAAGAAGA-3'

The 3' untranslated region of *OsCam2*

OsCam2-3'UTR-R	5'-GCCAAGTAAGTTGAAGCATC-3'
OsCam2-3'UTR-F	5'-ATATCAAGAATTTCGGTTTG-3'

The 3' untranslated region of *OsCam3*

OsCam3-3'UTR-F	5'-GCCAAGTAAGTTGAAGCATC-3'
OsCam3-3'UTR-R	5'-ATATCAAGAATTTCGGTTTG-3'

The 3' untranslated region of *OsCML1*

OsCML1-3'UTR-F	5'-GAGGATAGAGGAGAAGAGGG-3'
OsCML1-3'UTR-R	5'-GCCAGTGTTATTTCTCGATC-3'

The 50 µl of reaction mixture contained 5 U of *Taq* DNA polymerase, 200 µM dNTPs, 1x PCR buffer, 2.5 mM $MgCl_2$, 50-100 pmole of DNA template, and 10 pmole of each primer. PCR amplification was performed as follows: pre-denaturation at 94 °C for 5 minutes, denaturation at 94°C for 2 minutes, annealing at 55°C for 1 minute, and extension at 72°C for 2 minutes. The final extension step was performed at 72 °C for 10 minutes. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining and UV transilluminator and photographed. The resulting PCR products were purified by the QIAquick Gel Extraction Kit (see in 2.3.10).

b) Restriction enzyme digestion

For the preparation of Elongation Factor1α probe, the clone of Elongation Factor1α (Accession number: AU091878) was digested with *BstE*II This digested

DNA was separated by agarose gel electrophoresis and purified by the QIAquick Gel Extraction Kit for use in the next experiment.

2.3.13 Southern Blot Analysis

a) Genomic DNA preparation

Oryza sativa L. tissues were ground in liquid nitrogen using chilled mortars and pestles. The plant material was kept frozen and ground to a fine powder. Then, the ground tissues were added into 700 µl of hot extraction buffer (60 °C) (100mM Tris-HCl (pH8.0), 40mM EDTA (pH7.5), 1.4 M NaCl and 2% (w/v) CTAB) and three microlitres of 2-mercaptoethanol. After that, the mixture was inverted several times to mix and incubated at 60°C for 30 minutes. During the incubation time, the mixture was inverted gently every 10 minutes. Then, the mixture was left at room temperature for 5 minutes. Then, 500 µl of chloroform:isoamyalcohol (24:1) was added and mixed gently for 5 minutes. The mixture was centrifuged at 14,000×g for 10 minutes at 4 °C. The upper aqueous phase was transferred to a fresh microcentrifuge tube. Genomic DNA was precipitated by the addition of 0.1 volumes of 3 M NaOAc and 0.6 volumes of isopropanol. The suspension was mixed gently and allowed to stand for 30 minutes on ice (or at -20 °C). Then, the pellet was collected by centrifugation at 14,000×g for 10 minutes at 4 °C. The pellet was allowed to dry for 10-15 minutes. After that, the genomic DNA was resuspended with 100 µl of TE buffer. Two microlitres of 10mg/ml RNase A was added and the mixture was incubated at 37°C for 1 hour. Then, one volume of phenol:chloroform: isoamyalcohol (25:24:1) was added. The sample was mixed thoroughly and

immediately centrifuged for 1 minute. The upper aqueous phase was transferred to a fresh tube. One volume of chloroform was added and mixed gently. The mixture was centrifuged at 14,000×g for 1 minute at 4 °C and the top aqueous phase was collected. 0.1 volumes of 3 M NaOAc and 2.5 volumes of ice cold absolute ethanol were added and the suspension was mixed gently and allowed to stand for 10-30 minutes on ice (or at -20 °C). The genomic DNA was collected by centrifuging at 14,000×g for 10 minutes at 4 °C. The pellet was rinsed with 200 µl of 70% ethanol and centrifuged at 14,000×g for 5 minutes at 4 °C. The pellet was allowed to dry for 10-15 minutes. The genomic DNA was resuspended with 50 µl of sterile water. The concentration was estimated by measuring the absorbance at 260 nm, and calculated in µg/ml unit, using the following equation:

$$[\text{DNA}] = A_{260} \times \text{dilution factor} \times 50^*$$

* The absorbance at 260 nm (A_{260}) of 1.0 corresponds to the DNA of approximately 50 µg/ml (Sambrook et al., 1989).

b) Agarose gel electrophoresis

Ten micrograms of the digested genomic DNA were mixed with 10% (v/v) of DNA gel loading Buffer (0.1M EDTA/NaOH pH 7.5, 50% (v/v) of glycerol, 1% (w/v) of SDS, 0.5 (w/v) of xylene cyanol FF, and 0.5 (w/v) of Bromophenol blue. and loaded into the 1.0% (w/v) TAE agarose gel. Electrophoresis was carried out at constant 100 volts. The gel was run until the tracking dye reached 1/4 from the bottom of the gel. After electrophoresis, the gel was stained with ethidium bromide solution (5-10 µg/ml in distilled water) for 3-5 minutes and the destained with

distilled water 2-3 times. The resolved DNA bands were visualized on an UV transilluminator and photographed.

c) Southern blotting

After electrophoresis, the gel to be blotted was removed from the electrophoresis chamber and soaked in 5-10 gel volumes of denaturation solution (1.5M NaCl, 0.5N NaOH) for 20 minutes with gentle agitation, once and in 5-10 gel volumes of neutralization solution (0.5M Trisma base, 3M NaCl) for 15 minutes with gentle agitation, twice. Then, the soaked gel was transferred to positively charged nylon membrane by a vacuum blotter in 20x SSPE transfer buffer (2.98 M NaCl and 0.2M $NaH_2PO_4.H_2O$) for 3 hours. When transfer is complete, the nylon membrane was carefully removed from the gel by flat-tipped forceps. The RNA was immobilized by UV cross-linking in Bio-Rad GS Gene Linker™ UV Chamber. The blot was stored at room temperature.

d) Oligolabeling

200 ng of DNA was mixed with 60 ng/µl of random hexanucleotide in a final volume of 10 µl. The mixture was incubated in boiling water for 3-5 minutes. Then, the denatured DNA was chilled on ice for 30 seconds, and any condensation was collected by a 2-second spin in a minicentrifuge. While holding the tube behind a plexiglass shield, the labeling was started by adding 2 µl of 1x klenow buffer, 5 µl of [α-^{32}P]dCTP, 2 µl of H_2O and 1 µl of 5 -6 units of klenow. The mixture was incubated for 60 minutes at room temperature and the reaction was stopped with 25 mM Na_2EDTA. One microlitre of sample was taken for total radioactivity by spotting

onto filter paper held in glass scintillation vial. The remainder of the solution was passed through a spin column (Bio-Rad) to remove the unincorporated [α-^{32}P]dCTP. Before applying the sample, the excess liquid in the spin column was removed and the column was packed by spinning for 2 minutes at 2500 rpm. Then the sample was loaded to the center of the packed resin and collected by spinning for 4 minutes at 2500 rpm. One microlitre of sample was taken for total radioactivity by spotting onto filter paper held in glass scintillation vial. Radioactivity of the samples taken was counted in a liquid scintillation counter. Then the efficiency of radiolabelling was calculated as follows:

$$\% \text{ incorporate} = \frac{\text{incorporated cpm}}{\text{total cpm}} \times \frac{\text{final volume}}{\text{initial volume}}$$

To prepare for hybridization, the DNA solution was incubated in boiling water for at least 5 minutes, before immediately added to the hybridization mixture.

e) Southern blot hybridization and autoradiography

The membrane was rehydrated in 500 ml of boiling 20 mM Tris/HCl pH 8.0. The solution was allowed to cool to room temperature for 15-20 minutes. During this time, the prehybridization solution containing 50%(v/v) deionized formamide, 5x SSPE/NaOH (pH 7.4), and 20mM $Na_2EDTA.2H_2O$, 1x Denhardt's solution (100x Denhardt's solution consists of BSA, Ficoll, and polyvinylpyrrolidone(PVP) at 2%[w/v] each), 0.2% (w/v) sodium dodecyl sulfate (SDS) and 100 µg/ml denatured DNA stock (calf thymus DNA and TF buffer, pH 8.0) was prepared and warmed to a desired temperature. The prehybridization solution was added to the blotted filter

placed in a plastic bag. The plastic bag was then sealed and incubated in a hybridization oven at the desire temperature overnight. When prehybridization was completed, the prehybridization solution was discarded and replaced with the hybridization solution (same as prehybridized solution, but without denatured DNA stock) previously equilibrated to a desired temperature, then the prepared denature ^{32}P-oligolabeled DNA probes was added. After removing bubbles the bag was sealed by a heat-sealer and incubated for at least 16 hours at the desired temperature. When hybridization was completed, the filter was removed and immediately washed in 2x SSPE, 0.1% SDS twice and once in 1x SSPE, 0.1% SDS at room temperature. If the general level of cpm was too high, washes would be repeated in 1x SSPE, 0.1% SDS at a higher temperature. After that the damp filter was wrapped in plastic wrap and placed in an x-ray cassette with a KODAK BioMax MS film scientific imaging film. The film was exposed overnight at -80 °C. When the blot contained barely enough cpm, exposures of 4 to 7 days were necessary in some cases. For detection, the X-ray film was washed in a developer solution, water, a fixer solution, and water respectively for 1 minute in every step and air-dried.

2.2.14 Northern Blot Analysis

a) Northern blotting

After electrophoresis, the gel to be blotted was removed from the electrophoresis chamber and soaked in 250 ml of 10 mM Na_2HPO_4/NaH_2PO_4, pH 7.0 twice for 15-20 minutes each and then transferred to positively charged nylon membrane by a vacuum blotter in 20x SSPE transfer buffer (2.98 M NaCl and 0.2M

$NaH_2PO_4.H_2O$) for 3 hours. When transfer is complete, the nylon membrane was carefully removed from the gel by flat-tipped forceps. The RNA was immobilized by UV cross-linking in Bio-Rad GS Gene Linker™ UV Chamber. The blot was stored at room temperature.

b) Oligolabeling

The probe-labeling was performed as indicated in 2.3.13(d).

c) Northern blot hybridization and autoradiography

The membrane was rehydrated in 500 ml of boiling 20 mM Tris/HCl pH 8.0. The solution was allowed to cool to room temperature for 15-20 minutes. During this time, the prehybridization solution containing 50%(v/v) deionized formamide, 5x SSPE/NaOH (pH 7.4), and 20mM $Na_2EDTA.2H_2O$, 1x Denhardt's solution (100x Denhardt's solution consists of BSA, Ficoll, and polyvinylpyrrolidone(PVP) at 2%[w/v] each), 0.2% (w/v) sodium dodecyl sulfate (SDS) and 100 μg/ml denatured DNA stock (calf thymus DNA and TF buffer, pH 8.0) was prepared and warmed to a desired temperature. The prehybridization solution was added to the blotted filter placed in a plastic bag. The plastic bag was then sealed and incubated in a hybridization oven at the desire temperature overnight. When prehybridization was completed, the prehybridization solution was discarded and replaced with the hybridization solution (same as prehybridized solution, but without denatured DNA stock) previously equilibrated to a desired temperature, then the prepared denature ^{32}P-oligolabeled DNA probes was added. After removing bubbles the bag was sealed by a heat-sealer and incubated for at least 16 hours at the desired temperature. When

hybridization was completed, the filter was removed and immediately washed in 2x SSPE, 0.1% SDS twice and once in 1x SSPE, 0.1% SDS at room temperature. If the general level of cpm was too high, washes would be repeated in 1x SSPE, 0.1% SDS at a higher temperature. After that the damp filter was wrapped in plastic wrap and placed in an x-ray cassette with a KODAK BioMax MS film scientific imaging film. Film exposure and signal detection was performed as indicated in 2.3.13(e).