3.0 MATERIALS AND METHODS

3.1 Plant Materials

The seeds of *Oryza rufipogon* (IRGC105491) and *Oryza sativa* ssp. *indica* cv. MR219 were obtained from the GenBank division of the Malaysian Agricultural Research and Development Institute (MARDI) in Seberang Perai, Penang, Malaysia. Seeds of BC_2F_7 materials were provided by a National Rice Breeding program which involved *Oryza rufipogon* as a donor parent crossed with Malaysian rice variety, *Oryza sativa* ssp *indica* cv. MR219 as the recurrent parent as shown in Figure 3.1. BC_2F_7 line 7 was identified as a relatively high yield progeny line; whereas, BC_2F_7 line 23 was identified as a relatively low yield progeny line among backcross progeny under this program (Wickneswari pers. comm. 2010). *Oryza rufipogon*, *Oryza sativa* ssp. *indica* cv. MR219, BC_2F_7 line 7 and BC_2F_7 line 23 were planted in cycles. Samples were collected during the vegetative, reproduction and ripening phrases (Table 3.1).

3.2 Growth Conditions

The rice seeds were soaked in water on wet tissue paper in Petri dishes for three days. After that, they were transferred to a plastic tray with loam soil for 20 to 21 days for further growth. The plastic tray was flooded with water and placed under shade. After 23-24 days, each rice seedling was transferred to a flower pot containing loam soil. After four weeks, 1.5 g of Sodium: Potassium: Phosphorus (15:15:15) fertilizer pellets were added to each plot every two weeks until the milk grain stage.

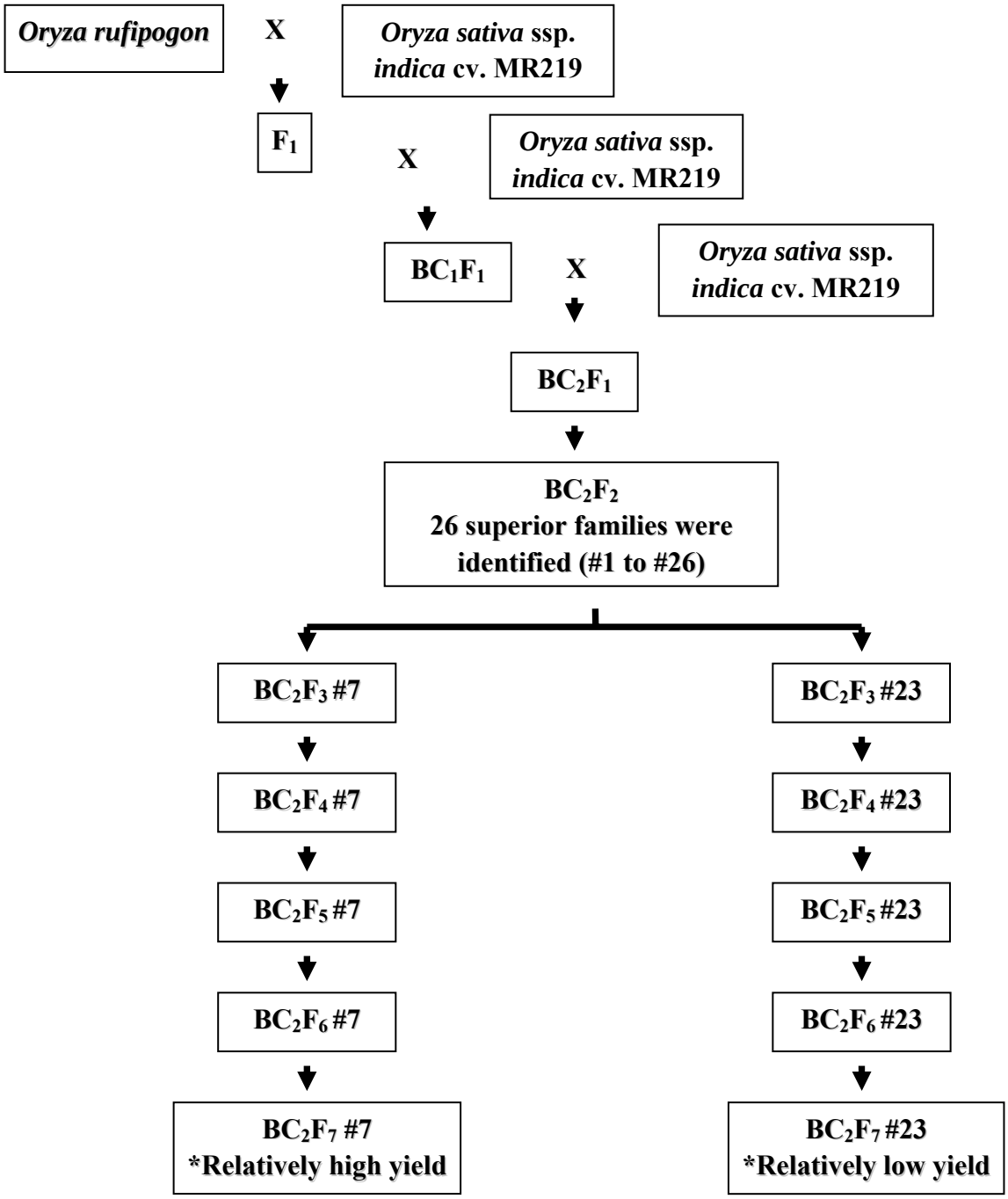

Figure 3.1: Schematic representation of advanced backcross progenies (*Oryza rufipogon* (IRGC105491) × *Oryza sativa* ssp. *indica* cv. MR219) under national breeding program (Sabu *et al*., 2006). *Relative yield across a three location trial over two growing seasons when BC_2F_7 line 7 the highest and BC_2F_7 line 23 the lowest grain yield were identified out of 26 backcross families (Appendix H; Wickneswari pers. comm. 2010).

No.	Line	Tissue	Stage	Phase
1	*Oryza rufipogon*	Leaf	8th day's seedling	Vegetative
2	*Oryza rufipogon*	Whole plant	8th day's seedling	Vegetative
3	*Oryza rufipogon*	Young panicle	Booting	Reproductive
4	*Oryza rufipogon*	Panicle	Heading	Reproductive
5	*Oryza rufipogon*	Panicle	Flowering	Reproductive
6	*Oryza rufipogon*	Grain	Milk grain	Ripening
7	MR219	Leaf	8th day's seedling	Vegetative
8	MR219	Whole plant	8th day's seedling	Vegetative
9	MR219	Young panicle	Booting	Reproductive
10	MR219	Panicle	Heading	Reproductive
11	MR219	Panicle	Flowering	Reproductive
12	MR219	Grain	Milk grain	Ripening
13	BC_2F_7 #7	Leaf	8th day's seedling	Vegetative
14	BC_2F_7 #7	Whole plant	8th day's seedling	Vegetative
15	BC_2F_7 #7	Young panicle	Booting	Reproductive
16	BC_2F_7 #7	Panicle	Heading	Reproductive
17	BC_2F_7 #7	Panicle	Flowering	Reproductive
18	BC_2F_7 #7	Grain	Milk grain	Ripening
19	BC_2F_7 #23	Leaf	8th day's seedling	Vegetative
20	BC_2F_7 #23	Whole plant	8th day's seedling	Vegetative
21	BC_2F_7 #23	Young panicle	Booting	Reproductive
22	BC_2F_7 #23	Panicle	Heading	Reproductive
23	BC_2F_7 #23	Panicle	Flowering	Reproductive
24	BC_2F_7 #23	Grain	Milk grain	Ripening

Table 3.1: List of rice samples used in this study. Samples were collected at the listed developmental stages from *Oryza rufipogon*, *Oryza sativa* ssp. *indica* cv. MR219 (MR219), BC_2F_7 line 7 and BC_2F_7 line 23.

3.3 Plasmid Vectors and Bacteria Strains

The plasmid vectors and bacteria strains used in this study are listed in Table 3.2.

No.	Plasmid Vectors or Bacteria Strains	Source
1	pGEM-T Easy Vector System	Promega, USA
2	pJET1.2/blunt cloning vector	Fermentas, Lithuania
3	pENTR™ Direction TOPO® vector	Invitrogen, California
4	pANDA vector	Prof. Ko Shimamoto, NAIST, Japan
5	*Escherichia coli* strain DH5α	Promega, USA
6	*Escherichia coli* strain DB3.1	Invitrogen, California

Table 3.2: List of plasmid vectors and bacterial strains.

3.4 Primer Design

The Primer Express 2.0 software (Applied Biosystems, USA) was used to design all primers that were used in this study. Primers of putative *RPK1* were designed according to sequences from Song *et al*. (2009). Meanwhile, primers of putative *CLV1* were designed according to the gene sequence of *Oryza sativa* ssp. *japonica* (gi:18677097). Different sets of primers were designed for different applications, including the amplification of open reading frames (ORFs), for amplification of RLM-RACE, for amplification of probe for Southern hybridization, for amplification of real time qRT-PCR, and for the construction of RNAi constructs. All primer sequences are shown in Table 3.3, Table 3.4, Table 3.5 and Table 3.6.

Primer Name	Primer Sequence
Southern hybridization RPK1	Forward: 5’ GCGGAAGACACCTTCTTGGTCAGG 3’ Reverse: 5’ GGAATTCCTTTGCTCCCAGCTCAA 3’
Southern hybridization CLV1	Forward: 5’ AGTGAACGAGAAGAGCGATGTG 3’ Reverse: 5’ CACGAAGAGCACACCATATATATTAGTTC 3’

Table 3.3: Primers sequences used to synthesize probes for Southern hybridization.

Primer Name	Primer Sequence
5’RACE-RPK1 Outer	Reverse: 5’AACTGCAGCGGATACGACCACAC 3’
5’RACE-RPK1 Inner	Reverse: 5’ TGCAGCGGATACGACCACACT 3’
3’RACE-RPK1 Outer	Forward: 5’AGGTTCTTTGGCTGCCTGTATG 3’
3’RACE-RPK1 Inner	Forward: 5’TCATCGTCTGCCCCCTTTGTG 3’
5' RACE Outer (Ambion, USA)	Forward: 5’GCTGATGGCGATGAATGAACACTG 3’
5' RACE Inner (Ambion, USA)	Forward: 5’CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG3’
3' RACE Outer (Ambion, USA)	Reverse: 5’ GCGAGCACAGAATTAATACGACT 3’
3' RACE Inner (Ambion, USA)	Reverse: 5’CGCGGATCCGAATTAATACGACTCACTATAGG 3’
ORF-RPK1	Forward: 5’ CTTCTCTCGCTAACCCACAGTGT 3’ Reverse: 5’ TGAGGCACAATGATCACAAAGG 3’

Table 3.4: Primers sequences used to amplify ORF and RLM-RACE cDNA sequence.

Primer Name	Primer Sequence
Real time qRT-PCR-RPK1	Forward: 5’ AGGTTCTTTGGCTGCCTGTATG 3’ Reverse: 5’ CCAACAAAACAATACCCAGGAAA 3’
Real time qRT-PCR-CLV1	Forward 1: 5’ TTCGGTGTGGTACTATTGGAGATC 3’ Forward 2: 5’ TTCGGTGTGGTACTACTGGAGATC 3’ Reverse: 5’ ACCCAGTCCACGATGTTGTTC 3’
Actin (Jain *et al*., 2006)	Forward: 5’ CAGCCACACTGTCCCCATCTA 3’ Reverse: 5’ AGCAAGGTCGAGACGAAGGA 3’
Eukaryotic elongation factor 1-alpha (eEF-1α; Jain *et al*., 2006)	Forward: 5’ TTTCACTCTTGGTGTGAAGCAGAT 3’ Reverse: 5’ GACTTCCTTCACGATTTCATCGTAA 3’
Ubiquitin 5 (UBQ5; Jain *et al*., 2006)	Forward: 5’ ACCACTTCGACCGCCACTACT 3’ Reverse: 5’ ACGCCTAAGCCTGCTGGTT 3’

Table 3.5: Primer sequences used for real time qRT-PCR analysis.

Primer Name	Primer Sequence
RPK1 pANDA	Forward: 5’ **CACC**AGGTTCTTTGGCTGCCTGTATG 3’ Reverse: 5’ CCAACAAAACAATACCCAGGAAA 3’
CLV1 pANDA	Forward: 5’ **CACC**AGTGAACGAGAAGAGCGATGTG 3’ Reverse: 5’ GTTGTTCCCCTCCCCGTACT 3’
Gus linker (Miki and Shimamoto, 2004)	Forward: 5’ CATGAAGATGCGGACTTACG 3’ Reverse: 5’ ATCCACGCCGTATTCGG 3’
Gus linker2 (pGUS1)	Forward: 5’ CATCCGGTCAGTGGCAGTGAA 3’ Reverse: 5’ TCGAGCATCTCTTCAGCGTAAGG 3’
Gus linker3 (pGUS2)	Forward: 5’ TACGTTAGCCGGGCTGCACTCAA 3’ Reverse: 5’ CAACGCGCAATATGCCTTGCGA 3’

Table 3.6: Primer sequences used for amplification for cloning into RNAi vector, pANDA.

3.5 DNA Extraction

DNA was isolated according to a Cetyl Trimethyl Ammonium Bromide (CTAB) DNA isolation technique (Stewart and Via, 1993). About 2 g of fresh leaf sample were ground under liquid nitrogen by mortar and pestle. After that, the sample was transferred into a 50 ml polycarbonate centrifuge tube containing 10 ml of pre-warmed CTAB buffer (Appendix A). Two percent of β-mercaptoethanol (β-ME) was added into the mixture and mixed vigorously by inversion. The mixture was incubated in a water bath for 60 min at 65 °C. After incubation, it was left on the bench for 10 min. Next, an equal volume of chloroform: isoamyl-alcohol (24:1) was added and mixed vigorously by inversion. The mixture was centrifuged at 2200 × g for 15 min. The upper aqueous phase was transferred into a new 50 ml Falcon tube. This step was repeated twice. Two volumes of absolute ethanol were added to precipitate the DNA pellet at -20 °C overnight. The next day, the mixture was centrifuged at 2200 × g for 15 min. The DNA pellet was washed with 10 ml of cold 70 % (v/v) ethanol and centrifuged at 2200 × g for 15 min. After that, the supernatant was discarded and the pellet was air-dried for 20 min. The DNA pellet was resuspended in 1 ml distilled water and 2 µl RNase (10 mg/ml) and was stored at -20°C. The concentration and purity of the DNA were determined by spectrophotometer (Eppendorf, Germany). The DNA extracted was analyzed by 0.7 % (w/v) agarose gel electrophoresis containing ethidium bromide.

3.6 Total RNA Extraction

Total RNA was isolated during grain development according to Gao *et al*. (2001). Extraction buffer (Appendix A) was preheated at 56 °C. After that, 2 % (w/v) of PVP-40, 2 % (w/v) of BSA and 10 mM of DTT were added. About 0.4 g fresh rice samples (see Table 3.1) were ground under liquid nitrogen by mortar and pestle. Then, the sample was transfered to a 2 ml centrifuge tube containing extraction buffer. Next, 3.5 µl of 100 mg of RNA grade Proteinase K was added immediately into the homogenate sample and incubated at 37 °C for 10 min. An equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) was added and mixed by vortex for 2 min. After that, the mixture was incubated at 56 °C for 20 min and then it was centrifuged at 12,000 x g for 15 min at 4 °C. Supernatant was transfer to new 2 ml centrifuge tubes and an equal volume of chloroform: isoamyl-alcohol (24:1) was added. The mixture was mixed by vortex. After that, the mixture was centrifuged at 12,000 × g for 15 min at 4 °C. This step was repeated twice. One third volume of LiCl was added into the 2 ml centrifuge tube with supernatant for precipitation overnight at 4 °C. The next day, the mixture was centrifuged at 12,000 × g for 15 min at 4 °C. Supernatant was discarded. The RNA pellet was rinsed with 1 ml of cold LiCl and centrifuged at 12,000 × g for 15 min at 4 °C. After that, the pellet was resuspended with 100 ml DEPC-treated distilled water (DEPC- dH_2O), 0.1 volumes of 3 M of sodium acetate and 2.5 volume of cold absolute ethanol. The mixture was precipitated the RNA pellet at -20 °C for overnight. The next day, the mixture was centrifuged at 12,000 × g for 15 min at 4 °C for recover RNA. The supernatant was discarded and washed with 1

ml of cold 70 % (v/v) ethanol and centrifuged at 12,000 × g for 5 min at 4 °C. After that, supernatant was discarded and the RNA pellet was air-dried for 15 min. The RNA pellet was resuspended with 30 µl of DEPC-dH_2O and stored at -80 °C. Concentration of total RNA was measured by spectrophotometer (Eppendorf, Germany). The integrity of total RNA extracted was confirmed by 1 % (w/v) agarose gel electrophoresis containing ethidium bromide.

3.7 First Strand cDNA Synthesis from Deoxyribonuclease I Treated Total RNA

3.7.1 Deoxyribonuclease I Treatment

Deoxyribonuclease I (DNase I) Amplification Grade (Invitrogen, California) was used to remove DNA from total RNA. A total of 2 µg/µl of total RNA sample, 1 × DNase I Reaction Buffer, 2 U of DNase I, Amp Grade (1 U/µl) were added to DEPC-dH_2O to 10 µl into 0.5 ml microcentrifuge tube. The mixture was incubated for 15 min at room temperature. After that, 1 µl of 25 mM EDTA solution was added into the 0.5 ml microcentrifuge tube and heated for 10 min at 65 °C to inactivate the DNase I reaction.

3.7.2 First Strand cDNA Synthesis

High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, USA) were used for cDNA synthesis. Oligo (dT) 12-18 primer (Invitogen, California) was used to replace the random primer provided (Applied Biosystems, USA). Total volume of 2.0 µl of 10 × RT Buffer, 0.8 µl of 25 × dNTP Mix (100mM), 1.0 µl of

oligo (dT) 12-18 primer (0.5 µg/ul), 50 U of MultiScribe™ Reverse Transcriptase (50 U/ul) and 20 U of RNase OUT™ Ribonuclease Inhibitor (Invitogen, California) (40 U/ul) were added with dH_2O to 10 µl into a 0.5 ml microcentrifuge tube. A total volume of 10 ul of DNase I treated total RNA (0.2 µg/ul) was added into the mixture and mixed gently with a pipette. Lastly, the mixture was placed in a DNA Thermocycler (Eppendorf, Germany). The program for cDNA synthesis was 25 °C for 10 min, 37 °C for 2 hr, and 85 °C for 5 sec.

3.8 Polymerase Chain Reaction (PCR)

GoTaq® Flexi DNA Polymerase (Promega, USA) was used to amplify DNA. Then, 50 ng DNA sample, 1 × Green GoTaq® Flexi Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs mixture, 0.2 µM of each forward and reverse primer (see section 3.4), 2.5 U GoTaq® Flexi DNA Polymerase (5 U/µl) were added to PCR grade dH_2O to 25 µl into a 0.5 ml microcentrifuge tube. A negative control with dH_2O template was also included for each primer pair. DNA was initially denatured at 95 °C for 2 min, followed by 30 cycles 30 sec at 95 °C, 30 sec at 58 °C, and 1 min at 72 °C in a DNA Thermocycler (Eppendorf, Germany). After 30 cycles, the reaction was followed by a final extension at 72 °C for 10 min.

3.9 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

For general RT-PCR purpose, GoTaq® Flexi DNA Polymerase (Promega, USA) was used to amplify cDNA. Then, 50 ng of cDNA sample, 1 × Green GoTaq® Flexi

Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs mixture, 0.2 µM of each forward and reverse primer (see section 3.4), 2.5 U GoTaq® Flexi DNA Polymerase (5 U/µl) were added with PCR grade dH_2O to 25 µl into a 0.5 ml microcentrifuge tube. A negative control with sample minus RT was also included for each primer pair. cDNA was initially denatured at 95 °C for 2 min, followed by 30 cycles of 30 sec at 95 °C, 30 sec at 58 °C, and 1 min at 72 °C in a DNA Thermocycler (Eppendorf, Germany). After 30 cycles, the reaction was followed by a final extension at 72 °C for 10 min.

For amplification of long fragments and GC rich content, Long PCR Enzyme Mix (Fermentas, Lithuania) was used to amplify the open reading frame (ORF). Then, 50 ng cDNA sample, 1 × Long PCR Buffer with 1.5 mM $MgCl_2$, 0.2 mM dNTPs mixture, 4 % DMSO, 0.2 µM of each forward and reverse primer (see section 3.4), 2.5 U Long PCR Enzyme Mix (5 U/µl) were added with PCR grade dH_2O to 25 µl into a 0.5 ml microcentrifuge tube. A negative control with sample minus RT was also included for each primer pair. cDNA was initially denatured at 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 45 sec at 58 °C, and 3 min at 68 °C in a DNA Thermocycler (Eppendorf, Germany). After 30 cycles, the reaction was followed by a final extension at 68 °C for 10 min.

3.10 Gel Extraction

DNA fragments of interest were purified from agarose gels using the QIAquick Gel Extraction Kit (QIAGEN, Germany). The slice of agarose gel containing the PCR

band was excised by clean scalpel and transferred to a 1.5 ml microcentrifuge tube. Three volumes of agarose lysis buffer were added to 1 volume of gel in the 1.5 ml microcentrifuge tube. After that, the tube was incubated at 50 °C for 10 min until the gel slice was completely dissolved. The tube was vortexed every 2-3 min during incubation to help dissolve the gel. The mixture was transferred to QIAquick column and centrifuged for 1 min. The flow-through was discarded and the spin column was placed back in the same 2 ml collection tube. Then, 750 µl of washing buffer was added to the QIAquick column and it was centrifuged for 1 min at 10,400 × g. The flow-through was discarded and the QIAquick column was placed back in the same 2 ml collection tube. The QIAquick column was centrifuged for an additional 1 min to dry the membrane. The collection tube was discarded and the QIAquick column was transferred to a clean 1.5 ml microcentrifuge tube. Elution was carried out using 30 µl of dH_2O. DNA was then recovered by centrifugation at 10,400 × g for 1 min. The sample was analyzed by gel electrophoresis.

3.11 Gene Cloning and Sequencing

3.11.1 Preparation of Competent Cells

A single colony of *E. coli* strain DH5α was used to inoculate 10 ml LB of broth and cultured overnight at 220 rpm and 37 °C. After 16-18 hr, 200 µl of overnight culture were added into a 50 ml polycarbonate centrifuge tube containing 20 ml LB broth. The mixture was cultured around 4 hr at 220 rpm and 37 °C until log phase (OD600 = 0.3-0.4). Then, the mixture was chilled in ice for 15 min before

centrifuging at 220 rpm around 4 min on 4 °C. Next, the supernatant was discarded. Then, 10 ml 0.1 M ice cold $CaCl_2$ was added into the tube to resuspended the pellet. After that, the tube was incubated on ice for 30 min. Later, the tube was centrifuged at 4800 × g for 5 min at 4 °C. Supernatant was discarded. Then, 2 ml of 85 mM ice cold $CaCl_2$ with 15 % glycerol were added into the tube to resuspended the pellet. Lastly, 200 µl of mixture was aliquoted into clean tube.

3.11.2 Preparation of Ligation Reactions

For sticky end cloning, ligation of DNA fragments of interest were carried out using T4 DNA Ligase (Promega, USA) overnight at 4 °C. The ligation reaction mixture contained 1 × Rapid Ligation Buffer, 3 Weiss units of T4 DNA ligase, purified PCR products and 50 ng of pGEM-T Easy vector in a molar ratio of 3:1. The reaction mixture was brought to a final volume of 10 µl with sterile dH_2O.

For blunt end cloning, ligation of unpurified PCR product were carried out using pJET1.2/blunt cloning vector (Fermentas, Lithuania) at room temperature for 20 min. The ligation reaction mixture contained 1 × Reaction Buffer, 3 Weiss units of T4 DNA ligase, 2 µl of unpurified PCR products and 50 ng of pJET1.2/blunt cloning vector. The reaction mixture was brought to a final volume of 20 µl with sterile dH_2O.

3.11.3 Transformation of *E. coli*

Frozen competent cells were thawed on ice. The ligation mixture was added into the competent cells and incubated for 30 min. Then, the mixture was incubated at 42 °C in water bath for exactly 45 sec. After that, the mixture was transferred immediately to ice to chill for 10 min. After this, an 800 µl aliquot of sterile LB of broth was added into the microcentrifuge tube. Next, the culture was shaken for 1/2-1 hr at 37 °C at 220 rpm. Then, 100 µl of transformed competent cells was transferred onto LB agar containing X-gal (80 µg/ml) and Ampicillin (50 µg/ml) or 50 µg/ml of Kanamycin and incubated overnight at 37 °C.

3.11.4 Plasmid DNA Preparation

Plasmid DNA was extracted using the QIAprep® Spin Minipreps system (QIAGEN, Germany). Putative positive colonies were selected randomly and checked by PCR with M13 primer set (see section 3.4). Positive colonies were inoculated into 10ml of LB medium containing Ampicillin (50 µg/ml) and incubated overnight at 37 °C by shaking incubator. Bacterial cells were centrifuged at 4800 × g for 15 min and the supernatant was discarded. Pelleted bacterial cells were resuspended in 250 µl cell resuspension buffer and transferred into 1.5 ml microcentrifuge tube. After that, 250 µl of lysis buffer was added into the mixture and mixed thoroughly by gently inverting the tube 4-6 times. This was followed by the addition of 350 µl of neutralization buffer and inverted gently the tube was inverted gently 4-6 times before centrifugation for 10 min at 17,900 × g.

The supernatant obtained after centrifugation was decanted into a QIAprep spin column that was placed on top of a collection tube which was then subjected to centrifugation at 17,900 × g for 1 min. The flow-through was discarded and the column was reinserted into the collection tube. The QIAprep spin column was then washed with 750 µl of wash buffer. The QIAprep spin column was centrifuged for an additional 1 min to dry the membrane. QIAprep spin column was transferred to a sterile 1.5 ml microcentrifuge tube. After that, 50 µl of elution buffer (10 mM Tris-Cl, pH 8.5) was added to the QIAprep spin column and incubated for 1 min at room temperature before being centrifuged at 17,900 × g for 1 min. The column was discarded and the eluted DNA was stored at -20 °C.

3.11.5 Sequencing Analysis

A commercial DNA sequencing service was provided by AbiBiotech Inc., Singapore.

3.12 Rapid Amplification of cDNA Ends (RACE) of Putative *RPK1*

FirstChoice® RLM-RACE Kit (Ambion, USA) was used to amplify the full length cDNA of the putative *RPK1* sequence. The rapid amplification of 5’ cDNA ends reactions were performed with 10 µg total RNA starting material. The RNA processing and 5’ adaptor ligation for cDNA synthesis was performed according to the manufacturer’s instructions. The outer 5' RLM-RACE PCR was performed by 5' RACE gene-specific outer primer and 5' RACE Outer Primer (see section 3.4); while

the inner 5' RLM-RACE PCR was performed by 5' RACE gene-specific inner primer and 5' RACE inner Primer (see section 3.4).

For the rapid amplification of 3’ cDNA ends, 1 μg total RNA was used as starting material. The 3’ adaptor ligation for cDNA synthesis was performed according to the manufacturer’s instructions. The outer 3' RLM-RACE PCR was performed by 3' RACE gene-specific outer primer and 3' RACE Outer Primer (see section 3.4); while the inner 3' RLM-RACE PCR was performed by 3' RACE gene-specific inner primer and 3' RACE inner Primer (see section 3.4).

The 5' and 3’ RLM-RACE PCRs were performed using AccuPrime™ GC-Rich DNA Polymerase (Invitrogen, California). The PCR cycling conditions were as follows: cDNA was initially denatured at 3 min at 95 °C, followed by 35 cycles of 95 °C for 30 sec, 30 sec at 58 °C, and 1 min at 72 °C in a DNA Thermocycler (Eppendorf, Germany). After 35 cycles, the reaction was followed by a final extension at 72 °C for 10 min. The 5' and 3’ RLM-RACE PCR product was analyzed by 1 % agarose gel electrophoresis, and then cloned into the CloneJET™ PCR Cloning Kit (Fermentas, Lithuania; see section 3.11.2).

3.13 Gene Sequence and Structure Analysis

Gene sequences were analyzed using GenScan (http://genes.mit.edu /GENSCAN.html) and GenView2 (http://zeus2.itb.cnr.it/~webgene/wwwgene.html).

The predicted peptide sequences obtained was analyzed using Simple Modular Architecture Research Tool (SMART; http://smart.embl-heidelberg.de/). The Kinase of Oruf_RPK1, OsI_RPK1, OsJ_RPK1 (gi:18677097) and OsJ_CLV1 (gi:125602183) were identified from SMART (http://smart.embl-heidelberg.de/) and were verified by a kinase domain prediction tool, KinG (http://hodgkin.mbu.iisc.ernet.in/;king; Krupa *et al*., 2004). Several well-studied protein kinases were selected and aligned with Oruf_RPK1, OsI_RPK1, OsJ_RPK1 (gi:18677097) and OsJ_CLV1 (gi:125602183) using ClustalW (Thompson *et al*., 1994; http://clustalw.genome.jp/). The characterization of the rice kinase was based on the presence or absence of conserved lysine kinase subdomain II, conserved arginine and aspartic acid (D) in kinase subdomain VIb and aspartic acid in kinase subdomain VII (Dardick and Ronald, 2006).

3.14 Base Substitution Mutation Analysis

Base substitution screening was detected by sequencing directly. RT-PCR products of putative *RPK1* gene sequences and putative *CLV1* gene sequences were purified from agarose gels using the QIAquick Gel Extraction Kit (QIAGEN, Germany). After that, purified RT-PCR products were sequenced directly. The sequencing data of putative *RPK1* and putative *CLV1* gene sequences were analysed and used to identify base substitutions. This analysis included investigation of heterozygous and homozygous single nucleotide polymorphisms (SNPs).

3.15 Phylogenetic Analysis

A total of 14 orthologous RPK1 amino acid sequences and 16 orthologous CLV1 amino acid sequences were selected from the GenBank protein database and OrthoMCL database (Table 3. 3; Table 3.4; Chen *et al*., 2006). Significant hit values above 1^{e-50} were selected. The highly diverged regions of orthologous amino acid sequences were eliminated using program Gblocks 0.91b (http://www.phylogeny.fr/version2_cgi/one_task.cgi?task_type =gblocks; Castresana, 2000; Dereeper *et al*., 2008). After that, conserved region of orthologous amino acid sequences were used for further phylogenetic analysis. Neighbor–Joining phylogenetic trees were constructed using MEGA (Kumar *et al*., 2008). Confidence levels for the branches in the trees were estimated by bootstrap analysis with 1,000 replicates.

No.	Name	Abbreviation	Accession No.	Species
1	Probable inactive receptor kinase	At_LRR1	At5g16590	*Arabidopsis thaliana*
2	Probable inactive receptor kinase	At_RKL1	At1g48480	*Arabidopsis thaliana*
3	Receptor-like kinase protein	At_RLK	At4g23740	*Arabidopsis thaliana*
4	Receptor-like kinase protein	At_RLK902	At3g17840	*Arabidopsis thaliana*
5	Putative leucine-rich repeat transmembrane protein kinase	At_TMK	At5g58300	*Arabidopsis thaliana*
6	Leucine-rich repeat transmembrane protein kinase like 1	At_TMKL1	At3g24660	*Arabidopsis thaliana*
7	Putative Receptor-like Protein Kinase 1	Oruf_RPK1	-	*Oryza rufipogon*
8	Putative Receptor-like Protein Kinase 1	OsI_RPK1	-	*Oryza sativa ssp. indica*
9	Putative Receptor-like Protein Kinase 1	OsJ_RPK1	gi:18677097	*Oryza sativa ssp. japonica*
10	Receptor-like kinase like protein 1	OsJ_RLL1	gi:7573610	*Oryza sativa ssp. japonica*
11	Receptor-like kinase like protein 2	OsJ_RLL2	gi:15128407	*Oryza sativa ssp. japonica*
12	Receptor-like kinase like protein 3	OsJ_RLL3	gi:115464509	*Oryza sativa ssp. japonica*
13	Putative atypical receptor-like kinase MARK	OsJ_MARK	gi:108710729	*Oryza sativa ssp. japonica*
14	Putative atypical receptor-like kinase	Zm_MARK	gi:226498594	*Zea mays*

Table 3.7: List of orthologous RPK1 amino acid sequences.

No.	Name	Abbreviation	Accession No.	Species
1	BARELY ANY MERISTEM 1	At_BAM1	At5g65700)	*Arabidopsis thaliana*
2	BARELY ANY MERISTEM 2	At_BAM2	At3g49670	*Arabidopsis thaliana*
3	BARELY ANY MERISTEM 3	At_BAM3	At4g20270	*Arabidopsis thaliana*
4	CLAVATA1 Receptor-like Kinase	At_CLV1	At1g75820	*Arabidopsis thaliana*
5	CLAVATA1-like protein 1	Gm_CLL1	gi:25956280	*Glycine max*
6	CLAVATA1-like protein 2	Gm_CLL2	gi:7329124	*Glycine max*
7	CLAVATA1-like protein 3	Gm_CLL3	gi:9651943	*Glycine max*
8	CLAVATA1-like protein 4	Gm_CLL4	gi:9651945	*Glycine max*
9	CLAVATA1-like receptor kinase	Mt_CLV1	gi:58372544	*Medicago truncatula*
10	CLAVATA1-like protein1	OsJ_CLL1	gi:50726262	*Oryza sativa ssp. japonica*
11	Putative CLAVATA1 Receptor-like Kinase	OsJ_CLV1	gi:125602183	*Oryza sativa ssp. japonica*
12	FLORAL NUMBER 1	OsJ_FON1	gi:113596633	*Oryza sativa ssp. japonica*
13	Receptor kinase-like protein1	OsJ_RLL1	gi:31745227	*Oryza sativa ssp. japonica*
14	CLAVATA1-like protein 1	Pg_CLL1	gi:104642235	*Picea glauca*
15	CLAVATA1-like protein 1	Ps_CLL1	gi:24940244	*Pisum sativum*
16	CLAVATA1-like protein 1	Vv_CLL1	gi:225424960	*Vitis vinifera*

Table 3.8: List of orthologous CLV1 amino acid sequences.

3.16 Southern Hybridization Analysis

3.16.1 Labeling of Hybridization Probes

A DIG DNA Labelling and Dectection Kit (Roche, USA) was used for Southern hybridization analysis. First, 300 ng of plasmid DNA in a final volume of 16 µl was heated in a boiling water bath for 10 min. Next, the mixture was placed on ice immediately for 5 min. DIG-High Prime was mixed thoroughly and 4 µl was added into the denatured sample and incubated at 37 °C for overnight. After that, 2 µl 0.2 M EDTA (pH 8.0) was added into the sample to stop the reaction.

3.16.2 Preparation of Genomic DNA Blot

Thirty µg genomic DNA was digested with the restriction enzymes *Eco*RI (30 U), *Bam*HI (30 U) and *Hind*III (30 U) (Fermentas, Lithuania) separately at 37 °C overnight. The digested genomic DNA was purified by chloroform: isoamylalcohol (24:1). This step was repeated twice. After that, 0.1 volume of 3 M sodium acetate and 2.5 volume of cold absolute ethanol were added into the supernatant to precipitate the digested genomic DNA at -20 °C for overnight. The next day, the mixture was centrifuged at 12,000 × g for 15 min at 4 °C for recover DNA. The pellet was washed with 1 ml of cold 70 % (v/v) ethanol and air-dried for 20 min. The pellet was resuspended in 10 µl of sterile dH_2O.

The digested genomic DNA and DIG-labeled DNA Molecular Weight Marker VII (Roche, Germany) were electrophoresed on 0.7 % (w/v) agarose gel containing

ethidium bromide at 40 V for 4 hr and 30 min. After that, the gel was visualized by a Gel Documentation System (Alpha Innotech, USA). Subsequently, the gel was submerged in 250 mM HCl with shaking at room temperature for 10 min. Next, the gel was submerged in Denaturation Solution (0.5 M NaOH, 1.5 M NaCl) for 15 min at room temperature with gentle shaking. The denaturation step was repeated once. Then, the gel was submerged in Neutralization Solution (0.5 M Tris-HCl, pH 7.5; 1.5 M NaCl) for 15 min at room temperature. The neutralization step was repeated once. After that, the gel was equilibrated in 20 × SSC for at least 10 min and capillary blotted onto a nylon membrane (Roche, Germany) overnight. The nylon membrane was rinsed with 2 × SSC and baked at 80 °C for 2 hr.

3.16.3 Hybridization and Visualisation of Genomic DNA Blot

The membrane was soaked in 10 ml of DIG Easy Hyb (Roche, Germany) at 39 °C for 30 min during pre-hybridization. Subsequently, 800 ng of probe and 50 µl of 2 × SSC were mixed and boiled for 10 min. Next, the mix was placed on ice immediately for 5 min. Then, the mixture was added to 3.5 ml pre-warmed DIG Easy Hyb (Roche, Germany) before being added to the pre-hybridization buffer. The membrane was hybridized at 39 °C for 16 hr. After hybridization, the membrane was washed with 200 ml Low Stringency Buffer (2 × SSC containing 0.1 % SDS) for 5 min with shaking. This step was repeated once. Then, the membrane was washed with 20 ml preheated High Stringency Buffer for 15 min with shaking at 60 °C. This step was repeated once.

The membrane was transferred and washed in 100 ml Washing Buffer for 2 min at room temperature with shaking. Then, the membrane was transferred and incubated in 100 ml Blocking Solution for 30 min with shaking. Later, the membrane was transferred and incubated in 20 ml Antibody Solution for 30 min, with shaking. Next, the membrane was washed with 100 ml Washing buffer for 15 min. This step was repeated once. The membrane was equilibrated in 20 ml Detection Buffer for 3 min. Then, 200 µl of NBT stock solution was added into 10 ml of Detection Buffer to make Colour Substrate Solution. The 10 ml Colour Substrate Solution was added and covered whole membrane completely without shaking and light for 16 hr. About 50 ml of TE buffer was added to stop the colour reaction.

3.17 Quantification of Gene Expression

3.17.1 Real Time Quantitative Reverse Transcriptase PCR (qRT-PCR) Amplification Efficiency

All the endogenous controls and gene specific primer target sets were tested for amplification efficiency before performing real time qRT-PCR experiments. The amounts of the first strand cDNA of *Oryza rufipogon* were tested at 1 ng, 10 ng, 50 ng, 100 ng and 200 ng with 200 nM primer. The average C_T value was ploted against the log of cDNA concentration in order to generate a standard curve. The slope of the standard curve indicated the PCR amplification efficiency. The PCR efficiency between 90-110 % is considered acceptable. All calculations were according to the geNORM v3.4 software (Primer-Design, UK) manual as below:

Equation A for calculation of the slope of the standard curve:

$y = mx + c$

where y = C_T value; m = slope of the standard curve; x = log input of concentration cDNA; c = y-intercept of the standard line

Equation B for calculation of PCR amplification efficiency:

$E = (10^{-1/slope} - 1) \times 100$

Where E = PCR amplification efficiency; slope = m as mentioned in Equation A

3.17.2 Housekeeping Gene Selection

Actin, *eukaryotic elongation factor 1-alpha* (*eEF-1α*) and *ubiquitin 5* (*UBQ5*) were used as housekeeping genes. The geNORM v3.4 software (Primer-Design, UK) was used for the analysis of the average C_T value of housekeeping gene expression stability at different developmental stages in rice (Vandesompele *et al*., 2002). These included leaves and also the whole plant from the 8th day at the seedling stage, the panicles at the booting, heading and flowering stages, as well as grains at the milk grain stage. Gene stability measure (M) below 1.5 was considered to be stable. However, the two genes with lowest measure (M) were selected to normalize target gene expression.

3.17.3 Real Time Quantitative Reverse Transcriptase PCR (qRT-PCR)

An Applied Biosystems 7300 Real Time PCR System was used to perform relative quantitative (Comparative C_T Method) real time qRT-PCR experiments. A total of four replicates of each stage of each line were used for each gene specific primer set (see section 3.4) and housekeeping gene primer set (see section 3.4). Approximately 50 ng cDNA sample, 1 × SYBR Green PCR Master Mix (Applied Biosystems, USA), and 200 nM of each forward and reverse primer (see section 3.4) were added to PCR grade dH_2O to 25 µl in an 8 well optical reaction strip (Applied Biosystems, USA). Three replicates of a negative control with dH_2O as template was also included for each primer pair. The real time qRT-PCRs were performed under following conditions: 1 cycle of 10 min at 95 °C, followed by 40 cycles of 15 sec at 95 °C and 1 min at 60 °C. geNORM v3.4 software (Primer-Design, UK) was used to analyze relative expression of each transcript (Vandesompele *et al*., 2002) in order to obtain more accurate normalization. All calculations of real time qRT-PCR were according the geNORM v3.4 software manual as below:

Equation C for calculation of the relative sample quantity:

$$Q = E^{\text{deltaCt}}$$

$$Q = E^{(\text{minCt} - \text{sampleCt})}$$

Where Q = Relative sample quantity; E = PCR efficiency

Equation D for calculation of the normalized expression level of target:

Normalized expression level of target n = $Q_{targetn}$ / $NF_{targetn}$

Where $Q_{targetn}$ = relative sample quantity of target n; $NF_{targetn}$ = normalization factor of target n

Equation E for calculation of the rescaled normalized expression target:

$$\text{Rescaled normalized expression target} = \frac{\text{normalized expression level}_{\text{target n}}}{\text{normalized expression level}_{\text{calibrator}}}$$

3.17.4 Statistical Analysis of Real Time qRT-PCR

Data of putative *RPK1* and putative *CLV1* expression were analyzed by two-way repeated measures analysis of variance (ANOVA) with $p<0.01$ considered significant using Prism 5 (GraphPad Software, USA). Data of rescaled normalized expression target and standard error of rescaled normalized expression target generated from geNORM v3.4 software (Primer-Design, UK) were used for statistical analyses.

3.18 Construction of RNAi Vectors for Knockdown of Gene Expression

A Gateway system vector, pANDA (Miki and Shimamoto, 2004), was used to construct vectors for of study gene function of candidate yield-related genes based on the RNAi method. *OsI_RPK1* and *OsI_CLV1* were selected.

3.18.1 Subcloning of Gene of Interest into pENTR/D-TOPO Cloning Vector

For pENTR/D-TOPO Cloning, four bases "CACC' were added at the 5' end of the forward primers (see section 3.4) to provide the correct orientation of the PCR product. The RT-PCR product was subcloned into pENTR/D-TOPO cloning vector (Invitrogen, California). pENTR/D-TOPO cloning vector provided the correct orientation for blunt-end PCR product and it was used as donor for the gateway destination vector. Molar ratio of PCR product: pENTR/D-TOPO cloning vector (0.5:1 to 2:1) and 1 µl of salt solution were added with dH_2O to 6 µl into a 0.5 ml microcentrifuge tube. After that, the mixture was mixed gently and incubated for 15 min at room temperature. Then, 2 µl mixtures were transformed into *E. coli* strain DH5α (see section 3.11.3). Putative positive colonies were selected randomly and checked by PCR using M13 forward primer and gene of interest reverse primer (see section 3.4). Plasmids of positive colonies were isolated (see section 3.11.4). Lastly, the recombinant plasmids with genes of interested were sequenced (see section 3.11.5).

3.18.2 Cloning of pENTR/D-TOPO Cloning Vector Containing Gene of Interest into pANDA Vector

Plasmid DNA of pANDA vector and pENTR/D-TOPO cloning vector containing the sequences of interest were isolated. After that, plasmid of pENTR/D-TOPO cloning vector containing sequences of interest was mobilized into the pANDA vector by an LR Clonase reaction. Plasmid DNA of pENTR/D-TOPO cloning vector

containing sequences of interest (100 ng/µl), pANDA vector (300 ng/µl), 2 µl of 5 times LR Reaction Buffer, 2 µl of LR Clonase enzyme (Invitrogen, California) were mixed with TE buffer to a final volume of 10 µl into a 0.5 ml microcentrifuge tube. The LR reaction mixture was incubated at 25 °C overnight. After that, 1 µl of Proteinase K was added into the mixture to stop the reaction and it was incubated at 37 °C for 10 min. Next, 6 µl of LR reaction mixture was mixed with competent cells of *E. coli* strain DH5α (see section 3.11.3). The final binary vector has the *attB* sequences of 50 bp. Putative positive colonies were selected randomly and checked by PCR amplification with Gus linker primer and gene of interest primer (see section 3.4). Gus linker forward primer and gene of interest reverse primer were used to check the sense orientation. Gene of interest forward primer and Gus linker reverse primer were used to check the antisense orientation. Plasmids of positive colonies were isolated (see section 3.11.4). Lastly, the plasmids with genes of interested were sequenced (see section 3.11.5).

3.19 Plant Transformation

3.19.1 Preparation of *Agrobacterium tumefaciens* Competent Cells

A single colony of *Agrobacterium tumefaciens* strain EHA105 was used to inoculate 3 ml of LB medium containing 10 ug/ml rifampicin and cultured overnight at 220 rpm and 28 °C. After that, 0.5 ml of the overnight culture was inoculated into a 50 ml polycarbonate centrifuge tube containing volume of LB broth with 10 ug/ml rifampicin and cultured overnight at 220 rpm and 28 °C until log phase (OD 600 =

0.3-0.4). Then, the mixture was chilled in ice for 15 min and it was centrifuged at 3200 × g for 5 min at 4 °C. The supernatant was discarded, and then 110 ml of sterile ice-cold 100 mM $MgCl_2$ solution was added into the tube and the pellet resuspended carefully then incubated on ice for 1 hr. Another centrifugation step was repeated as above, then 2 ml of 20 mM sterile ice-cold $CaCl_2$ was added into the tube with the pellet and resuspended carefully. It was incubated on ice for 6 hr to yield the competent cell suspension. After that, 20 % glycerol was added into the tube to resuspended the pellet. Lastly, 200 µl of mixture was aliquoted into clean tube and stored at -80 °C.

3.19.2 Transformation of pANDA RNAi Construct Containing the Required Sequences into *Agrobacterium tumefaciens* Strain EHA105

The transformation protocol was based on the freeze and thaw method (Jyothishwaran *et al*., 2007). Frozen competent cells of *Agrobacterium tumefaciens* strain EHA105 were thawed on ice for 5 min. Then, 2 µg of pANDA vector containing sequences of interest was added and mixed carefully. After that, the competent cells were frozen in liquid nitrogen for 10 min and then thawed at 37 °C for 5 min. Next, 10 µl were transferred into 1 ml of pre-warmed LB medium and incubated for 1 hr at 28 °C with 220 rpm in water bath shaker. After that, 50 µl of the suspension was spread onto an LB agar plate containing 10 ug/ml rifampicin and 50 µg/ml kanamycin. The plate was incubated at 28 °C for 24 hr. Putative positive

colonies were selected randomly and checked by PCR amplification with a Gus linker primer (see section 3.4).

3.19.3 Transformation and Selection of Hygromycin Resistant Plants

Callus induction from mature rice seeds was according to Sivakumar *et al*. (2010). One month old callus were transformed based on *Agrobacterium tumefaciens*-mediated transformation method (Toki, 1997). Then, they were selected and regenerated on 50 μg/ml hygromycin medium. Regenerated hygromycin resistant plants were grown in a Physical Containment Level 2 (PCL 2) transgenic greenhouse under natural lighting conditions. Lastly, leaves of hygromycin resistant plants at flowering stage were analyzed by PCR with Gus linker primer (see section 3.4).