# 4.0 RESULTS

## 4.1 Characterization and Identification of Full Length cDNA of Putative *RPK1*

### 4.1.1 Isolation of Full Length cDNA and Sequence Analysis of Putative *RPK1*

Primers designed from putative *RPK1* 5' untranslated region (5' UTR) and 3' untranslated region (3' UTR) of *Oryza sativa* ssp. *japonica* (gi:18677097) and *Oryza rufipogon* IRGC105491 Song *et al*. (2009) successfully amplified a band of 2,419 bp from cDNA of *Oryza rufipogon* and *Oryza sativa* ssp. *indica* cv. MR219 respectively (Figure 4.1). These two fragments of gel extraction product (Figure 4.2) were successfully cloned into pGEM-T Easy vector (Promega, USA). DNA sequencing of *Oryza rufipogon* putative *RPK1* (*Oruf_RPK1*) and *Oryza sativa* ssp. *indica* cv. MR219 putative *RPK1* (*OsI_RPK1*) were analysed by using GenScan (http://genes.mit.edu/GENSCAN.html) and a 2,055 bp long fragment open reading frame (ORF) was identified in both sequences. Multiple alignment showed that the gene sequences of ORF *Oruf_RPK1* and *OsI_RPK1* showed 99 % identity with *Oryza sativa* ssp. *japonica* putative *RPK1* (*OsJ_RPK1*; gi:18677097; Figure 4.3). A total of eleven single nucleotide polymorphisms (SNPs) were identified among the ORF of *Oruf_RPK1*, *OsI_RPK1* and *OsJ_RPK1* (gi:18677097) as shown in Figure 4.3, including ten SNPs between *Oruf_RPK1* and *OsI_RPK1*, eight SNPs between *Oruf_RPK1* and *OsJ_RPK1* (gi:18677097), and five SNPs between *OsI_RPK1* and *OsJ_RPK1* (gi:18677097). Five out of the eleven SNPs are non-synonymous substitutions; whereas, the remaining SNPs are synonymous substitutions.

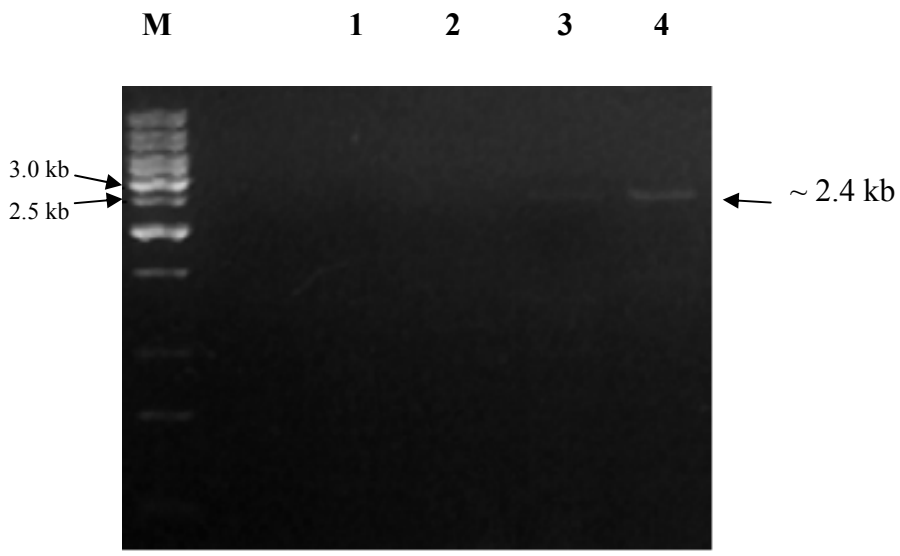



Figure 4.1: RT-PCR amplification of putative *RPK1*. The expected size of *Oruf_RPK1* and *OsI_RPK1* is around 2.4 kb. Lane M: 1 kb marker; Lane 1: DNase I treated total RNA of *Oryza rufipogon*; Lane 2: DNase I treated total RNA of *Oryza sativa* ssp. *indica* cv. MR219; Lane 3: *Oryza rufipogon* cDNA; Lane 4: *Oryza sativa* ssp. *indica* cv. MR219 cDNA.

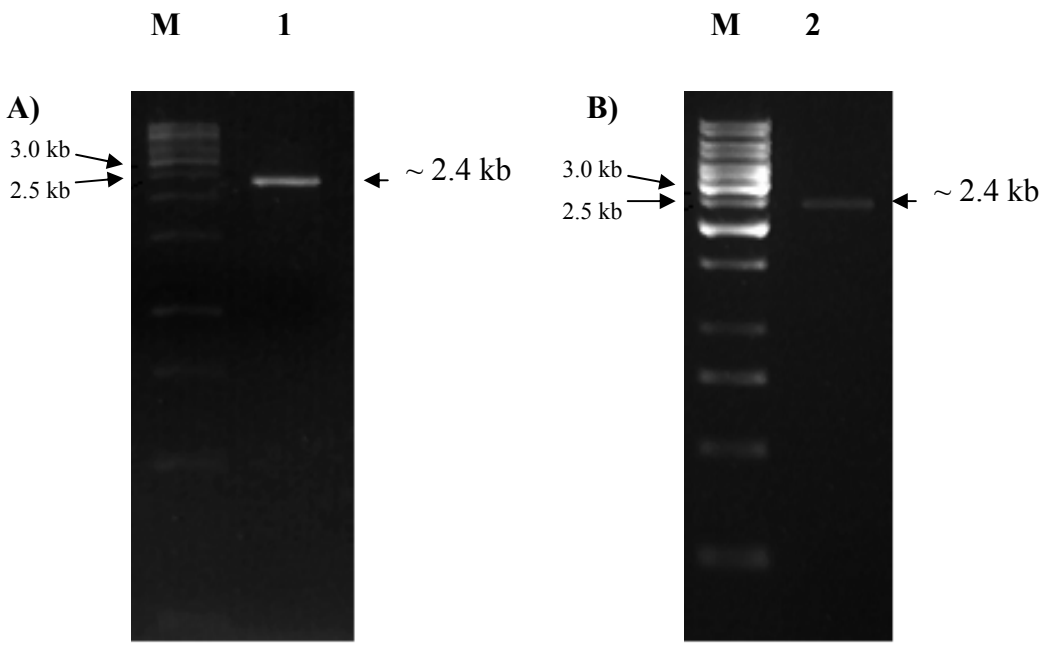
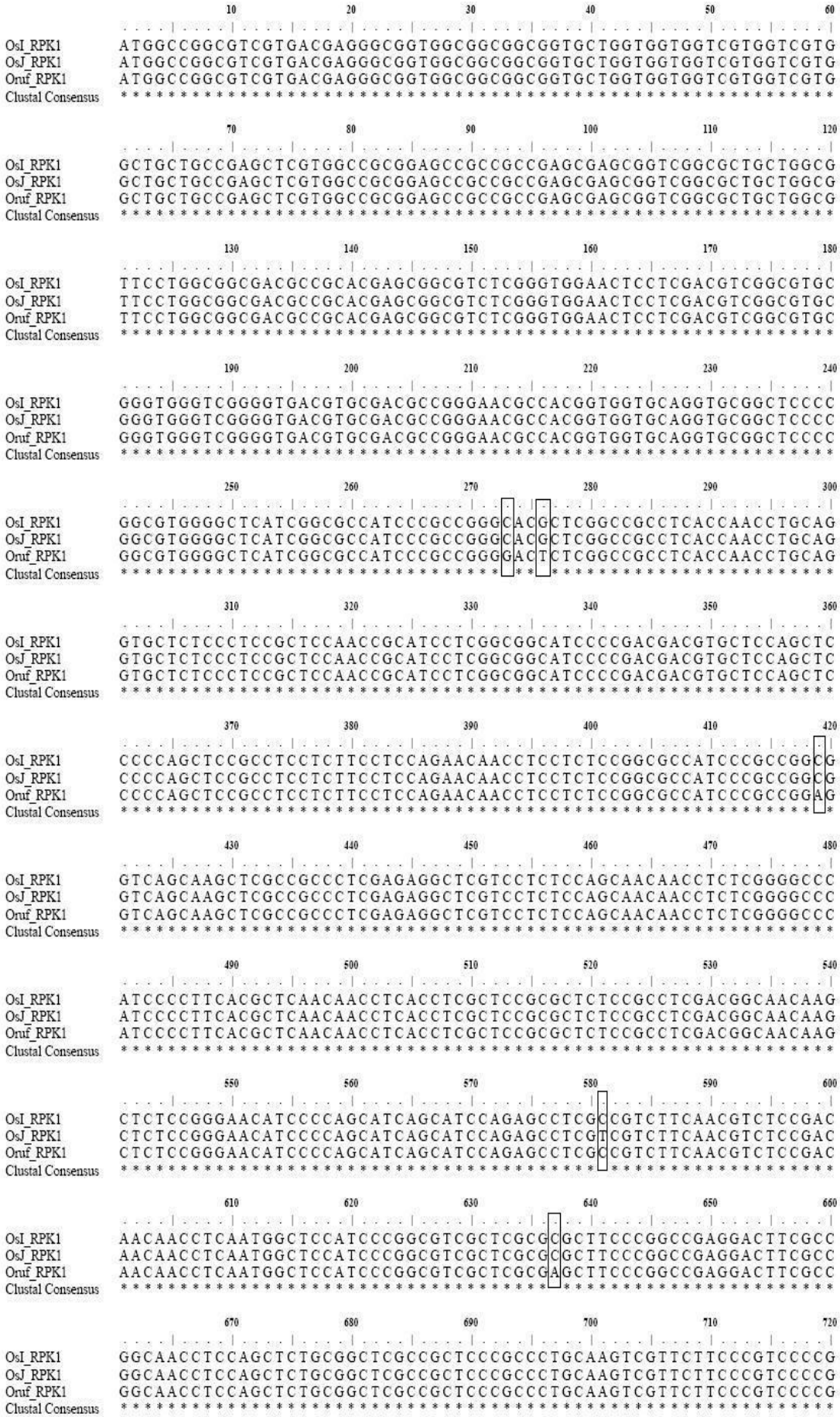
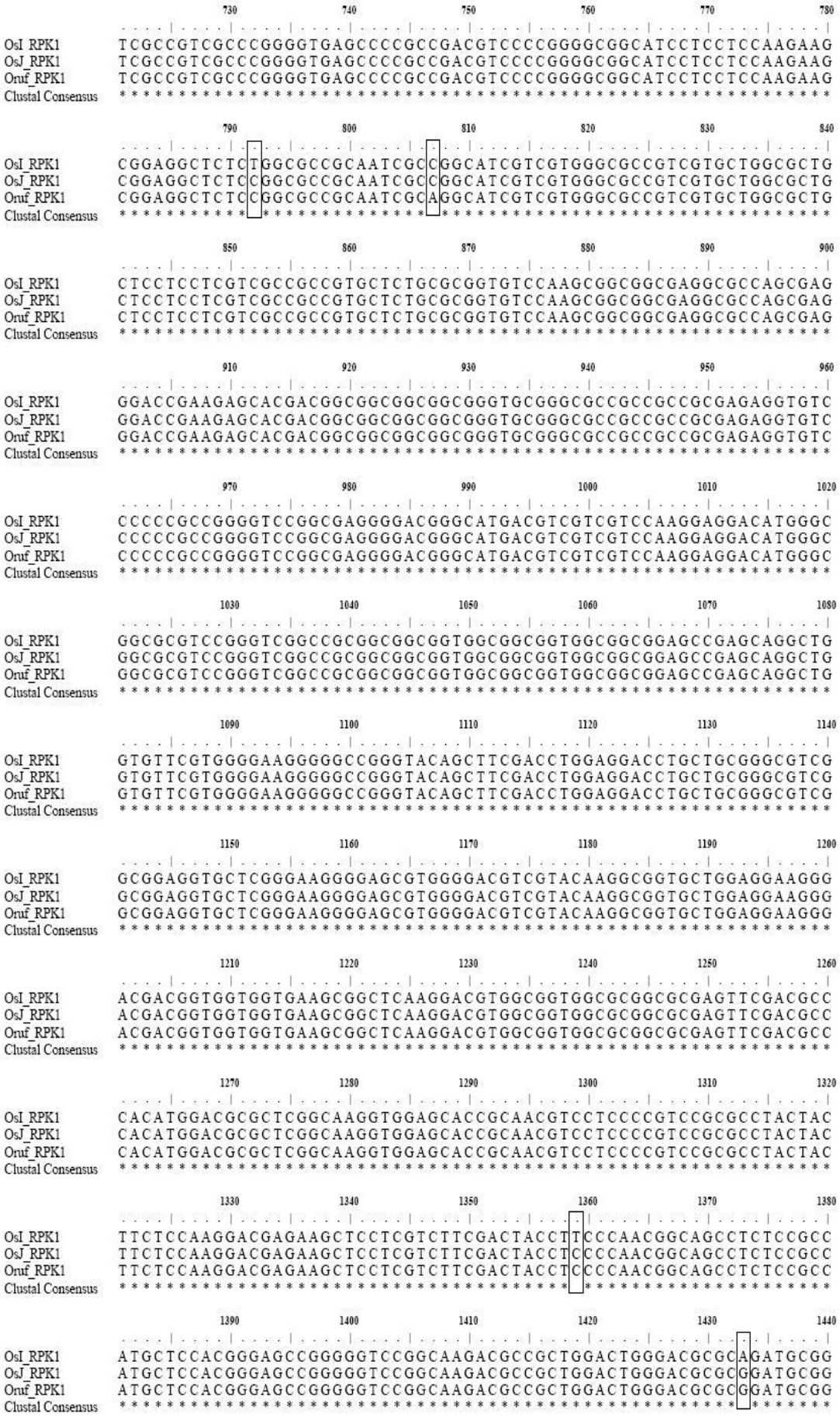



Figure 4.2: Gel extraction products of (A) *Oruf_RPK1* and (B) *OsI_RPK1*. Lane M: 1 kb marker; Lane 1: *Oruf_RPK1*; Lane 2: *OsI_RPK1*.


OsI_RPK1
OsJ_RPK1
Oruf_RPK1
Clustal Consensus


730 740 750 760 770 780
OsI_RPK1 TCGCCGTCGCCCGGGGTGAGCCCCGCCGACGTCCCCGGGGCGGCATCCTCCTCCAAGAAG
OsJ_RPK1 TCGCCGTCGCCCGGGGTGAGCCCCGCCGACGTCCCCGGGGCGGCATCCTCCTCCAAGAAG
Oruf_RPK1 TCGCCGTCGCCCGGGGTGAGCCCCGCCGACGTCCCCGGGGCGGCATCCTCCTCCAAGAAG
Clustal Consensus
790 800 810 820 830 840
OsI_RPK1 CGGAGGCTCTCTGGCGCCGCAATCGCCGGCATCGTCGTGGGCGCCGTCGTGCTGGCGCTG
OsJ_RPK1 CGGAGGCTCTCCGGCGCCGCAATCGCCGGCATCGTCGTGGGCGCCGTCGTGCTGGCGCTG
Oruf_RPK1 CGGAGGCTCTCCGGCGCCGCAATCGCAGGCATCGTCGTGGGCGCCGTCGTGCTGGCGCTG
Clustal Consensus
850 860 870 880 890 900
OsI_RPK1 CTCCTCCTCGTCGCCGCCGTGCTCTGCGCGGTGTCCAAGCGGCGGCGAGGCGCCAGCGAG
OsJ_RPK1 CTCCTCCTCGTCGCCGCCGTGCTCTGCGCGGTGTCCAAGCGGCGGCGAGGCGCCAGCGAG
Oruf_RPK1 CTCCTCCTCGTCGCCGCCGTGCTCTGCGCGGTGTCCAAGCGGCGGCGAGGCGCCAGCGAG
Clustal Consensus
910 920 930 940 950 960
OsI_RPK1 GGACCGAAGAGCACGACGGCGGCGGCGGCGGGTGCGGGCGCCGCCGCCGCGAGAGGTGTC
OsJ_RPK1 GGACCGAAGAGCACGACGGCGGCGGCGGCGGGTGCGGGCGCCGCCGCCGCGAGAGGTGTC
Oruf_RPK1 GGACCGAAGAGCACGACGGCGGCGGCGGCGGGTGCGGGCGCCGCCGCCGCGAGAGGTGTC
Clustal Consensus
970 980 990 1000 1010 1020
OsI_RPK1 CCCCCGCCGGGGTCCGGCGAGGGGACGGGCATGACGTCGTCGTCCAAGGAGGACATGGGC
OsJ_RPK1 CCCCCGCCGGGGTCCGGCGAGGGGACGGGCATGACGTCGTCGTCCAAGGAGGACATGGGC
Oruf_RPK1 CCCCCGCCGGGGTCCGGCGAGGGGACGGGCATGACGTCGTCGTCCAAGGAGGACATGGGC
Clustal Consensus
1030 1040 1050 1060 1070 1080
OsI_RPK1 GGCGCGTCCGGGTCGGCCGCGGCGGCGGTGGCGGCGGTGGCGGCGGAGCCGAGCAGGCTG
OsJ_RPK1 GGCGCGTCCGGGTCGGCCGCGGCGGCGGTGGCGGCGGTGGCGGCGGAGCCGAGCAGGCTG
Oruf_RPK1 GGCGCGTCCGGGTCGGCCGCGGCGGCGGTGGCGGCGGTGGCGGCGGAGCCGAGCAGGCTG
Clustal Consensus
1090 1100 1110 1120 1130 1140
OsI_RPK1 GTGTTCGTGGGGAAGGGGGCCGGGTACAGCTTCGACCTGGAGGACCTGCTGCGGGCGTCG
OsJ_RPK1 GTGTTCGTGGGGAAGGGGGCCGGGTACAGCTTCGACCTGGAGGACCTGCTGCGGGCGTCG
Oruf_RPK1 GTGTTCGTGGGGAAGGGGGCCGGGTACAGCTTCGACCTGGAGGACCTGCTGCGGGCGTCG
Clustal Consensus
1150 1160 1170 1180 1190 1200
OsI_RPK1 GCGGAGGTGCTCGGGAAGGGGAGCGTGGGGACGTCGTACAAGGCGGTGCTGGAGGAAGGG
OsJ_RPK1 GCGGAGGTGCTCGGGAAGGGGAGCGTGGGGACGTCGTACAAGGCGGTGCTGGAGGAAGGG
Oruf_RPK1 GCGGAGGTGCTCGGGAAGGGGAGCGTGGGGACGTCGTACAAGGCGGTGCTGGAGGAAGGG
Clustal Consensus
1210 1220 1230 1240 1250 1260
OsI_RPK1 ACGACGGTGGTGGTGAAGCGGCTCAAGGACGTGGCGGTGGCGCGGCGCGAGTTCGACGCC
OsJ_RPK1 ACGACGGTGGTGGTGAAGCGGCTCAAGGACGTGGCGGTGGCGCGGCGCGAGTTCGACGCC
Oruf_RPK1 ACGACGGTGGTGGTGAAGCGGCTCAAGGACGTGGCGGTGGCGCGGCGCGAGTTCGACGCC
Clustal Consensus
1270 1280 1290 1300 1310 1320
OsI_RPK1 CACATGGACGCGCTCGGCAAGGTGGAGCACCGCAACGTCCTCCCCGTCCGCGCCTACTAC
OsJ_RPK1 CACATGGACGCGCTCGGCAAGGTGGAGCACCGCAACGTCCTCCCCGTCCGCGCCTACTAC
Oruf_RPK1 CACATGGACGCGCTCGGCAAGGTGGAGCACCGCAACGTCCTCCCCGTCCGCGCCTACTAC
Clustal Consensus
1330 1340 1350 1360 1370 1380
OsI_RPK1 TTCTCCAAGGACGAGAAGCTCCTCGTCTTCGACTACCTTCCCAACGGCAGCCTCTCCGCC
OsJ_RPK1 TTCTCCAAGGACGAGAAGCTCCTCGTCTTCGACTACCTCCCCAACGGCAGCCTCTCCGCC
Oruf_RPK1 TTCTCCAAGGACGAGAAGCTCCTCGTCTTCGACTACCTCCCCAACGGCAGCCTCTCCGCC
Clustal Consensus
1390 1400 1410 1420 1430 1440
OsI_RPK1 ATGCTCCACGGGAGCCGGGGGTCCGGCAAGACGCCGCTGGACTGGGACGCGCAGATGCGG
OsJ_RPK1 ATGCTCCACGGGAGCCGGGGGTCCGGCAAGACGCCGCTGGACTGGGACGCGCGGATGCGG
Oruf_RPK1 ATGCTCCACGGGAGCCGGGGGTCCGGCAAGACGCCGCTGGACTGGGACGCGCGGATGCGG
Clustal Consensus

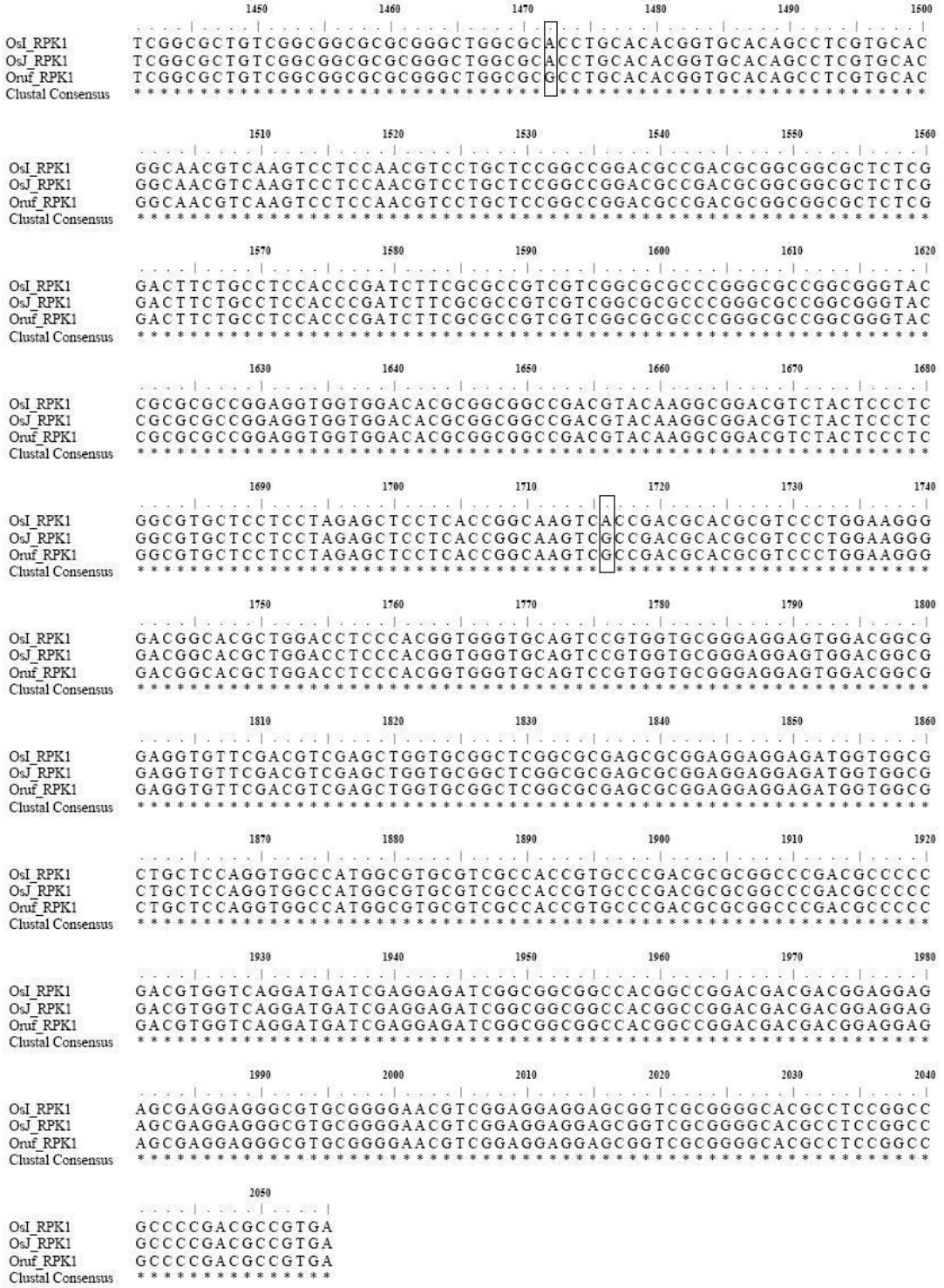



Figure 4.3: ClustalW ORF sequence alignment of *Oruf_RPK1*, *OsI_RPK1* and *OsJ_RPK1* (gi:18677097). Boxes indicate SNPs.

The genotype variation of putative *RPK1* of *Oryza rufipogon*, *Oryza sativa* ssp. *indica* cv. MR219, $BC_2F_7$ line 7 and $BC_2F_7$ line 23 were identified using a direct sequencing method. Purified RT-PCR products of putative *RPK1* were sequenced directly. The sequencing data of *Oryza rufipogon*, *Oryza sativa* ssp. *indica* cv. MR219, $BC_2F_7$ line 7 and $BC_2F_7$ line 23 shown in Figure 4.4. Putative *RPK1* of *Oryza rufipogon* and $BC_2F_7$ line 23 were identical to each other; whereas, *Oryza sativa* ssp. *indica* cv. MR219 and $BC_2F_7$ line 7 were identical to each other. Sequencing chromatogram of putative *RPK1* revealed that two bases of cytosine (C) and adenine (A) at position 791 and 807 respectively in *Oryza rufipogon* and $BC_2F_7$ line 23 were exchanged completely to thymine (T) and cytosine (C) in *Oryza sativa* ssp. *indica* cv. MR219 and $BC_2F_7$ line 7. Thus, $BC_2F_7$ line 23 is homozygous for the putative *RPK1* allele from *Oryza rufipogon*; whereas, $BC_2F_7$ line 7 is homozygous for the putative *RPK1* allele from *Oryza sativa* ssp. *indica* cv. MR219.

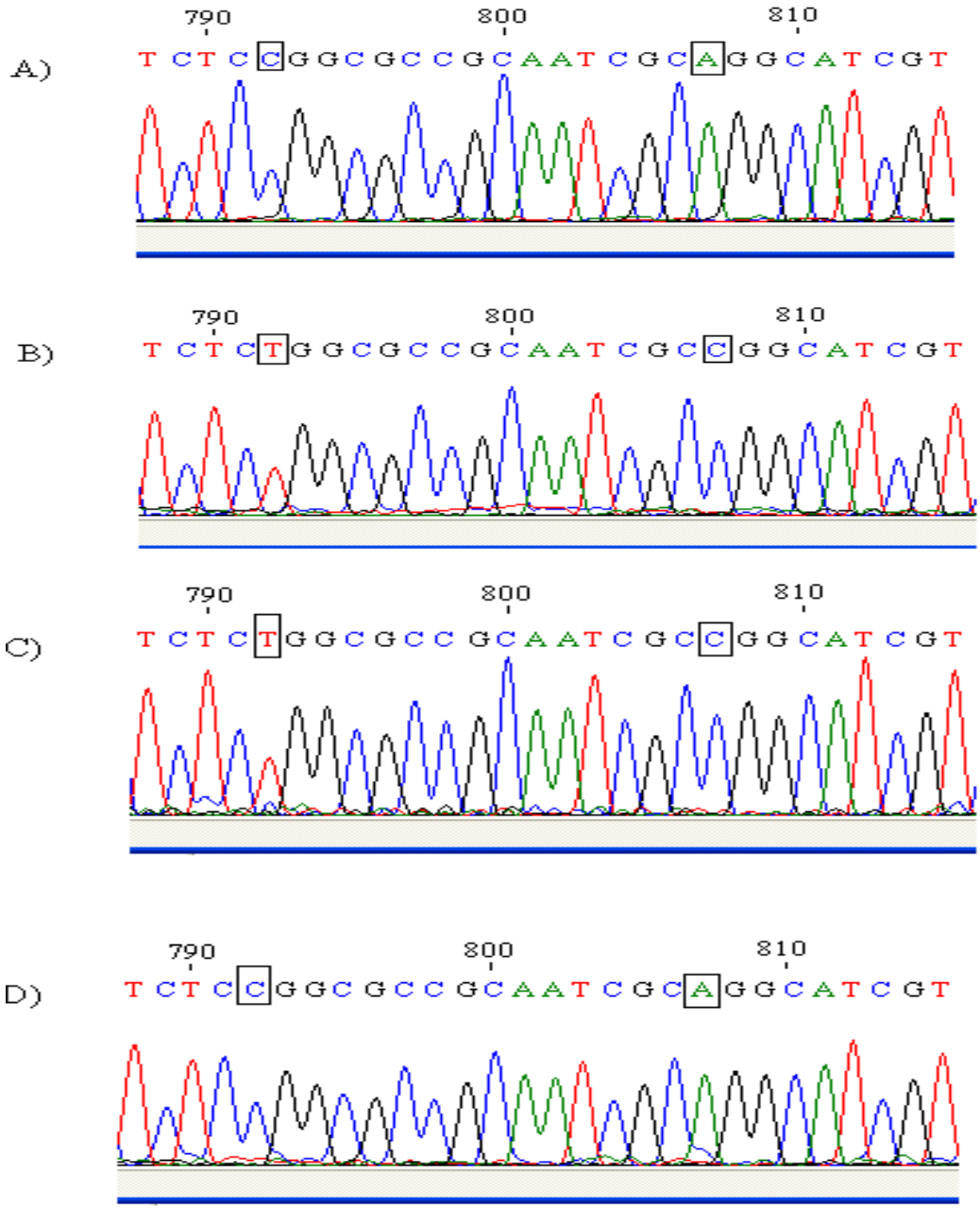



Figure 4.4: Base substitution of partial putative *RPK1* sequences revealed by sequencing from (A) *Oryza rufipogon*, (B) *Oryza sativa* ssp. *indica* cv. MR219, (C) $BC_2F_7$ line 7 and (D) $BC_2F_7$ line 23. Base substitutions are boxed. Sequencing chromatogram of *Oryza rufipogon* and $BC_2F_7$ line 23 were identical to each other; whereas, sequencing chromatogram of *Oryza sativa* ssp. *indica* cv. MR219 and $BC_2F_7$ line 7 were identical to each other.

The cDNA sequences of *Oruf_RPK1* (Figure 4.5) and *OsI_RPK1* (Figure 4.6) were successfully amplified and cloned into cloning vector pJET1.2/blunt (CloneJET™ PCR Cloning Kit, Fermentas, Lithuania) by RACE. The nucleotide sequence obtained for the *Oruf_RPK1* cDNA is 2,832 bp long with a 404 bp 5' UTR and a 373 bp 3' UTR. The nucleotide sequence of the *OsI_RPK1* cDNA is 2,576 bp long with a 103 bp 5' UTR and a 418 bp 3' UTR. The sequences of *Oruf_RPK1* and *OsI_RPK1* were compared with *OsJ_RPK1* (gi:18677097) as shown in Table 4.1. The sequences from these three rice types have some similar features: Their ORF lengths were 2,055 bp with 73 % GC content. Genomic DNA sequence of *Oruf_RPK1* (Song *et al*., 2009), *Oryza sativa* ssp. *indica* variety 9311 putative *RPK1* (gi:57015219) and *OsJ_RPK1* (gi:18677097) were included in the analysis. The 5' and 3' splice sites of the introns were identified according to GT-AG rules (Sheth *et al*., 2006; Figure 4.7). Two exons and one intron were identified. The putative *RPK1* of the three different species are interrupted by various lengths of intron sequences: The length of the *Oruf_RPK1* intron is 3,752 bp, while the *OsI_RPK1* (gi:57015219) intron is 3,345 bp and the *OsJ_RPK1* (gi:18677097) intron is 3,357 bp.

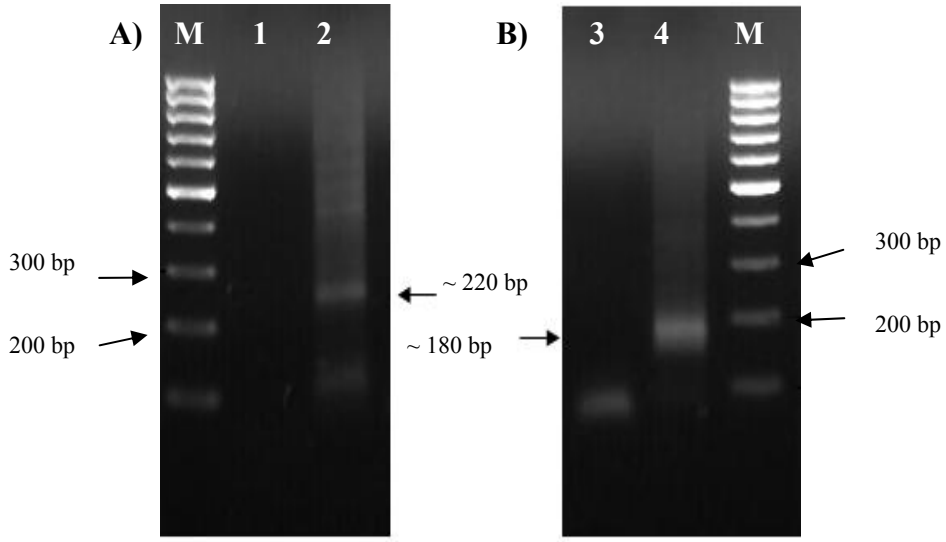



Figure 4.5: The inner 5' and 3’ RLM-RACE PCR amplification of *Oruf_RPK1*. (A) The expected size of inner 5’ RLM-RACE PCR *Oruf_RPK1* is around 220 bp. (B) Inner 3’ RLM-RACE PCR *Oruf_RPK1* give a band of the expected size of around 180 bp. Lane M: 100 bp marker; Lane 1 and 3: Minus-template control; Lane 2: Outer 5’ RLM-RACE PCR of *Oryza rufipogon*; Lane 4: Outer 3’ RLM-RACE PCR of *Oryza rufipogon*.

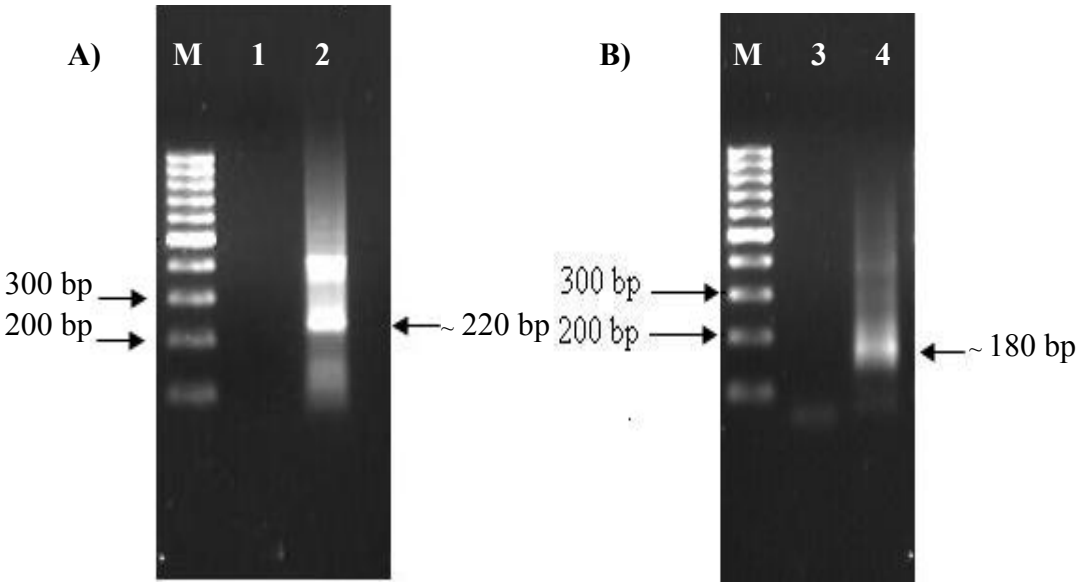



Figure 4.6: The inner 5' and 3' RLM-RACE PCR amplification of *OsI_RPK1*. (A) The expected size of inner 5' RLM-RACE PCR *OsI_RPK1* is around 220 bp. (B) Inner 3' RLM-RACE PCR *OsI_RPK1* give a band of the expected size of around 180 bp. Lane M: 100 bp marker; Lane 1 and 3: Minus-template control; Lane 2: Outer 5' RLM-RACE PCR of *Oryza sativa* ssp. *indica* cv. MR219; Lane 4: Outer 3' RLM-RACE PCR of *Oryza sativa* ssp. *indica* cv. MR219.

| Feature | *Oryza rufipogon* | *Oryza sativa* ssp. *indica* cv. MR219 | *Oryza sativa* ssp. *japonica* |
|---|---|---|---|
| **Genomic DNA** | 6,584 bp [a] | 5,400 bp [b] | 5,411 bp [c] |
| **cDNA** | 2,832 bp | 2,576 bp | 2,535 bp [c] |
| **5' UTR** | 404 bp | 103 bp | 120 bp |
| **3' UTR** | 386 bp | 418 bp | 359 bp |
| **ORF** | 2,055 bp | 2,055 bp | 2,055 bp |
| **GC content** | 73 % | 73 % | 73 % |
| **Intron** | 1 (3,752 bp) [a] | 1 (3,345 bp) [b] | 1 (3,357 bp) [c] |
| **Exon** | 2 (1,390 bp + 665 bp) | 2 (1,390 bp + 665 bp) | 2 (1,390 bp + 665 bp) |
| **Amino acid** | 684 residues | 684 residues | 684 residues |

Table 4.1: Comparative sequence analysis of *Oruf_RPK1*, *OsI_RPK1* and *OsJ_RPK1* (gi:18677097) cDNA.

[a] The gene sequence for genomic DNA and predicted intronic region were retrieved from previously reported *Oruf_RPK1* by Song *et al*. (2009).

[b] The gene sequence for genomic DNA and predicted intronic region were retrieved from *Oryza sativa* ssp. *indica* variety 9311 putative *RPK1* (gi:57015219).

[c] The gene sequence for genomic DNA and cDNA were retrieved from *OsJ_RPK1* (gi:18677097).

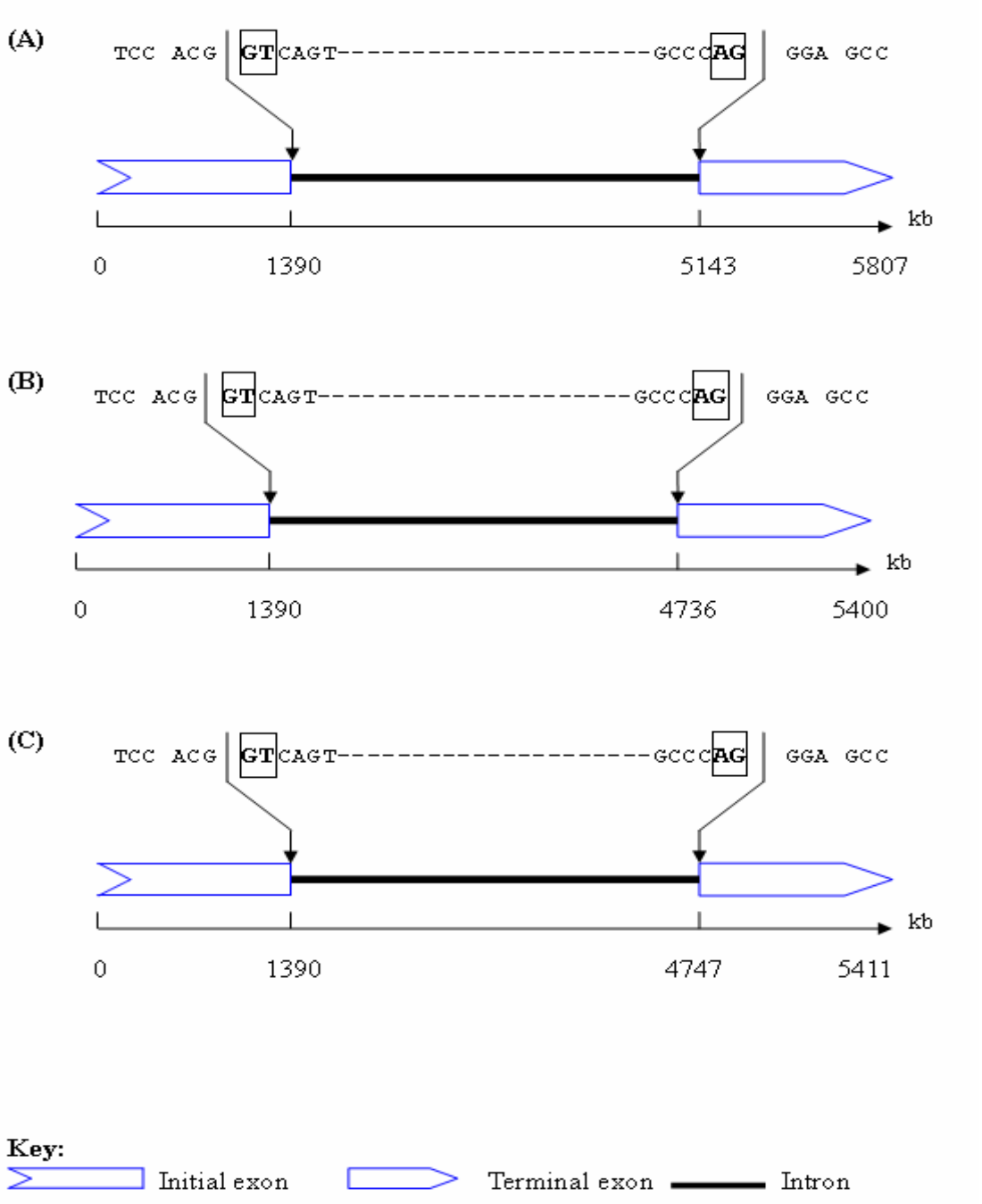



Figure 4.7: Predicted 5' and 3' splice sites of putative *RPK1*. Genomic DNA sequence of (A) *Oruf_RPK1* (Song *et al*., 2009), (B) *Oryza sativa* ssp. *indica* putative *RPK1* (gi:57015219) and (C) *OsJ_RPK1* (gi:18677097) were aligned with cDNA sequence of *Oruf_RPK1*, *OsI_RPK1* and *OsJ_RPK1* (gi:18677097) respectively. Donor and acceptor sites are boxed.

### 4.1.2 Structure Analysis of Putative RPK1

The *Oruf_RPK1*, *OsI_RPK1* and *OsJ_RPK1* (gi:18677097) ORF sequences were predicted to encode a polypeptide of 684 amino acids using GenScan (Appendix B). Amino acid sequences of Oruf_RPK1 and OsI_RPK1 showed 99 % identity with OsJ_RPK1 (gi:18677097) as shown in Figure 4.8. The Simple Modular Architecture Research Tool (SMART) analysis showed that the Oruf_RPK1, OsI_RPK1 and OsJ_RPK1 (gi:18677097) contained seven conserved domains, namely signal peptide, leucine-rich repeat N terminus (LRRNT_2), LRR motif, transmembrane helix and serine/threonine protein kinase. The LRRNT_2 and LRR motif belong to extracellular receptor domains; whereas, serine/threonine protein kinase belongs to cytoplasmic protein kinase domains (Figure 4.8). The SMART is used as a relational database management system (RDBMS; http://www.PostgreSQL.org), which is able to cross-references to other domain databases. The signal peptide was predicted using SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP; Nielsen *et al*., 1997; Bendtsen *et al*., 2004; Emanuelsson *et al*., 2007). The transmembrane helix was predicted using Transmembrane Protein Topology with a Hidden Markov Model (TMHMM) Server 2.0 (http://www.cbs.dtu.dk/services/TMHMM/; Krogh *et al*., 2001). The LRRNT_2 was predicted using protein domain families (PFAM) database (Bateman *et al*., 2004). The remaining conserved domains were identified using Basic Local Alignment Search Tool (BLAST) database (Karlin and Altschul, 1993). Interestingly, amino acid sequence of signal peptide, LRRNT_2, LRR1, LRR2 and LRR3 showed 100 % identity among these three species. However, two amino acid

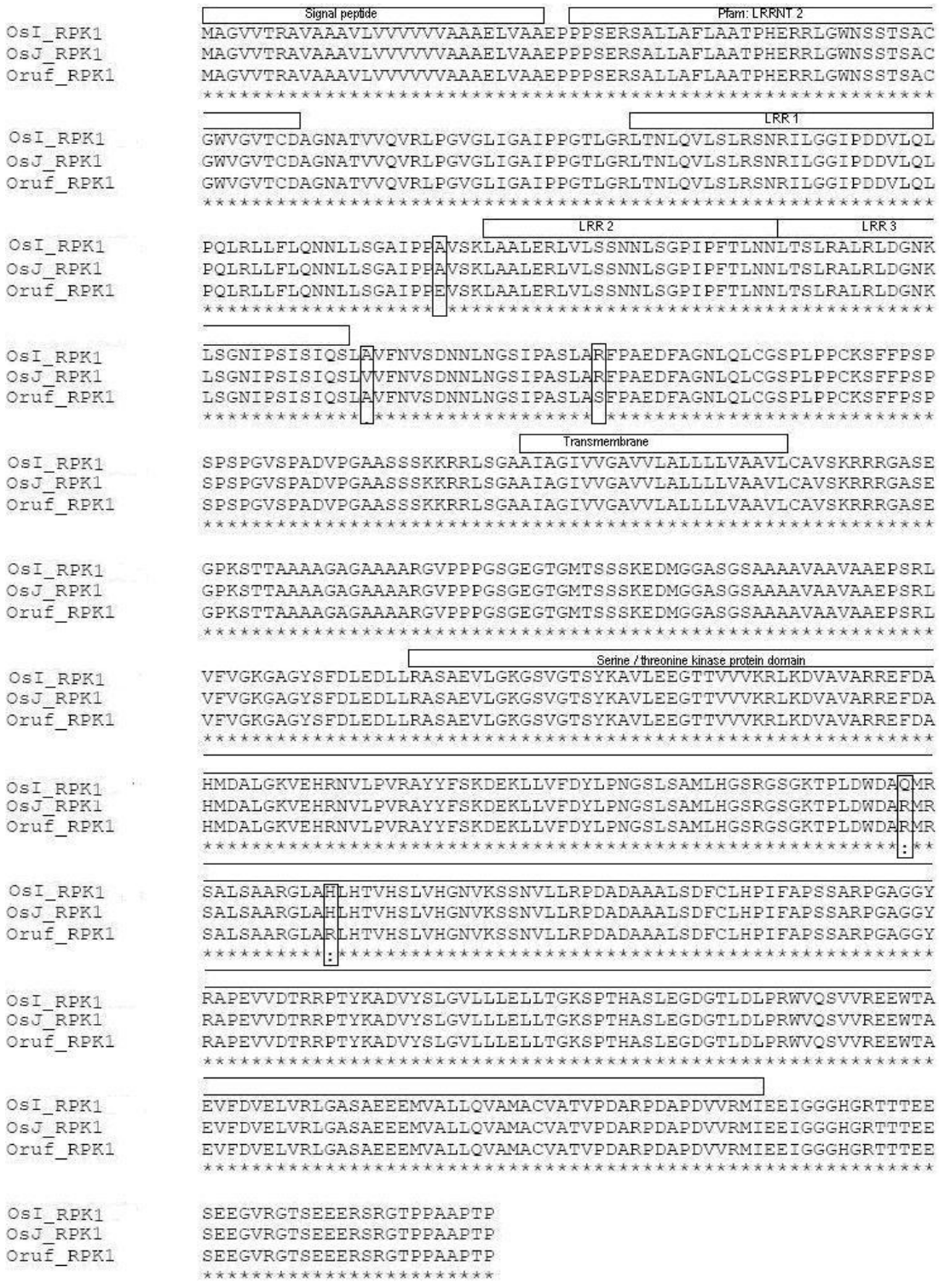


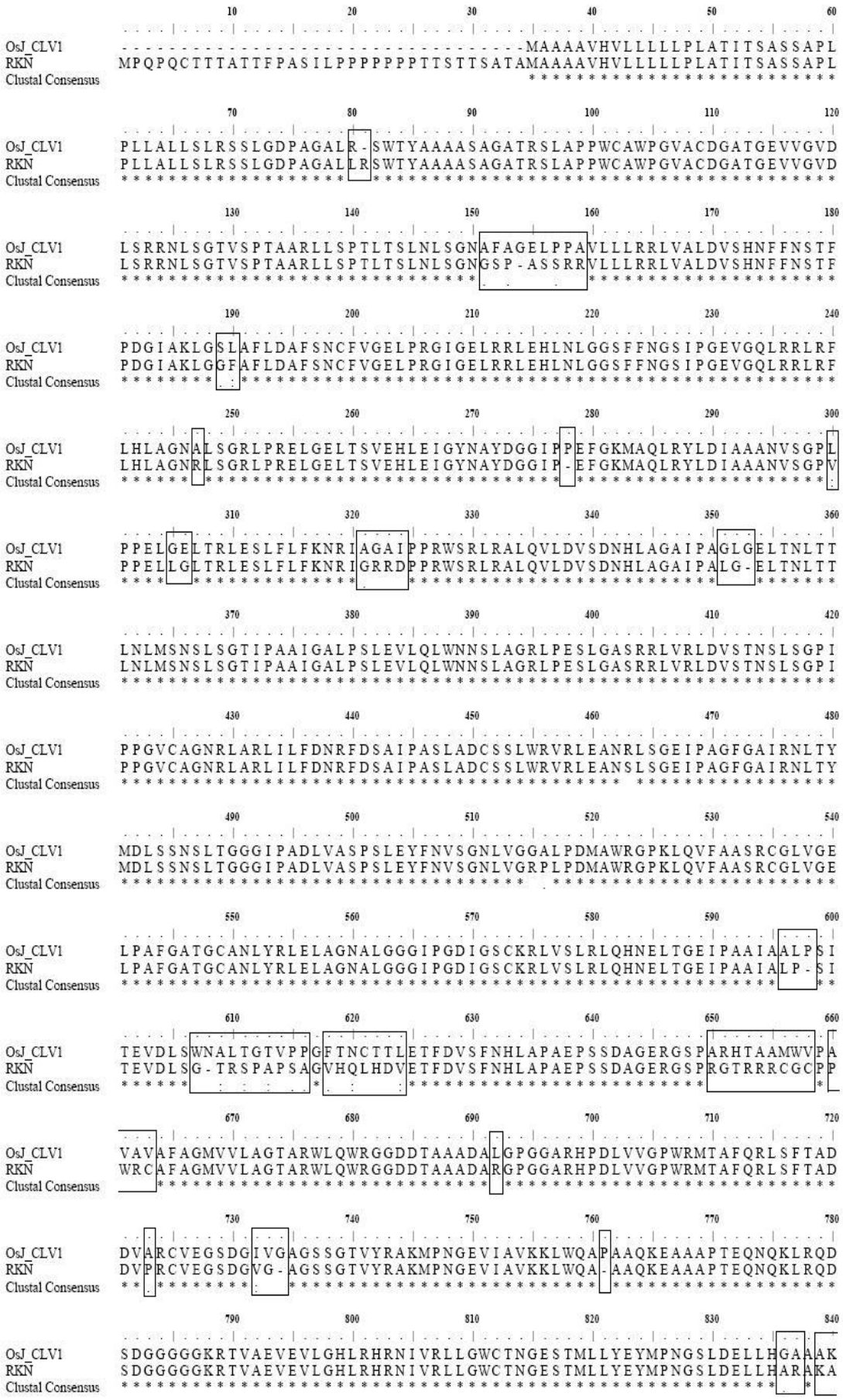

Figure 4.8: ClustalW alignment of amino acid sequence and gene structure prediction of Oruf_RPK1, OsI_RPK1 and OsJ_RPK1 (gi:18677097). Functional domains identified by SMART analysis are indicated above the sequence. Amino acid substitutions are boxed.

substitutions were detected on the serine/threonine protein kinase domain. A total of five amino acid substitutions were all non-synonymous substitution identified from the amino acid sequences of Oruf_RPK1, OsI_RPK1 and OsJ_RPK1 (gi:18677097) as shown in Figure 4.8. Four amino acid substiutions were identified between Oruf_RPK1 and OsI_RPK1, and between Oruf_RPK1 and OsJ_RPK1 (gi:18677097) respectively, whilst, two amino acid substiutions were identified between OsI_RPK1 and OsJ_RPK1 (gi:18677097). The five amino acid substitutions were of either polar side chain to polar side chain or non-polar side chain to non-polar side chain.

## 4.2 Characterization and Identification of Full Length cDNA of Putative *CLV1*

### 4.2.1 Isolation of Full Length cDNA and Sequence Analysis of Putative *CLV1*

The Receptor-like Protein Kinase (RKN) used for primer design in this study is a patented sequence (WO/2000/004761) claimed to have abilities in increasing growth and yield in rice (Zhong *et al*., 2000; Appendix B). However, the patent document did not name any species of rice. The amino acid sequences derived from this sequence showed high similarity (90 % identity) to the *Oryza sativa* ssp. *japonica* putative CLV1 (OsJ_CLV1; gi:125602183) on chromosome 8 (Figure 4.9). Primers designed from *OsJ_CLV1* (gi:125602183) successfully amplified bands of 402 bp from the cDNA of *Oryza rufipogon* and *Oryza sativa* ssp. *indica* cv. MR219 respectively (Figure 4.10). These two fragments isolated as gel extraction products were successfully cloned into pGEM-T Easy vector (Promega, USA).


OsJ_CLV1
RKN
Clustal Consensus

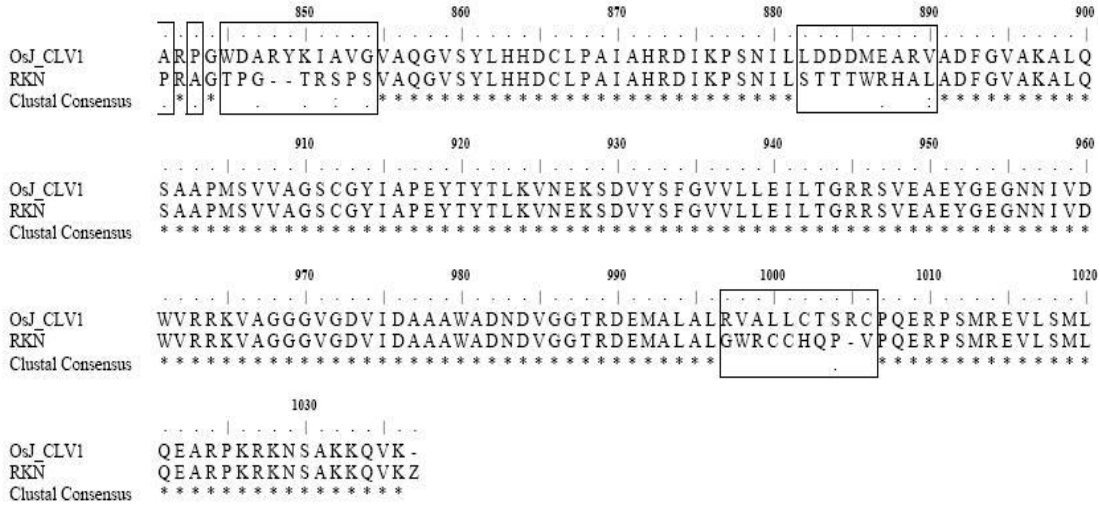



Figure 4.9: ClustalW amino acid sequence alignment of OsJ_CLV1 (gi:125602183) and RKN patent sequence (WO/2000/004761). The amino acid substitutions and deletions are boxed.

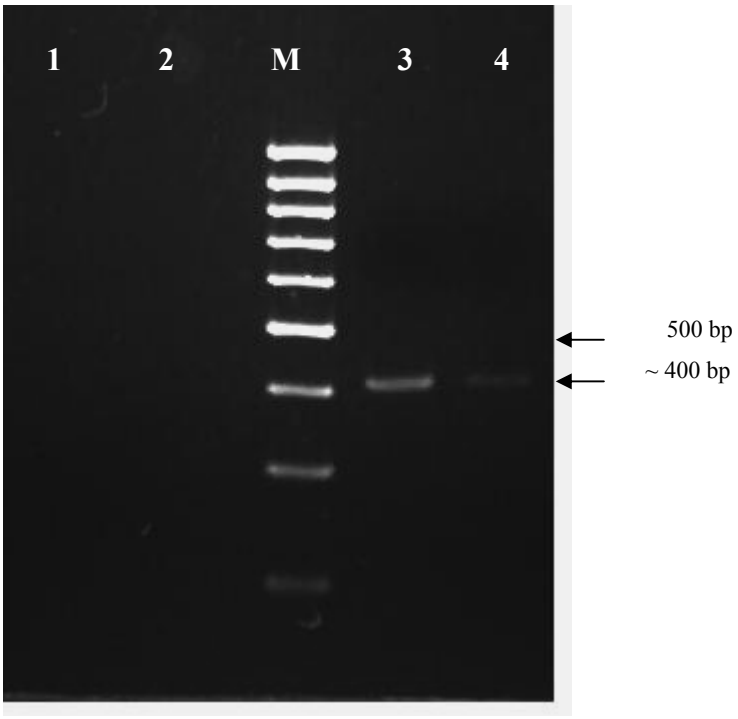



Figure 4.10: RT-PCR amplification of putative *CLV1*. The expected size of *Oruf_CLV1* and *OsI_CLV1* is around 400 bp. Lane M: 100 bp marker; Lane 1: DNase I treated total RNA of *Oryza rufipogon*; Lane 2: DNase I treated total RNA of *Oryza sativa* ssp. *indica* cv. MR219; Lane 3: *Oryza rufipogon* cDNA; Lane 4: *Oryza sativa* ssp. *indica* cv. MR219 cDNA.

Sequencing results showed that the gene sequences of *Oryza rufipogon* putative *CLV1* (*Oruf_CLV1*) and *Oryza sativa* ssp. *indica* cv. MR219 putative *CLV1* (*OsI_CLV1*) showed 99 % and 100 % identity respectively with partial gene sequence of *OsJ_CLV1* (gi:125602183; 2,640-3,006) as shown in Figure 4.11. The partial gene sequences of *OsJ_CLV1* (gi:125602183; 2,640-3,006) and *OsI_CLV1* were identical. However, four SNPs were identified between *Oruf_CLV1* and *OsI_CLV1*, and between *Oruf_CLV1* and *OsJ_CLV1* (gi:125602183; 2,640-3,006) respectively. One of the four SNPs is a non-synonymous substitution; whereas, the remaining SNPs are synonymous substitutions.

Purified putative *CLV1* RT-PCR products from *Oryza rufipogon*, *Oryza sativa* ssp. *indica* cv. MR219, $BC_2F_7$ line 7 and $BC_2F_7$ line 23 were sequenced directly. The sequencing data is showed in Figure 4.12. Putative *CLV1* of *Oryza rufipogon* and $BC_2F_7$ line 23 were identical to each other; whereas, putative *CLV1* of *Oryza sativa* ssp. *indica* cv. MR219 and $BC_2F_7$ line 7 were identical to each other. Sequencing chromatogram of cytosine (C) and adenine (A) at position 2,710 and 2,724 respectively in *Oryza rufipogon* and $BC_2F_7$ line 23 were exchanged completely to thymine (T) and guanine (G) in *Oryza sativa* ssp. *indica* cv. MR219 and $BC_2F_7$ line 7. Thus, $BC_2F_7$ line 23 is homozygous for the putative *CLV1* allele from *Oryza rufipogon*; whereas, $BC_2F_7$ line 7 is homozygous for the putative *CLV1* allele from *Oryza sativa* ssp. *indica* cv. MR219.

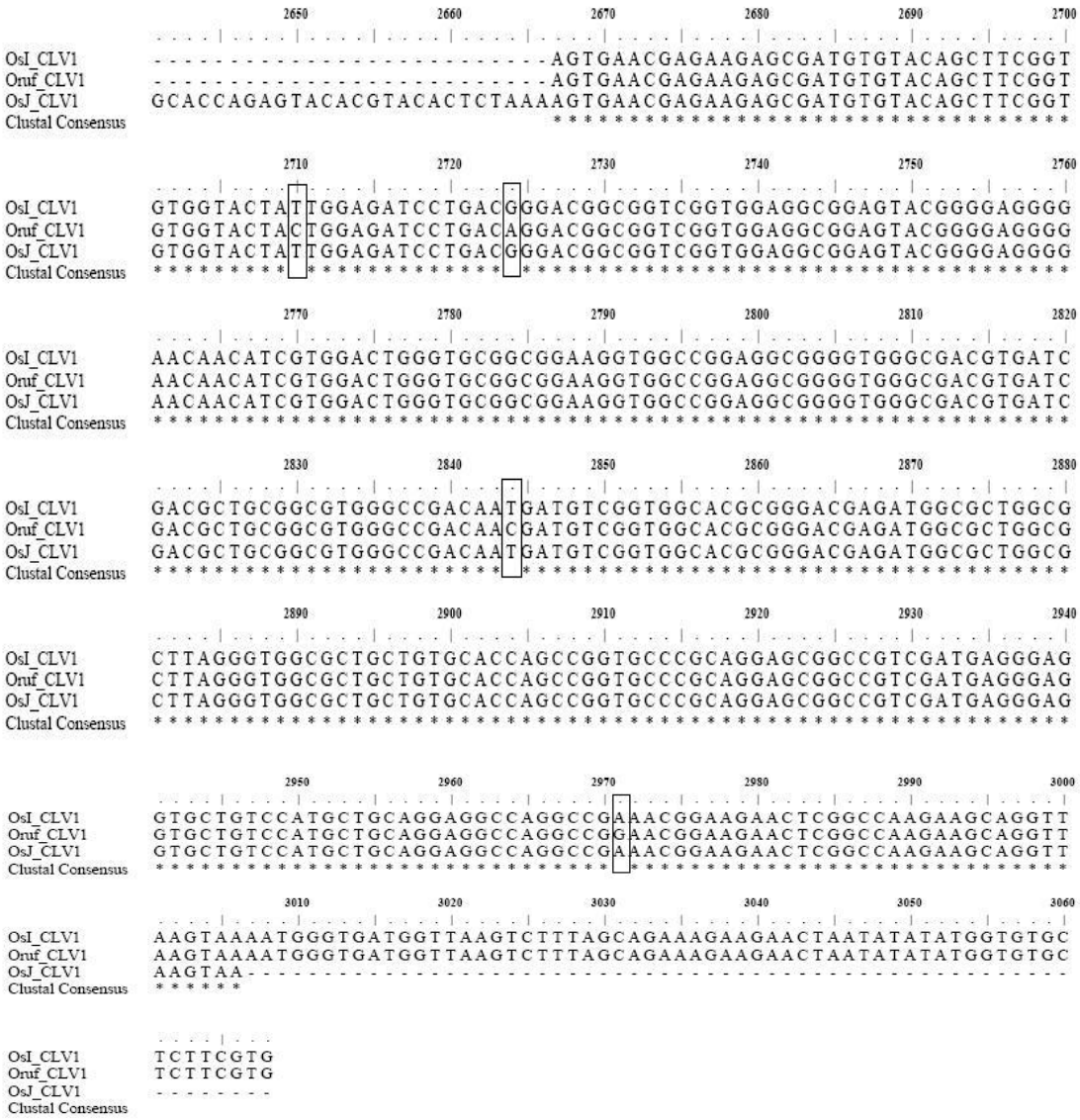



Figure 4.11: ClustalW gene sequence alignment of *Oruf_CLV1*, *OsI_CLV1* and *OsJ_CLV1* (gi:1256021832; 2,640-3,006). Boxes show the SNPs.

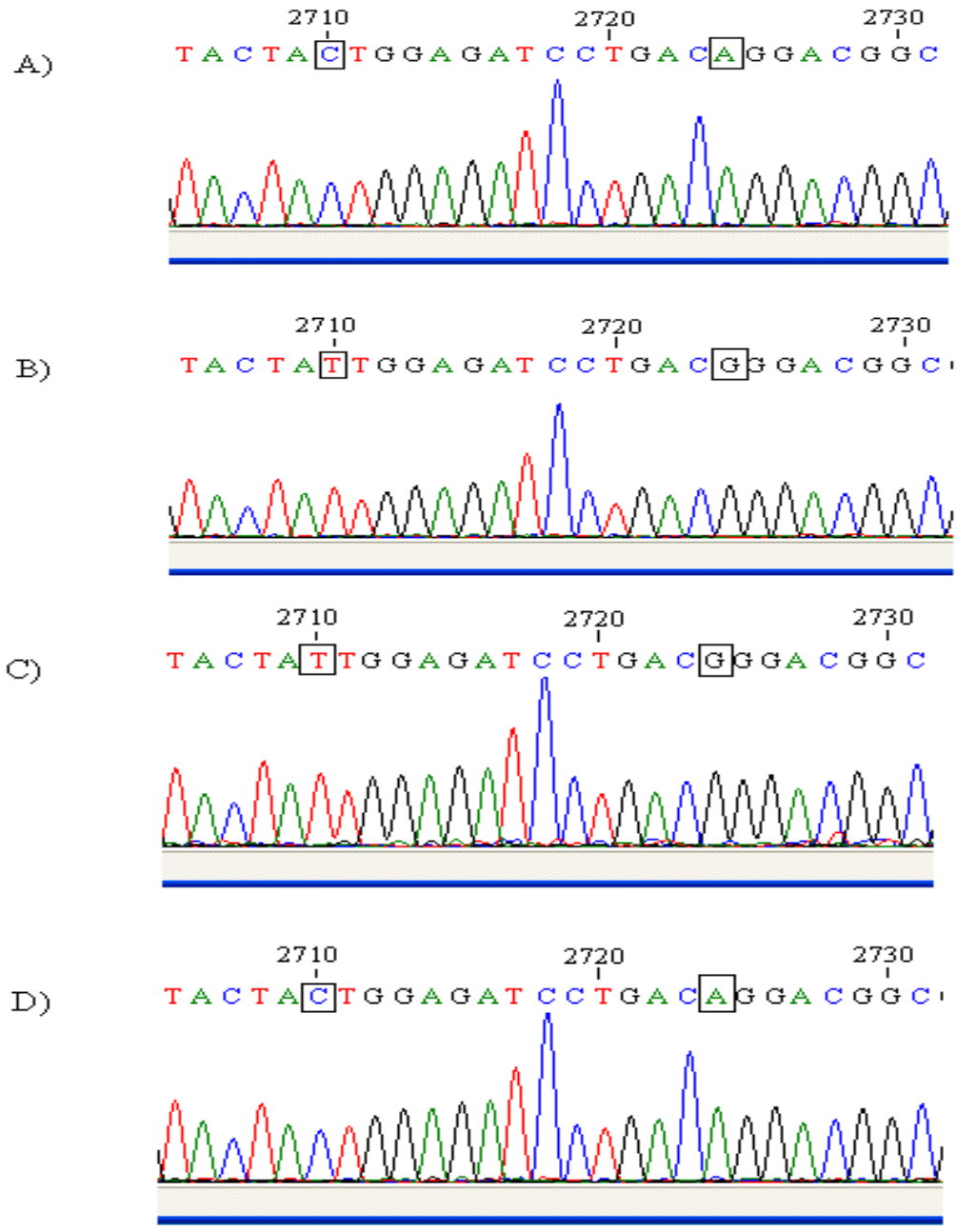
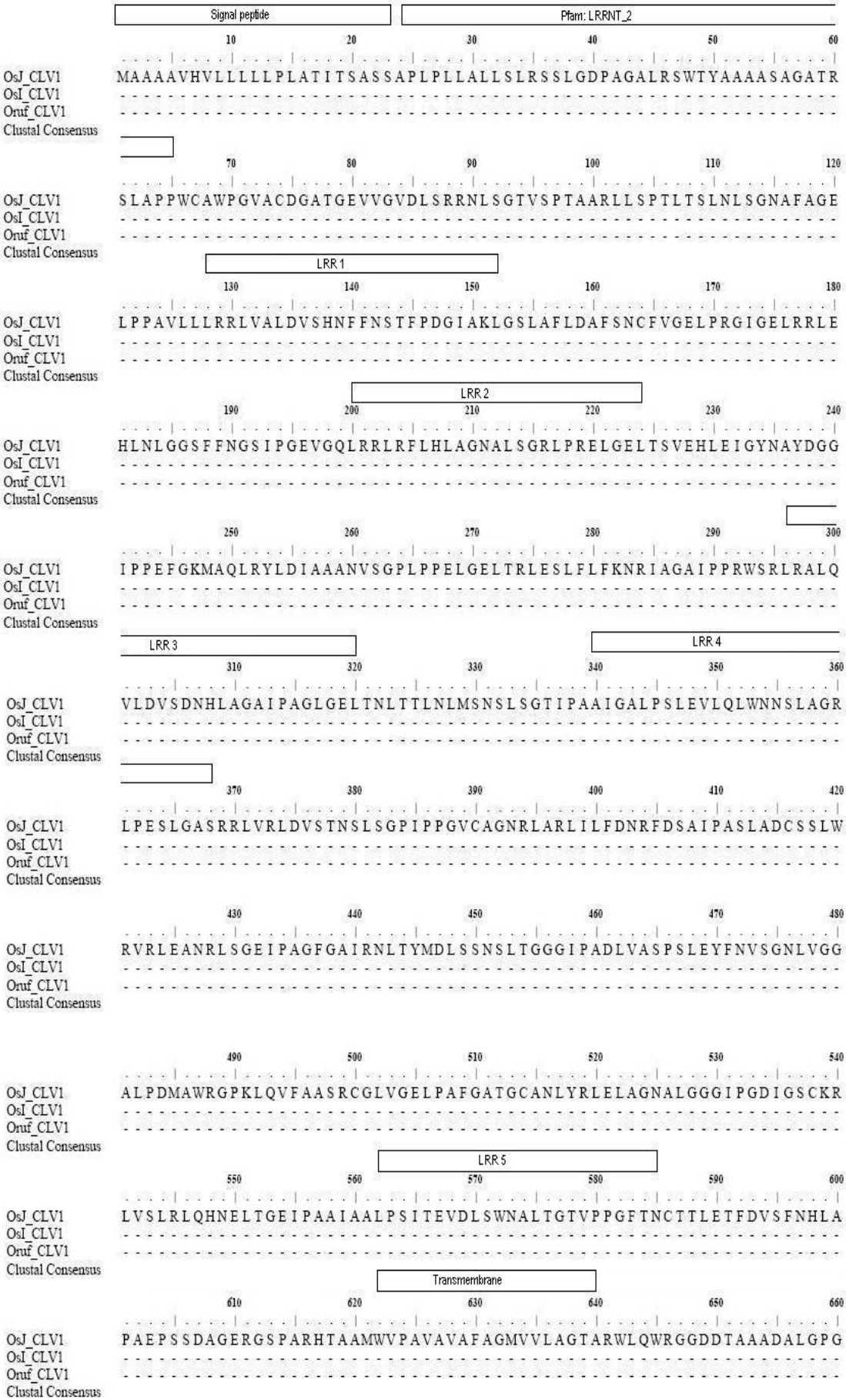



Figure 4.12: Base substitution of partial putative *CLV1* sequences revealed by sequencing from (A) *Oryza rufipogon*, (B) *Oryza sativa* ssp. *indica* cv. MR219, (C) $BC_2F_7$ line 7 and (D) $BC_2F_7$ line 23. Base substitutions are boxed. Sequencing chromatogram of *Oryza rufipogon* was identical to $BC_2F_7$ line 23; whereas, sequencing chromatogram of *Oryza sativa* ssp. *indica* cv. MR219 was identical to $BC_2F_7$ line 7.

Gene-specific outer and inner primers for 5' and 3’ RLM-RACE PCR were designed and based on the 5’ UTR and 3’ UTR of patented sequence (WO/2000/004761) and *OsJ_CLV1* (gi:125602183). However, PCR amplifications of 5' and 3’ RLM-RACE PCR of *Oruf_RPK1* and *OsI_RPK1* were unsuccessful.

### 4.2.2 Structure Analysis of Putative CLV1

Although the amplification of *Oruf_CLV1* and *OsI_CLV1* full length cDNA were unsuccessful, the partial predicted 112 amino acids for Oruf_CLV1 and OsI_CLV1 respectively were compared with the predicted 1,001 amino acids of OsJ_CLV1 (gi:125602183). Partial amino acid sequences of OsI_CLV1 showed 100 % identity with OsJ_CLV1 (gi:125602183), whilst partial amino acid sequences of Oruf_CLV1 showed 99 % identity with OsJ_CLV1 (gi:125602183) as shown in Figure 4.13. SMART analysis showed that the OsJ_CLV1 (gi:125602183) contained extracellular receptor domains and cytoplasmic protein kinase domains (Figure 4.13). Eight conserved domains were identified, namely signal peptide, LRRNT_2, LRR motif, and serine/threonine protein kinase. The LRRNT_2 and LRR motif belong to extracellular receptor domains; whereas, serine/threonine protein kinase belongs to cytoplasmic protein kinase domains (Figure 4.13). As mentioned in section 4.1.2 previously, the SMART able to cross-references to other domain databases. The signal peptide was predicted using SignalP 3.0 Server (http://www.cbs.dtu.dk/services /SignalP; Nielsen *et al*., 1997; Bendtsen *et al*., 2004; Emanuelsson *et al*., 2007. The LRRNT_2 was predicted using protein domain families (PFAM) database (Bateman


Signal peptide
Pfam: LRRNT_2
LRR 1
LRR 2
LRR 3
LRR 4
LRR 5
Transmembrane
OsJ_CLV1
OsI_CLV1
Oruf_CLV1
Clustal Consensus
MAAAAVHVLLLLLPLATITSASSAPLPLLALLSLRSSLGDPAGALRSWTYAAAASAGATR
SLAPPWCAWPGVACDGATGEVVGVDLSRRNLSGTVSPTAARLLSPTLTSLNLSGNAFAGE
LPPAVLLLRRLVALDVSHNFFNSTFPDGIAKLGSLAFLDAFSNCFVGELPRGIGELRRLE
HLNLGGSFFNGSIPGEVGQLRRLRFLHLAGNALSGRLPRELGELTSVEHLEIGYNAYDGG
IPPEFGKMAQLRYLDIAAANVSGPLPPELGELTRLESLFLFKNRIAGAIPPRWSRLRALQ
VLDVSDNHLAGAIPAGLGELTNLTTLNLMSNSLSGTIPAAIGALPSLEVLQLWNNSLAGR
LPESLGASRRLVRLDVSTNSLSGPIPPGVCAGNRLARLILFDNRFDSAIPASLADCSSLW
RVRLEANRLSGEIPAGFGAIRNLTYMDLSSNSLTGGGIPADLVASPSLEYFNVSGNLVGG
ALPDMAWRGPKLQVFAASRCGLVGELPAFGATGCANLYRLELAGNALGGGIPGDIGSCKR
LVSLRLQHNELTGEIPAAIAALPSITEVDLSWNALTGTVPPGFTNCTTLETFDVSFNHLA
PAEPSSDAGERGSPARHTAAMWVPAVAVAFAGMVVLAGTARWLQWRGGDDTAAADALGPG

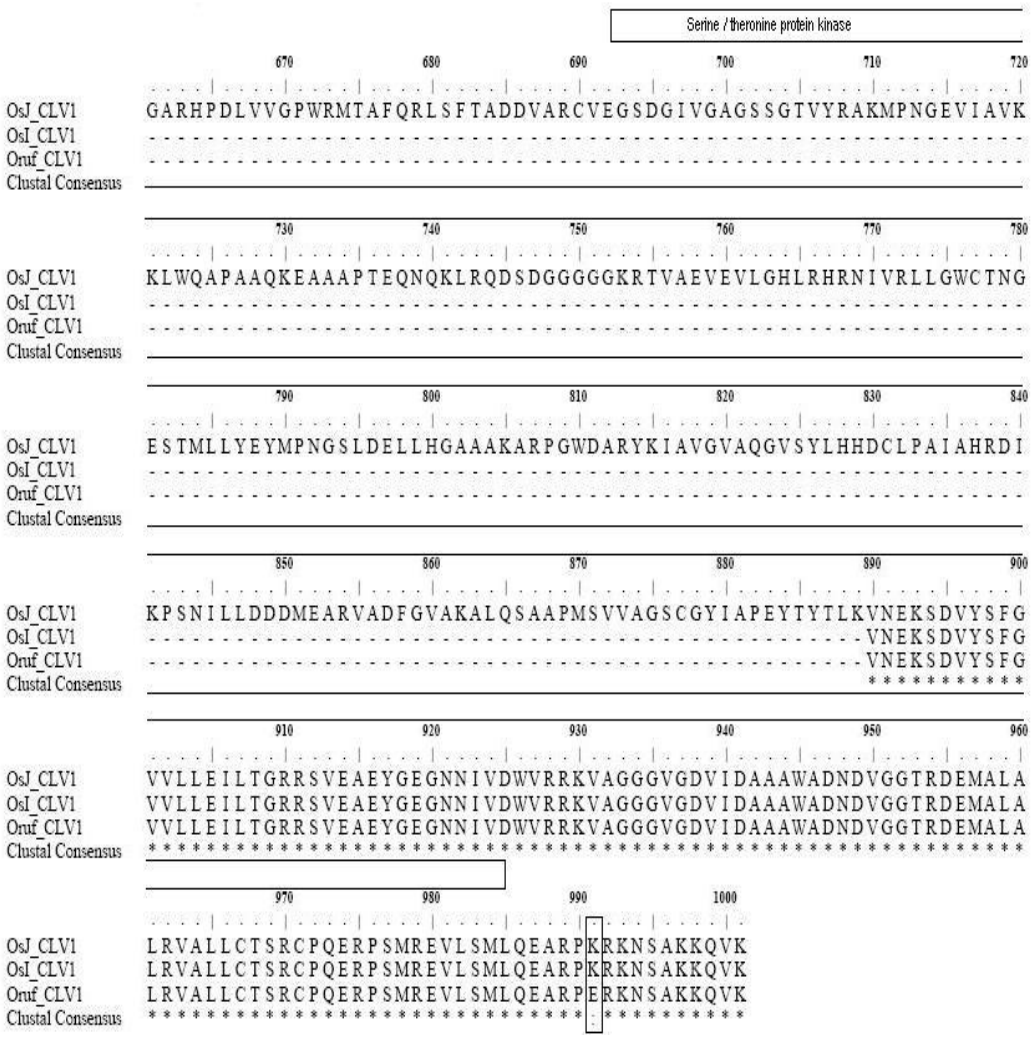



Figure 4.13: ClustalW alignment of amino acid sequence and gene structure prediction of Oruf_CLV1, OsI_CLV1 and OsJ_CLV1 (gi:125602183). Functional domains identified by SMART analysis are indicated above the sequence. The amino acid substitutions are boxed.

*et al*., 2004). The transmembrane helix was predicted using Transmembrane Prediction Server (http://www.ch.embnet.org/software/TMPRED_form.html; Hofmann and Stoffel, 1993). The remaining conserved domains were identified using Basic Local Alignment Search Tool (BLAST) database (Karlin and Altschul, 1993). There was only one different amino acid identified between Oruf_CLV1 and OsI_CLV1, and this also occurs between Oruf_CLV1 and OsJ_CLV1 (gi:125602183). This substitution of lysine to glutamine acid converses a polar side chain.

### 4.3 Characterization of Kinase of Putative RPK1 and Putative CLV1

A number of known RLK kinases were aligned with the predicted amino acid sequences of the kinase domains of Oruf_RPK1 (382-643), OsI_RPK1 (382-643), OsJ_RPK1 (gi:18677097; 382-643) and OsJ_CLV1 (gi:125602183; 699-952). *Arabidopsis thaliana* CLAVATA1 At_CLV1 (sp:Q9SYQ8.3; 692-968), rice FLORAL NUMBER1 OsJ_FON1 (gi:56790017; 708-915) and *Arabidopsis thaliana* ERECTA At_ERECTA (gi:1345132: 653-910) are known to belong to the RD kinase class (Clark *et al*., 1997; Dardick and Ronald, 2006; Afzal *et al*., 2008). Meanwhile, rice bacterial blight resistance protein OsI_Xa21 (gi:1122443; 708-922) and *Arabidopsis thaliana* flagellin perception At_FLS2 (sp:Q9FL28.1; 882-1152) are known to belong to the non-RD kinase class (Song *et al*., 1995, Gomez-Gomez and Boller, 2000; Dardick and Ronald, 2006; Afzal *et al*., 2008). The *Solanum lycopersicum* receptor-like protein kinase Sl_PEPRK1 (gi:3015488; 372-638) and *Glycine max* receptor-like kinase protein Gm_RHG1 (gi:206584433; 578-834) were

identified to belong to the kinase minus class (Muschietti *et al*., 1998; Dardick and Ronald, 2006; Afzal and Lightfoot, 2007; Afzal *et al*., 2008). Based on the absence of a conserved arginine (R) and aspartic acid (D) in the kinase subdomain VI, Oruf_RPK1 (382-643), OsI_RPK1 and OsJ_RPK1 (gi:18677097; 382-643) most probably belong to the RD minus kinase class (shown in box B; Figure 4.14); whereas, OsJ_CLV1 (gi:125602183; 699-952) most probably belong to the RD kinase class based on the presence of conserved arginine (R) and aspartic acid (D) residues in the kinase subdomain VIb (shown in box B; Figure 4.14).

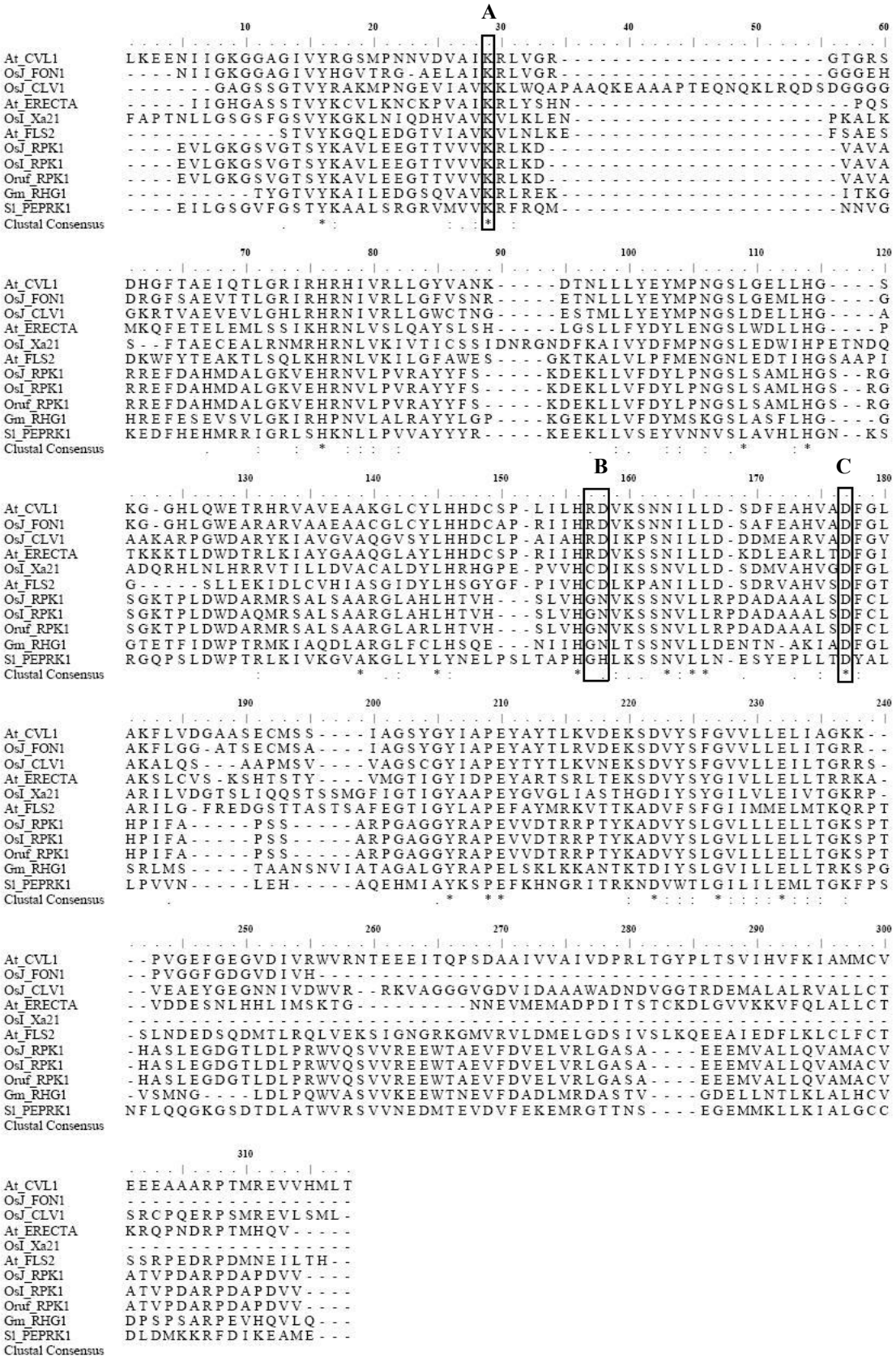



Figure 4.14: ClustalW amino acid alignment of kinase domains with known RLKs. Characterization of kinase was based on presence or absence conserved lysine (K)

kinase subdomain II (shown in box A), conserved arginine (R) and aspartic acid (D) in kinase subdomain VIb (shown in box B), and conserved aspartic acid (D) in kinase subdomain VII (shown in box C; Dardick and Ronald, 2006; Afzal *et al*., 2008). At_CLV1 (sp:Q9SYQ8.3; 692-968), At_ERECTA (gi:1345132: 653-910), At_FLS2 (sp:Q9FL28.1; 882-1152) from *Arabidopsis thaliana*; Gm_RHG1 (gi:206584433; 578-834) from *Glycine max*; OsI_RPK1 (382-643), OsI_Xa21 (gi:1122443; 708-922), OsJ_CLV1 (gi:125602183; 699-952), OsJ_FON1 (gi:56790017; 708-915), OsJ_RPK1 (gi:18677097; 382-643) from *Oryza sativa*; Oruf_RPK1 (382-643) from *Oryza rufipogon*; Sl_PEPRK1 (gi:3015488; 372-638) from *Solanum lycopersicum*.

**4.4 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 the OrthoMCL database (Chen *et al*., 2006). The relatively conserved regions of orthologous RPK1 amino acid sequences and orthologous CLV1 amino acid sequences were screened by Gblocks 0.91 (Appendix C; Appendix D; Castresana, 2000; Dereeper *et al*., 2008). The Gblocks 0.91 screened sequences were used to construct phylogenetic trees (Appendix E; Appendix F). Figure 4.15 shows phylogenetic tree putative RPK1 amino acid sequences. The Oruf_RPK1, OsI_RPK1 and OsJ_RPK1 (gi:18677097) are grouped together with At_TMK (At5g58300), At_RLK (At4g23740), OsJ_RLL1 (gi:7573610), OsJ_RLL2 (gi:15128407) and OsJ_

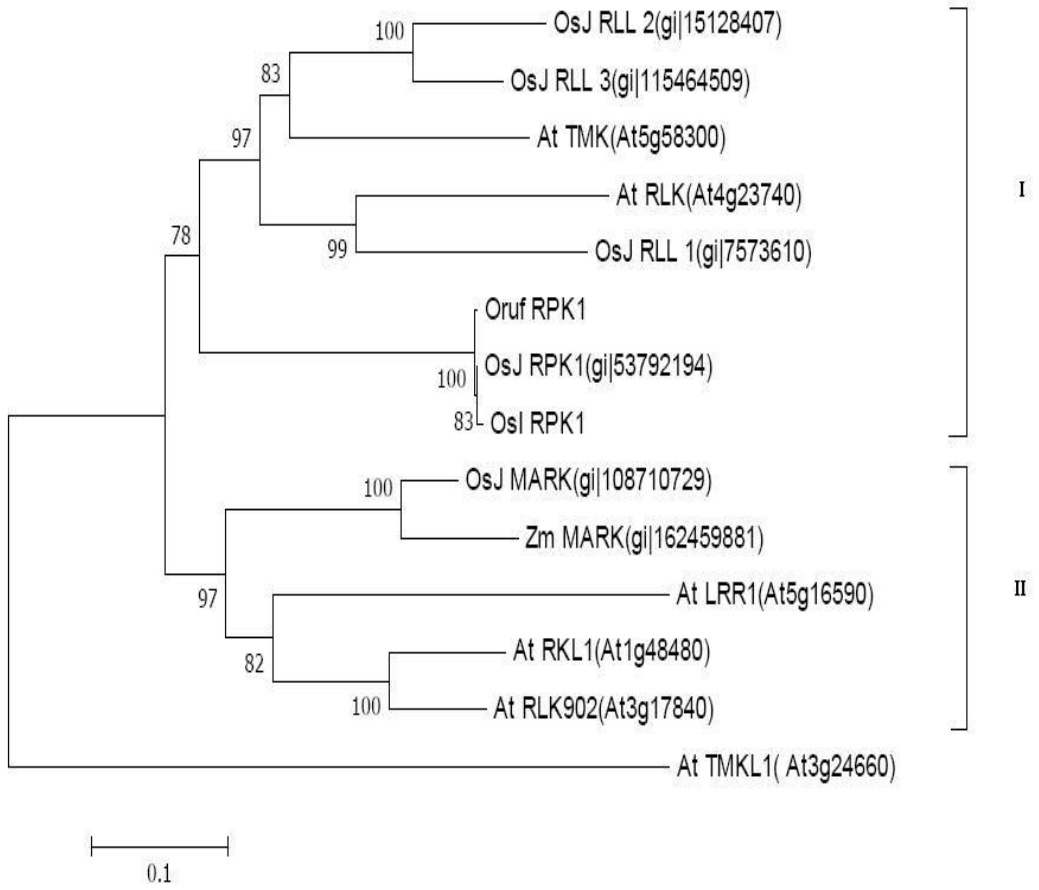



Figure 4.15: Phylogenetic analysis of orthologous RPK1 amino acid sequences from *Arabidopsis thaliana*, *Oryza sativa*, *Oryza rufipogon* and *Zea mays*. The tree was formatted with MEGA 4.0.2 program. The bootstrap value greater than 50 % was selected from the neighbor-joining method with Poisson correction. At_LRR1 (At5g16590), At_RKL1 (At1g48480), At_RLK (At4g23740), At_RLK902 (At3g17840), At_TMK (At5g58300) and At_TMKL1 (At3g24660) from *Arabidopsis thaliana*; OsJ_MARK (gi:108710729), OsI_RPK1, OsJ_RPK1 (gi:18677097) OsJ_RLL1 (gi:7573610), OsJ_RLL2 (gi:15128407) and OsJ_RLL3 (gi:115464509) from *Oryza sativa*; Oruf_RPK1 from *Oryza rufipogon*; Zm_MARK (gi:226498594) from *Zea mays*.

RLL3 (gi:115464509) in group I. The phylogenetic tree of orthologous CLV1 amino acid sequences as shown in Figure 4.16. Three well-supported groups (designated as group I, group II and group III) were formed from 16 orthologous putative CLV1 amino acid sequences. The OsJ_CLV1 (gi:125602183) are grouped together with Pg_CLL1 (gi:104642235) and OsJ_CLL1 (gi:50726262) in group III.

Most of the orthologous RPK1 and CLV1 amino acid sequences are known to belong to LRR-III and LRR-XI subfamilies in the plant RLK/*Pelle* family respectively (Shiu *et al*., 2002; Shiu *et al*., 2004). The phylogenetic analysis of these sequences (Figure 4.15 and 4.16) is in agreement with the suggestion that Oruf_RPK1, OsI_RPK1 and OsJ_RPK1 (gi:18677097) could belong to the LRR-III subfamily in the plant RLK/*Pelle* family; whereas, OsJ_CLV1 (gi:125602183) may be grouped in the LRR-XI subfamily in the plant RLK/*Pelle* family. The partial 402 bp long gene sequences of *Oruf_CLV1* and *OsI_CLV1* were not selected for the construction of phylogenetic tree because only full length amino acid sequence was selected in this study. It is suggested that Oruf_CLV1 and OsI_CLV1 belong to the LRR XI subfamily in the plant RLK/*Pelle* family too.

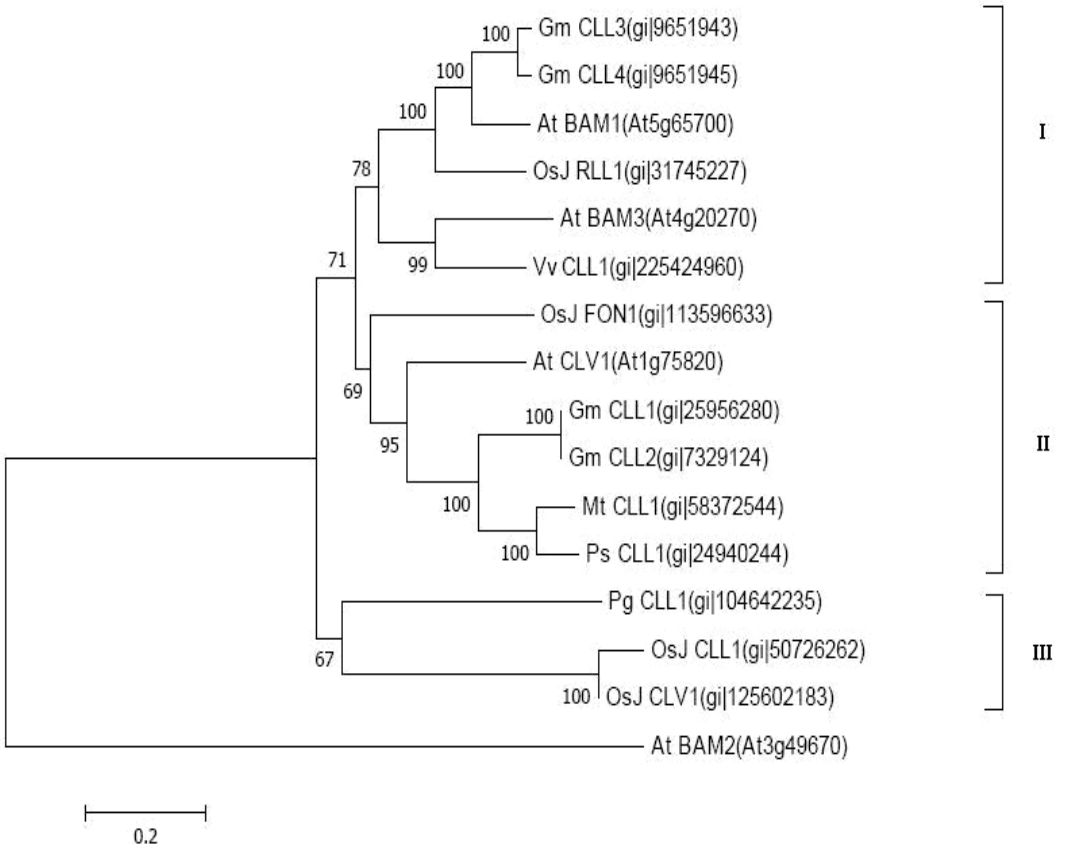



Figure 4.16: Phylogenetic analysis of orthologous CLV1 amino acid sequences from *Arabidopsis thaliana*, *Oryza sativa*, *Glycine max*, *Medicago truncatula*, *Picea glauca*, *Pisum sativum* and *Vitis vinifera*. The tree was formatted with MEGA 4.0.2 program. The bootstrap value greater than 50 % was selected from the neighbor-joining method with Poisson correction. At_BAM1 (At5g65700), At_BAM2 (At3g49670), At_BAM3 (At4g20270) and At_CLV1 (At1g75820) from *Arabidopsis thaliana*; Gm_CLL1 (gi:25956280), Gm_CLL2 (gi:7329124), Gm_CLL3 (gi:9651943) and Gm_CLL4 (gi:9651945) from *Glycine max*; Mt_CLV1 (gi:58372544) from *Medicago truncatula*; OsJ_CLL1 (gi:50726262), OsJ_CLV1 (gi:125602183), OsJ_FON1 (gi:113596633), OsJ_LRK1 (gi:255677496) and OsJ_RLL1 (gi:31745227) from *Oryza sativa*; Pg_CLL1 (gi:104642235) from *Picea glauca*; Ps_CLL1 (gi:24940244) from *Pisum sativum*; Vv_CLL1 (gi:225424960) from *Vitis vinifera*.

## 4.5 Gene Expression Study of Putative *RPK1* and Putative *CLV1*

### 4.5.1 Validation of Comparative $C_T$ Method Real Time qRT-PCR

The total RNA of *Oryza rufipogon* (accession number IRGC105491), *Oryza sativa* ssp. *indica* cv. MR219, $BC_2F_7$ line 7 and $BC_2F_7$ line 23 was successfully extracted from the leaves and the whole plant at the seedling stage, the panicles at the booting, heading and flowering stages, and grains at the milk grain stage (Figure 4.17A). After that, the total RNA from different tissues at different developmental stages was treated with DNase I to remove DNA (Figure 4.17B). Next, all treated total RNAs were reverse transcribed to synthesize cDNA. The cDNA of different tissues at different developmental stages in rice were used during the real time qRT-PCR experiments.

Housekeeping genes are used to normalize target genes in a real time qRT-PCR experiment. The normalization of real time qRT-PCR data against housekeeping genes is to obtain an accurate and reliable gene expression profile. *Actin*, *eEF-1*α and *UBQ5* were selected to examine gene expression stability at different developmental stages in rice using geNORM v3.4 software (Primer-Design, UK) in this study. Measure M value of *Actin* was 0.360, whereas measure M value of *eEF-1*α and *UBQ5* were 0.18 (Figure 4.18). Thus, *eEF-1α* and *UBQ5* were selected as pair-wise housekeeping genes to normalize the gene expression of putative *RPK1* and putative *CLV1*. The *eEF-1*α and *UBQ5* were found to be the most stable housekeeping genes

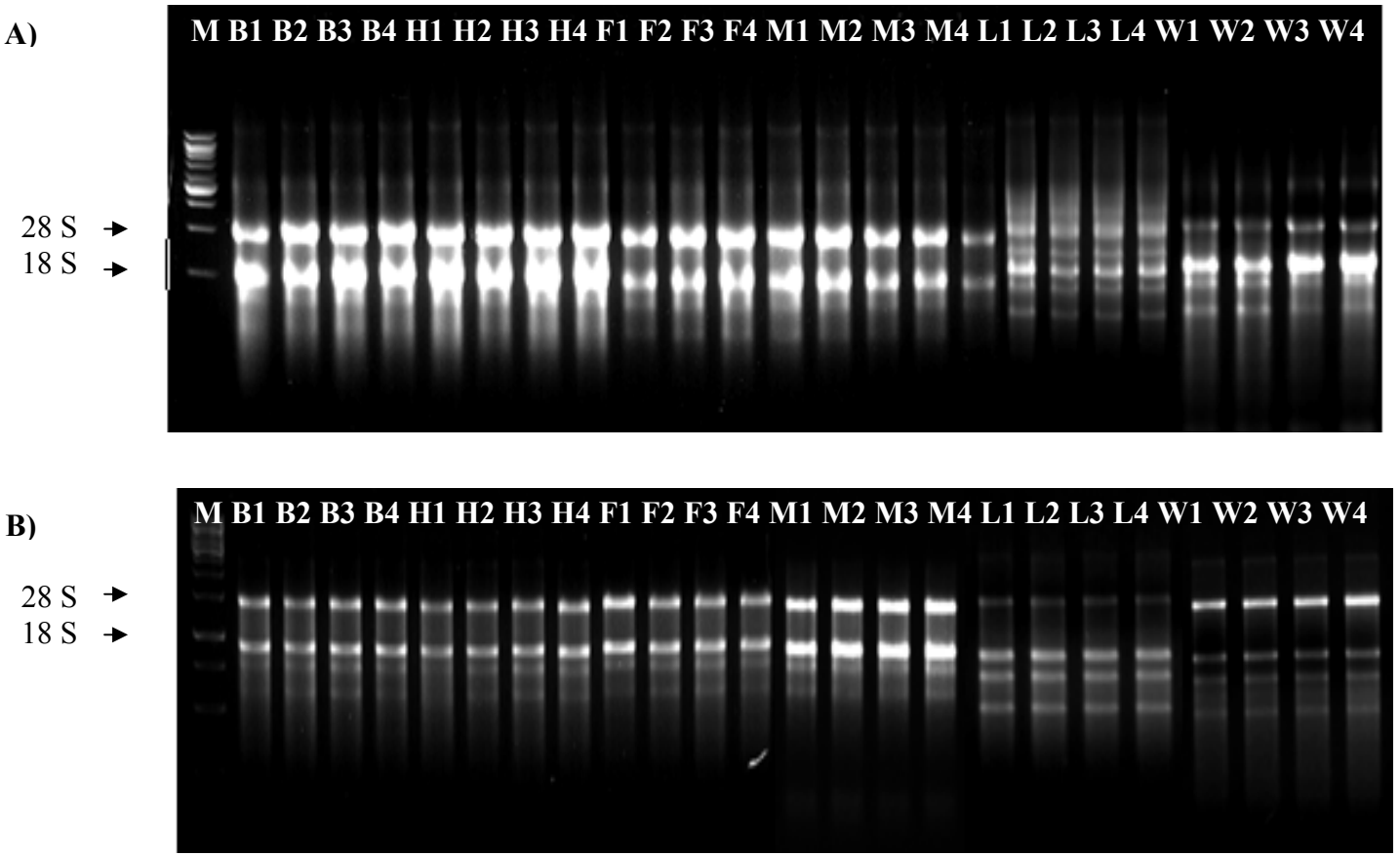



Figure 4.17: Agarose gel electrophoresis of (A) total RNA and (B) DNaseI treated total RNA of *Oryza rufipogon* (1), *Oryza sativa* ssp. *indica* cv. MR219 (2), $BC_2F_7$ line 7 (3) and $BC_2F_7$ line 23 (4) at different stages. M: 1 kb DNA marker; B: Booting stage; H: Heading stage; F: Flowering stage; M: Milk grain stage; L: Leaf of 8th seedling day; W: Whole plant of 8th seedling day.

**(A)**

| Change Data | Actin | eEF-1α | UBQ5 | Normalisation Factor |
|---|---|---|---|---|
| Booting | 1.87E+01 | 1.80E+01 | 1.82E+01 | 0.9364 |
| Heading | 1.86E+01 | 1.82E+01 | 1.82E+01 | 0.9385 |
| Flowering | 2.07E+01 | 1.74E+01 | 1.75E+01 | 0.9451 |
| Milk grain | 3.33E+01 | 1.67E+01 | 1.70E+01 | 1.0811 |
| Leaf of Seedling | 2.32E+01 | 1.97E+01 | 2.01E+01 | 1.0726 |
| Whole plant | 2.13E+01 | 1.94E+01 | 2.02E+01 | 1.0384 |
| | | | | |
| M < 1.5 | 0.360 | 0.189 | 0.189 | |

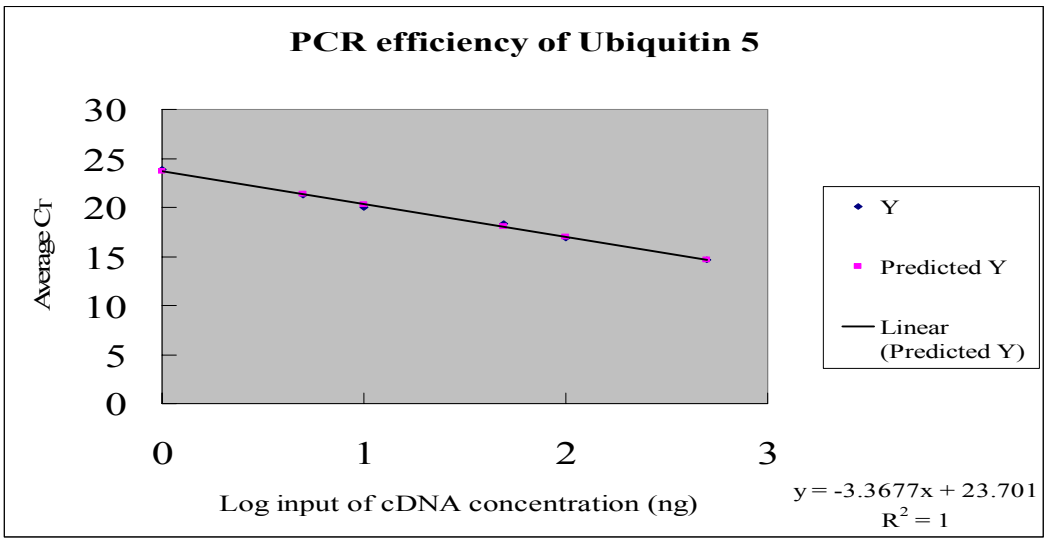
**(B)**


Figure 4.18: Selection of housekeeping genes by geNORM v3.4. (A) Calculation of gene stability measure (M) of *Actin*, *eEF-1α* and *UBQ5* at different developmental stages in rice. (B) Average expression stability values of *Actin*, *eEF-1α* and *UBQ5*.

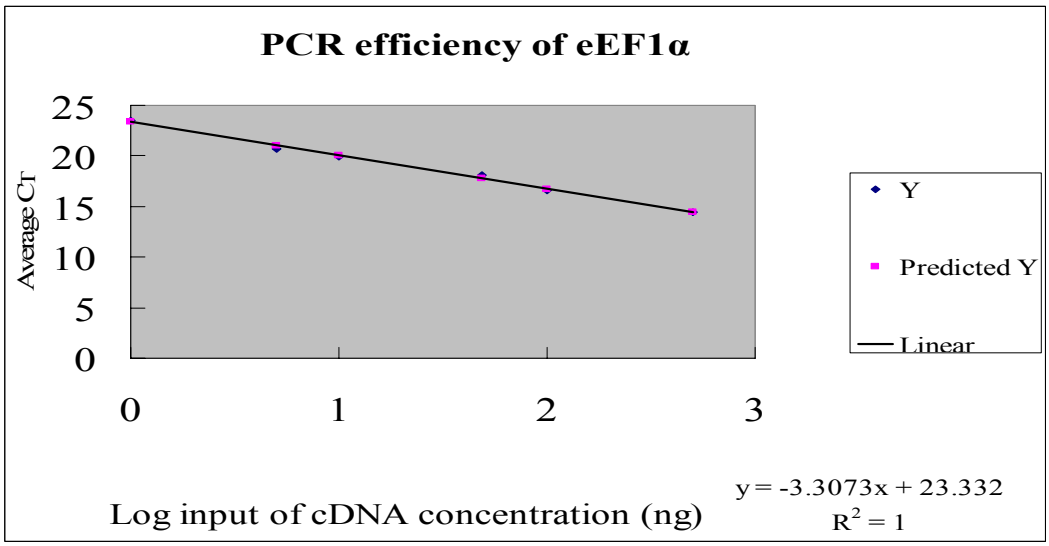
across developmental stages, showing the lowest gene stability measure M values (Figure 4.18).

While, PCR amplification efficiency was used to measure the overall performance of real time qRT-PCR experiment. The PCR amplification efficiency of the housekeeping gene (*eEF-1α* and *UBQ5*) and gene specific primer target set (putative *RPK1* and putative *CLV1*) were within 90-110 % with the standard curve slope between -3.587 to -3.103 (Figure 4.19). The PCR amplification efficiency results suggest that they are suitable for investigation of gene expression of putative *RPK1* and putative *CLV1*. Two different sets of forward primers were designed for putative *CLV1* (see section 3.4). The lengths of the two different forward primers of putative *CLV1* (I) and putative *CLV1* (II) were 24 bases. The 16$^{th}$ base of putative *CLV1* (I) forward primer was substituted from thymine (T) to cytosine (C) for the putative *CLV1* (II) primer. Putative *CLV1* (I) forward primer was used for *Oryza rufipogon* and $BC_2F_7$ line 23 samples, whereas, putative *CLV1* (II) forward primer was used for *Oryza sativa* ssp. *indica* cv. MR219 and $BC_2F_7$ line 7 samples.

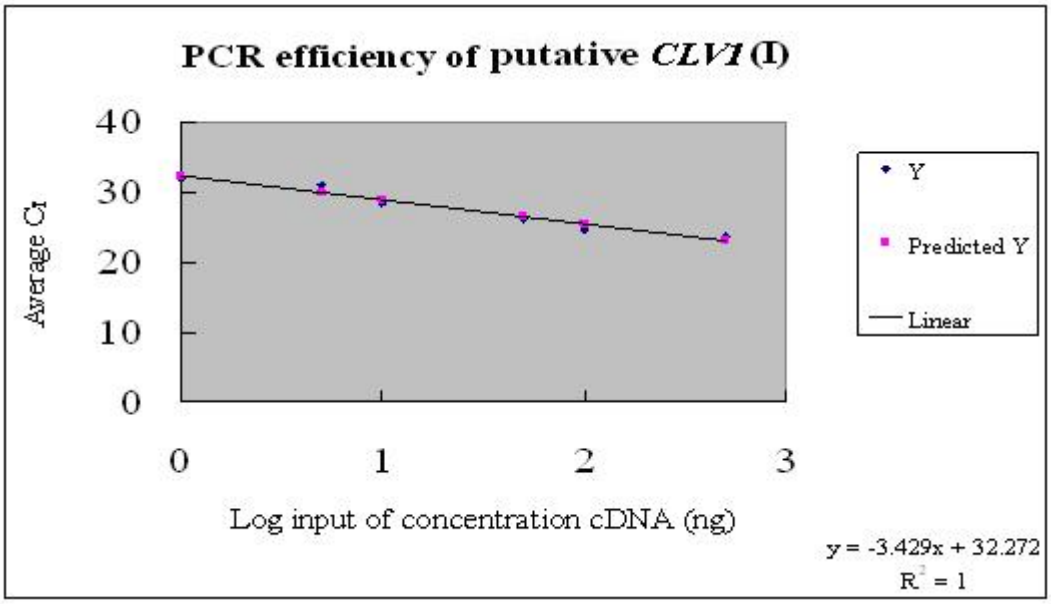
(A)


PCR efficiency of Ubiquitin 5
Average Ct
30
25
20
15
10
5
0
0
1
2
3
Log input of cDNA concentration (ng)
Y
Predicted Y
Linear (Predicted Y)
y = -3.3677x + 23.701
R2 = 1

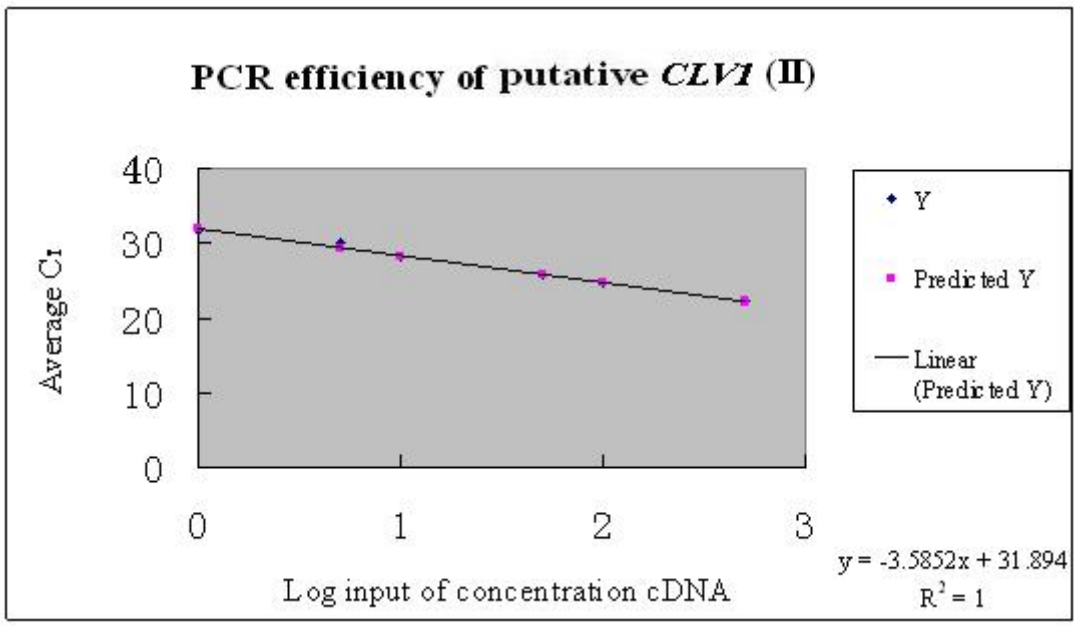
(B)


PCR efficiency of eEF1α
Average Ct
25
20
15
10
5
0
0
1
2
3
Log input of cDNA concentration (ng)
Y
Predicted Y
Linear
y = -3.3073x + 23.332
R2 = 1

(C)


PCR efficiency of putative CLV1 (I)
Average Ct
40
30
20
10
0
0
1
2
3
Log input of concentration cDNA (ng)
Y
Predicted Y
Linear
y = -3.429x + 32.272
R2 = 1

**(D)**




**(E)**

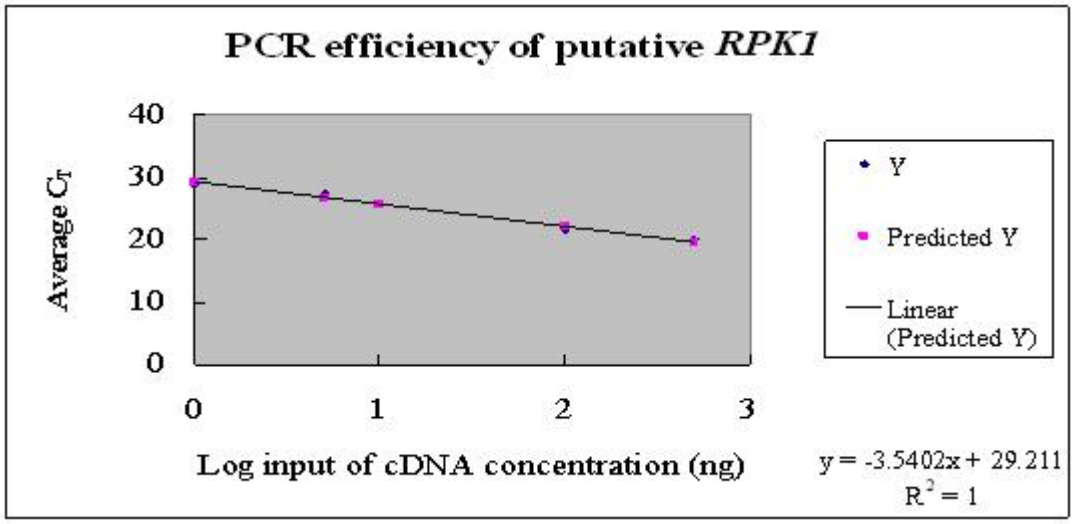



Figure 4.19: PCR efficiency of targets and housekeeping genes. The PCR efficiencies of (A) *UBQ5*, (B) *eEF-1*α, (C) putative *CLV1* (I), (D) putative *CLV1* (II) and (E) putative *RPK1* were plotted.

### 4.5.2 Expression Profiles of Putative *RPK1*

Figure 4.20 shows real time qRT-PCR gene expression profiling of putative *RPK1* of *Oryza rufipogon*, *Oryza sativa* ssp. *indica* cv. MR219, $BC_2F_7$ line 7 and $BC_2F_7$ line 23 at different developmental stages. Leaf of 8th day seedling stage was selected as calibrator for expression profiling of putative *RPK1*. This is because putative *RPK1* transcript leaf of 8th day seedling stage had the lowest expression compared with other stages. Gene expression of putative *RPK1* was the highest in the panicle of booting stage, except in $BC_2F_7$ line 23. After the booting stage, gene expression decreased gradually until the flowering stage. According to sequencing data as shown in Figure 4.4, $BC_2F_7$ line 23 is homozygous for the putative *RPK1* allele from *Oryza rufipogon*; whereas, $BC_2F_7$ line 7 is homozygous for the putative *RPK1* allele from *Oryza sativa* ssp. *indica* cv. MR219. However, the putative *RPK1* expression profile did not observe correlation between *Oryza rufipogon* and $BC_2F_7$ line 23, and between *Oryza sativa* ssp. *indica* cv. MR219 and $BC_2F_7$ line 7 as shown in Figure 4.20. Statistical analysis using two-way ANOVA with rescaled normalized expression level from the putative *RPK1* of *Oryza rufipogon*, *Oryza sativa* ssp. *indica* cv. MR219, $BC_2F_7$ line 7 and $BC_2F_7$ line 23 collected at different developmental stages (Table 4.2) revealed that the lines, stages, and correlation between lines and stages were significant ($P<0.01$; Table 4.3).

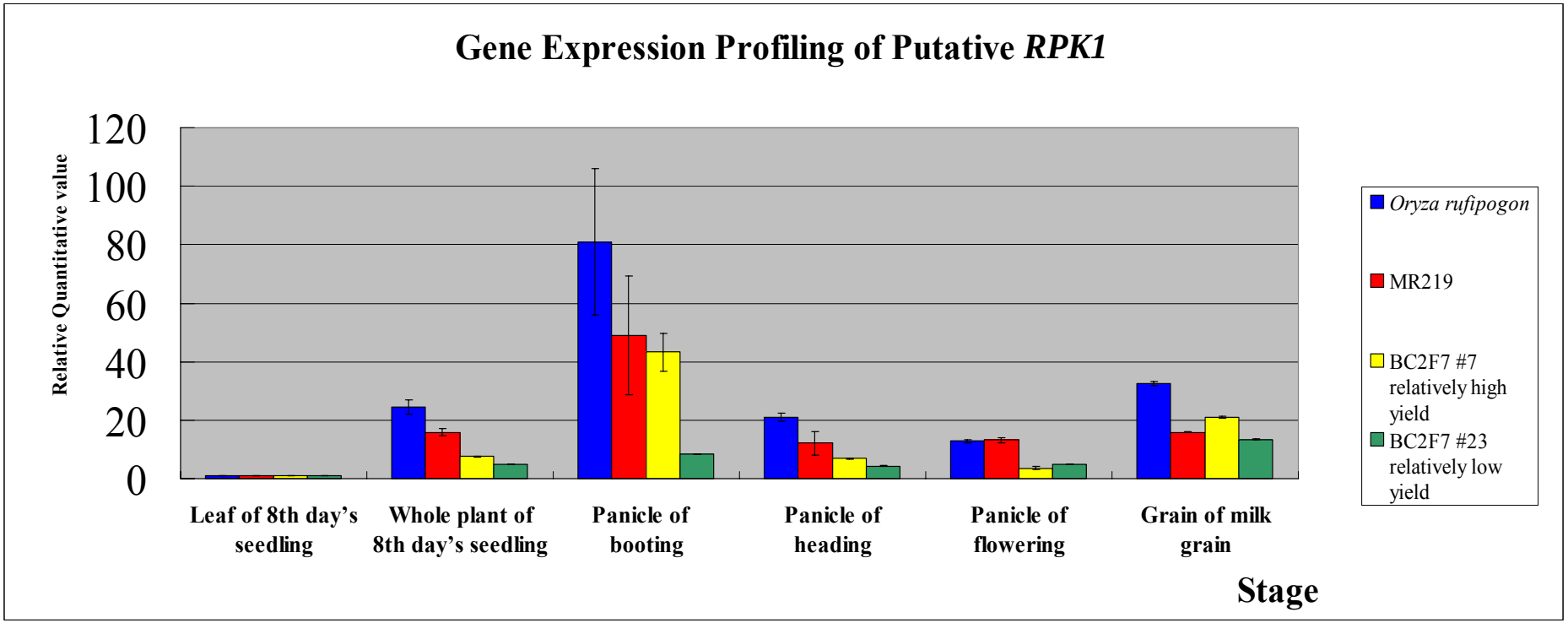



Figure 4.20: Comparative gene expression of putative *RPK1* between vegetative, reproductive and ripening phase. *Oryza rufipogon*, *Oryza sativa* ssp. *indica* cv. MR219, $BC_2F_7$ line 7 and $BC_2F_7$ line 23 were used on leaves and the whole plant at the seedling stage, the panicles at the booting, heading and flowering stages, and grains at the milk grain stage. Error bars indicate standard error of the means.

| **Sample and stage** | **Normalized Expression level of target (putative *RPK1)*** | **Standard error of normalized Expression level of target (putative *RPK1)*** | **Rescaled normalized expression level of target (putative *RPK1)*** | **Standard error of rescaled normalized expression level of target (putative *RPK1)*** |
|---|---|---|---|---|
| OR-L | 0.0300 | 0.0010 | 1 | 0.0010 |
| OR-W | 0.7351 | 0.1040 | 24.5033 | 2.5483 |
| OR-B | 2.4295 | 0.3088 | 80.9833 | 25.0076 |
| OR-H | 0.6303 | 0.0701 | 21.0100 | 1.4728 |
| OR-F | 0.3870 | 0.0392 | 12.9000 | 0.5057 |
| OR-M | 0.9767 | 0.0173 | 32.5567 | 0.5632 |
| MR-L | 0.0519 | 0.0022 | 1 | 0.0022 |
| MR-W | 0.8218 | 0.1152 | 15.8343 | 1.8241 |
| MR-B | 2.5419 | 0.4116 | 48.9769 | 20.1589 |
| MR-H | 0.6270 | 0.0578 | 12.0809 | 0.6983 |
| MR-F | 0.5345 | 0.0493 | 10.2987 | 0.5077 |
| MR-M | 0.8180 | 0.0147 | 15.7611 | 0.2317 |
| $BC_2F_7$#7 -L | 0.0331 | 0.0018 | 1 | 0.0018 |
| $BC_2F_7$#7-W | 0.2521 | 0.0324 | 7.6163 | 0.2468 |
| $BC_2F_7$#7-B | 1.4306 | 0.1469 | 43.2205 | 6.3491 |
| $BC_2F_7$#7-H | 0.2292 | 0.0305 | 6.9245 | 0.2112 |
| $BC_2F_7$#7-F | 0.1187 | 0.0184 | 3.6012 | 0.4275 |
| $BC_2F_7$#7-M | 0.6979 | 0.0139 | 21.0846 | 0.2931 |
| $BC_2F_7$#23 -L | 0.0749 | 0.0051 | 1 | 0.0051 |
| $BC_2F_7$#23-W | 0.3712 | 0.0537 | 4.9560 | 0.0598 |
| $BC_2F_7$#23-B | 0.6292 | 0.0392 | 8.4000 | 0.0697 |
| $BC_2F_7$#23-H | 0.3275 | 0.0116 | 4.3725 | 0.0107 |
| $BC_2F_7$#23-F | 0.3628 | 0.0544 | 4.8438 | 0.0501 |
| $BC_2F_7$#23-M | 1 | 0.0640 | 13.3500 | 0.1808 |

Table 4.2: Data of rescaled normalized expression level of putative *RPK1* and standard error of rescaled normalized expression level of putative *RPK1* of *Oryza rufipogon* (OR), *Oryza sativa* ssp. *indica* cv. MR219 (MR), $BC_2F_7$ line 7 and $BC_2F_7$ line 23 at different developmental stages. L: Leaf of 8th seedling day; W: Whole plant of 8th seedling day; B: Booting stage; H: Heading stage; F: Flowering stage; M: Milk grain stage.

| Item | Sum of Squares | Degrees of Freedom | Mean Squares | Variances | P value summary | Probability |
|---|---|---|---|---|---|---|
| Between lines | 6392 | 3 | 2131 | 14.01 | ** | < 1 % |
| Between stages | 19302 | 5 | 3860 | 42.29 | *** | < 1 % |
| Interaction | 6912 | 15 | 460.8 | 15.15 | *** | < 1 % |
| Residual (error) | 13030 | 72.0 | 181.0 | | | |
| Total | 45637 | 95.0 | | | | |

Table 4.3: Statistical analysis using two-way ANOVA with putative *RPK1* profile of *Oryza rufipogon*, *Oryza sativa* ssp. *indica* cv. MR219, $BC_2F_7$ line 7 and $BC_2F_7$ line 23 collected at different developmental stages. * indicates that the degrees of statistical significance.

### 4.5.3 Expression Profiles of Putative *CLV1*

Gene expression profiling of putative *CLV1* of *Oryza rufipogon*, *Oryza sativa* ssp. *indica* cv. MR219, $BC_2F_7$ line 7 and $BC_2F_7$ line 23 at different developmental stages shown in Figure 4.21. Putative *CLV1* transcript leaf of 8th day seedling stage had lowest expression compared with other stages, and was selected as calibrator for expression profiles. Gene expression of putative *CLV1* of $BC_2F_7$ line 7 was highly up-regulated at booting, heading and flowering stages compared to *Oryza rufipogon*, *Oryza sativa* ssp. *indica* cv. MR219 and $BC_2F_7$ line 23. According to sequencing data as shown in Figure 4.12, putative *CLV1* of *Oryza rufipogon* and $BC_2F_7$ line 23 were identical to each other; whereas, putative *CLV1* of *Oryza sativa* ssp. *indica* cv. MR219 and $BC_2F_7$ line 7 were identical to each other. However, the correlation of putative *CLV1* expression between *Oryza rufipogon* and $BC_2F_7$ line 23, and between *Oryza sativa* ssp. *indica* cv. MR219 and $BC_2F_7$ line 7 did not observe (Figure 4.21). Statistical analysis using two-way ANOVA with rescaled normalized expression level from the putative *CLV1* of *Oryza rufipogon*, *Oryza sativa* ssp. *indica* cv. MR219, $BC_2F_7$ line 7 and $BC_2F_7$ line 23 collected at different developmental stages (Table 4.4) revealed that the lines, stages, and correlation between lines and stages was extremely significant ($P<0.0001$; Table 4.5).

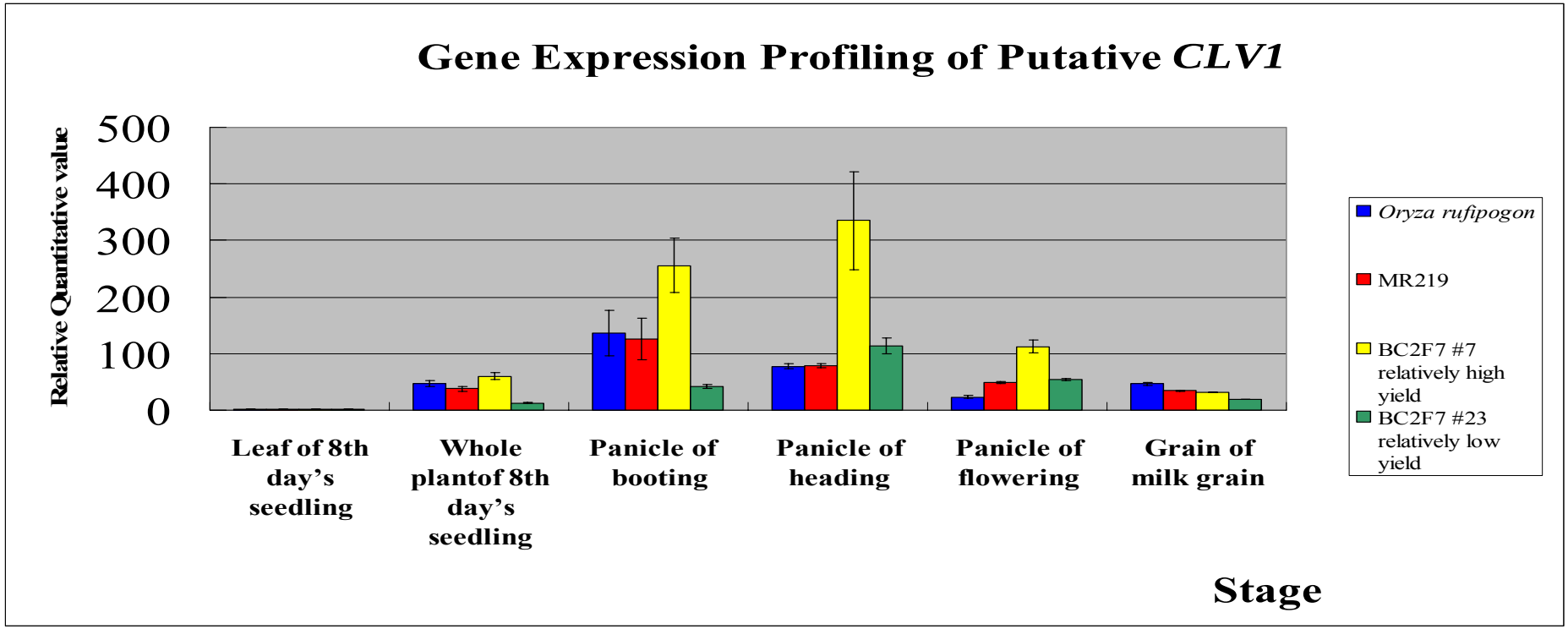



Figure 4.21: Comparative gene expression of putative *CLV1* between vegetative, reproductive and ripening phase. *Oryza rufipogon*, *Oryza sativa* ssp. *indica* cv. MR219, $BC_2F_7$ line 7 and $BC_2F_7$ line 23 were used on leaves and the whole plant at the seedling stage, the panicles at the booting, heading and flowering stages, and grains at the milk grain stage. Error bars indicate standard error of the means.

| Sample and stage | Normalized expression level of target (putative *CLV1*) | Standard error of normalized expression level of target (putative *CLV1*) | Rescaled normalized expression level of target (putative *CLV1*) | Standard error of rescaled normalized expression level of target (putative *CLV1*) |
|---|---|---|---|---|
| OR-L | 0.0083 | 0.0008 | 1 | 0.0008 |
| OR-W | 0.3744 | 0.0647 | 45.1084 | 2.9185 |
| OR-B | 2.4295 | 0.2682 | 292.7161 | 78.5065 |
| OR-H | 1.0828 | 0.0561 | 130.4578 | 7.3187 |
| OR-F | 0.8993 | 0.0132 | 108.3493 | 1.4302 |
| OR-M | 0.2172 | 0.0057 | 26.1686 | 0.1492 |
| MR-L | 0.0209 | 0.0033 | 1 | 0.0033 |
| MR-W | 0.7864 | 0.1020 | 37.6268 | 3.8379 |
| MR-B | 2.6300 | 0.2869 | 125.8373 | 36.1027 |
| MR-H | 1.6578 | 0.0438 | 79.3206 | 3.4742 |
| MR-F | 1.0281 | 0.0411 | 49.1914 | 2.0218 |
| MR-M | 0.7176 | 0.0231 | 34.3349 | 0.7931 |
| $BC_2F_7$#7 -L | 0.0025 | 0.00004 | 1 | 0.00004 |
| $BC_2F_7$#7-W | 0.1269 | 0.0393 | 50.7600 | 1.9949 |
| $BC_2F_7$#7-B | 1.6802 | 0.3626 | 672.0800 | 134.9162 |
| $BC_2F_7$#7-H | 1.8671 | 0.0946 | 746.8400 | 70.6511 |
| $BC_2F_7$#7-F | 0.8405 | 0.0563 | 336.2000 | 18.9281 |
| $BC_2F_7$#7-M | 0.4762 | 0.0178 | 190.4800 | 3.3905 |
| $BC_2F_7$#23 -L | 0.0256 | 0.0070 | 1 | 0.0070 |
| $BC_2F_7$#23-W | 0.3192 | 0.0865 | 12.4688 | 1.0786 |
| $BC_2F_7$#23-B | 1.0490 | 0.1491 | 40.9766 | 6.1096 |
| $BC_2F_7$#23-H | 1.5913 | 0.0763 | 62.1602 | 4.5904 |
| $BC_2F_7$#23-F | 0.8509 | 0.0574 | 33.2383 | 1.9079 |
| $BC_2F_7$#23-M | 0.6455 | 0.0195 | 25.2148 | 0.4917 |

Table 4.4: Data of rescaled normalized expression level of putative *CLV1* and standard error of rescaled normalized expression level of putative *CLV1* of *Oryza rufipogon* (OR), *Oryza sativa* ssp. *indica* cv. MR219 (MR), $BC_2F_7$ line 7 and $BC_2F_7$ line 23 at different developmental stages. L: Leaf of 8th seedling day; W: Whole plant of 8th seedling day; B: Booting stage; H: Heading stage; F: Flowering stage; M: Milk grain stage.

| Item | Sum of Squares | Degrees of Freedom | Mean Squares | Variances | P value summary | Probability |
|---|---|---|---|---|---|---|
| Between lines | 1.389e+006 | 3 | 463072 | 34.55 | *** | < 1 % |
| Between stages | 1.088e+006 | 5 | 217690 | 27.07 | *** | < 1 % |
| Interaction | 1.169e+006 | 15 | 77960 | | *** | < 1 % |
| Residual (error) | 374322 | 72.0 | 5199 | | | |
| Total | 4.021e+006 | 95.0 | | | | |

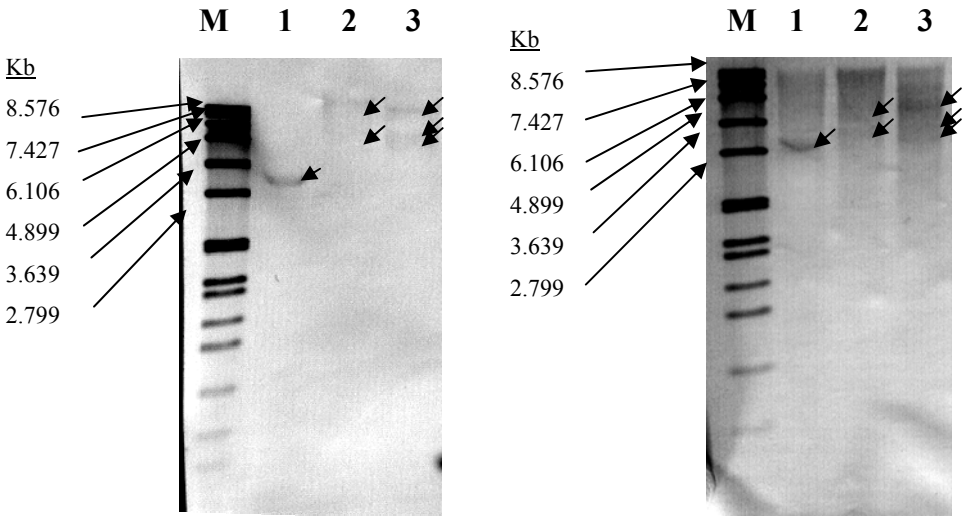
Table 4.5: Statistical analysis using two-way ANOVA with and putative *CLV1* profile of *Oryza rufipogon*, *Oryza sativa* ssp. *indica* cv. MR219, $BC_2F_7$ line 7 and $BC_2F_7$ line 23 collected at different developmental stages. * indicates that the degrees of statistical significance.

## 4.6 Southern Hybridization Analysis

Genomic DNA isolated from young leaves of *Oryza rufipogon* and *Oryza sativa* ssp. *indica* cv. MR219 was single digested with *Eco*RI, with *Bam*HI and with *Hind*III. The putative *RPK1* and putative *CLV1* cDNA fragments from *Oryza rufipogon* and *Oryza sativa* ssp. *indica* cv. MR219 respectively were used as probes for Southern hybridization. The length of putative *RPK1* probe was 320 bp, while the length of putative *CLV1* probe was 402 bp.

### 4.6.1 Gene Structure of Putative *RPK1*

Southern hybridization analysis showed the presence of one to three hybridized bands differing in size and with weak intensities (Figure 4.22). The putative *RPK1* probe did not contain *Eco*RI, *Bam*HI or *Hind*III restriction sites. The putative *RPK1* is probably present at one to three copies in the genomes of *Oryza rufipogon* and *Oryza sativa* ssp. *indica* cv. MR219.




**(A)** **(B)**

Figure 4.22: Southern hybridization analysis of putative *RPK1*. Genomic DNA of (A) *Oryza rufipogon* and (B) *Oryza sativa* ssp. *indica* cv. MR219 were digested with *Eco*RI (lane 1), *Bam*HI (lane 2) and *Hind*III (lane 3). Lane M: DIG-labeled DNA Molecular Weight Marker VII (Roche, Germany); Lane 1: Genomic DNA digested with *Eco*RI; Lane 2: Genomic DNA digested with *Bam*HI; Lane 3: Genomic DNA digested with *Hind*III.

### 4.6.2 Gene Structure of Putative *CLV1*

Southern hybridization analysis showed the presence of three to six hybridized bands differing in size and with strong and weak intensities (Figure 4.23). The putative *CLV1* probe did not contain *Eco*RI, *Bam*HI or *Hind*III restriction sites. The putative *CLV1* may be present at three to five copies in the genomes of *Oryza rufipogon* and *Oryza sativa* ssp. *indica* cv. MR219.

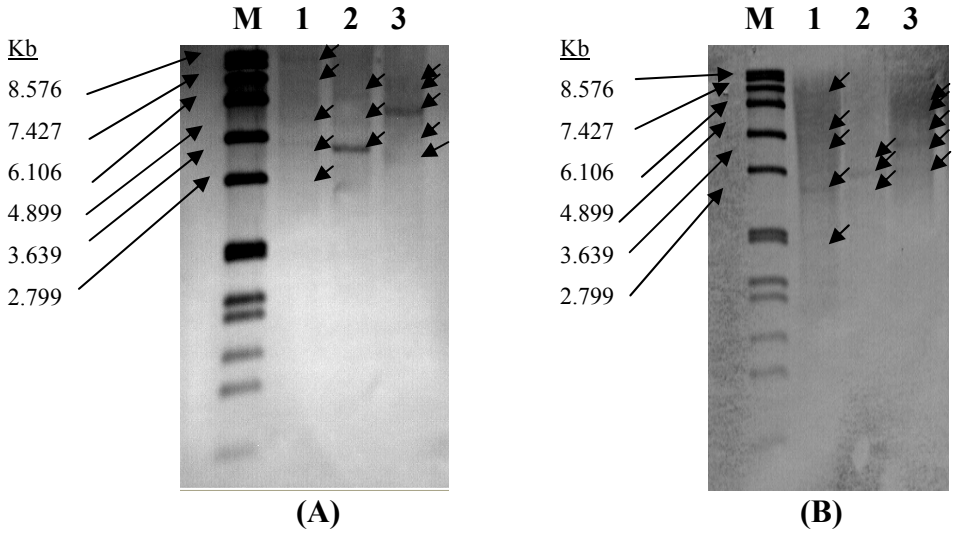



Figure 4.23: Southern hybridization analysis of putative *CLV1*. Genomic DNA of (A) *Oryza rufipogon* and (B) *Oryza sativa* ssp. *indica* cv. MR219 were digested with *Eco*RI (lane 1), *Bam*HI (lane 2) and *Hind*III (lane 3). Lane M: DIG-labeled DNA Molecular Weight Marker VII (Roche, Germany); Lane 1: Genomic DNA digested with *Eco*RI; Lane 2: Genomic DNA digested with *Bam*HI; Lane 3: Genomic DNA digested with *Hind*III.

## 4.7 Construction of RNAi Vectors for Knockdown of Gene Expression

### 4.7.1 Cloning of Putative *RPK1* and Putative *CLV1* Sequences into pANDA Vector

The putative *RPK1* and putative *CLV1* were successfully amplified from the cDNA of *Oryza rufipogon* (Figure 4.24). RT-PCR products of 105 bp for putative *RPK1* and 104 bp putative *CLV1* were amplified; each including the four bases "CACC" at the 5' end from each forward primer (see section 3.4) to allow for orientation based cloning into pENTR/D-TOPO (Invitrogen, California). After transformation in *E. coli*, putative positive colonies of pENTR/D-TOPO vector containing the sequences were confirmed by colony PCR (Figure 4.25) and DNA sequencing (Appendix G). The length of inserts in the putative positive clones of pENTR/D-TOPO vector containing the sequences of interest were 247 bp, including M13 forward (-20) priming site and *attL1* region.

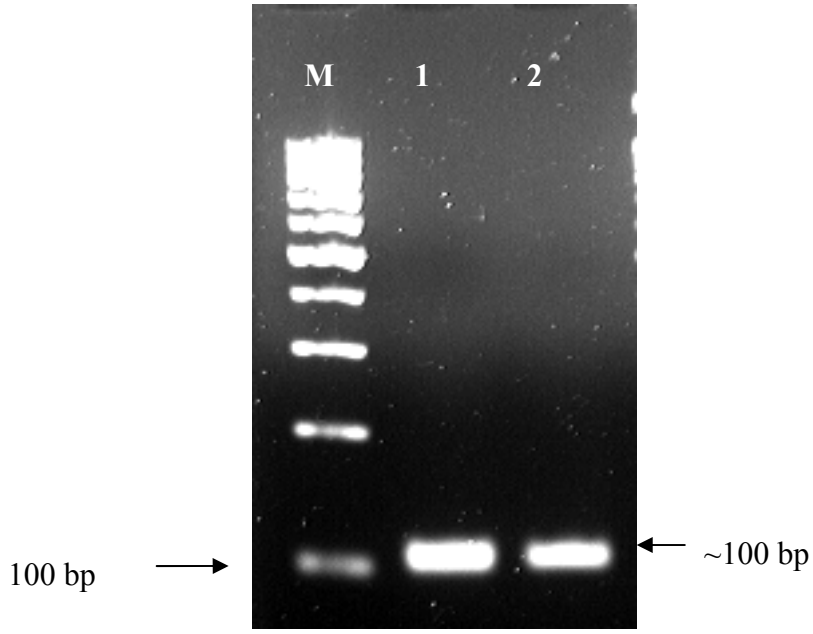



Figure 4.24: RT-PCR amplification of genes of interest. Lane M: 100 bp marker; Lane 1: RT-PCR amplified putative *RPK1* sequences; Lane 2: RT-PCR amplified putative *CLV1* sequences.

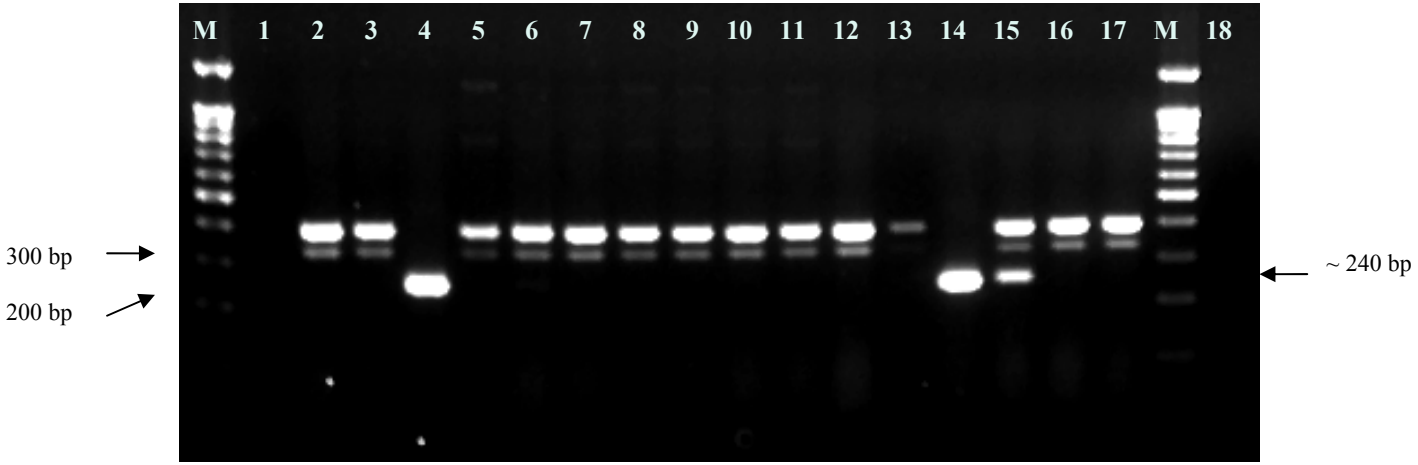



Figure 4.25: PCR confirmation of pENTR/D-TOPO cloning vector. Lane M: 100 bp marker; Lane 1: $H_2O$ template; Lane 1-9: Putative subclone of putative *RPK1* sequences; Lane 4: Positive subclone of putative *RPK1* sequences; Lane 10-18: putative subclone of putative *CLV1* sequences; Lane 14: Positive subclone of putative *CLV1* sequences; Lane 18: $H_2O$ template.

Next, the pENTR/D-TOPO cloning vectors containing the sequences of interest were successfully mobilized into pANDA vector by an LR Clonase enzyme reaction. After transformation, putative positive colonies were confirmed by colony PCR (Figure 4.26) and DNA sequencing (Appendix G). Colony PCR was performed by using Gus linker primers and gene of interest primers: Gus linker forward and a gene of interest reverse primer were used to confirm the presence of the sense orientation, while a gene of interest reverse and Gus linker reverse primer were used to confirm the presence of the antisense orientation for each construct (Figure 4.26). The length of the PCR product of pANDA containing the gene sequences of interest was 984 bp, including 829 bp of Gus linker fragment and 50 bp *attB* sequences; whereas, the length of the PCR product antisense orientation of pANDA containing the gene sequences of interest was 887 bp, including 732 bp of Gus linker fragment and 50 bp *attB* sequences.

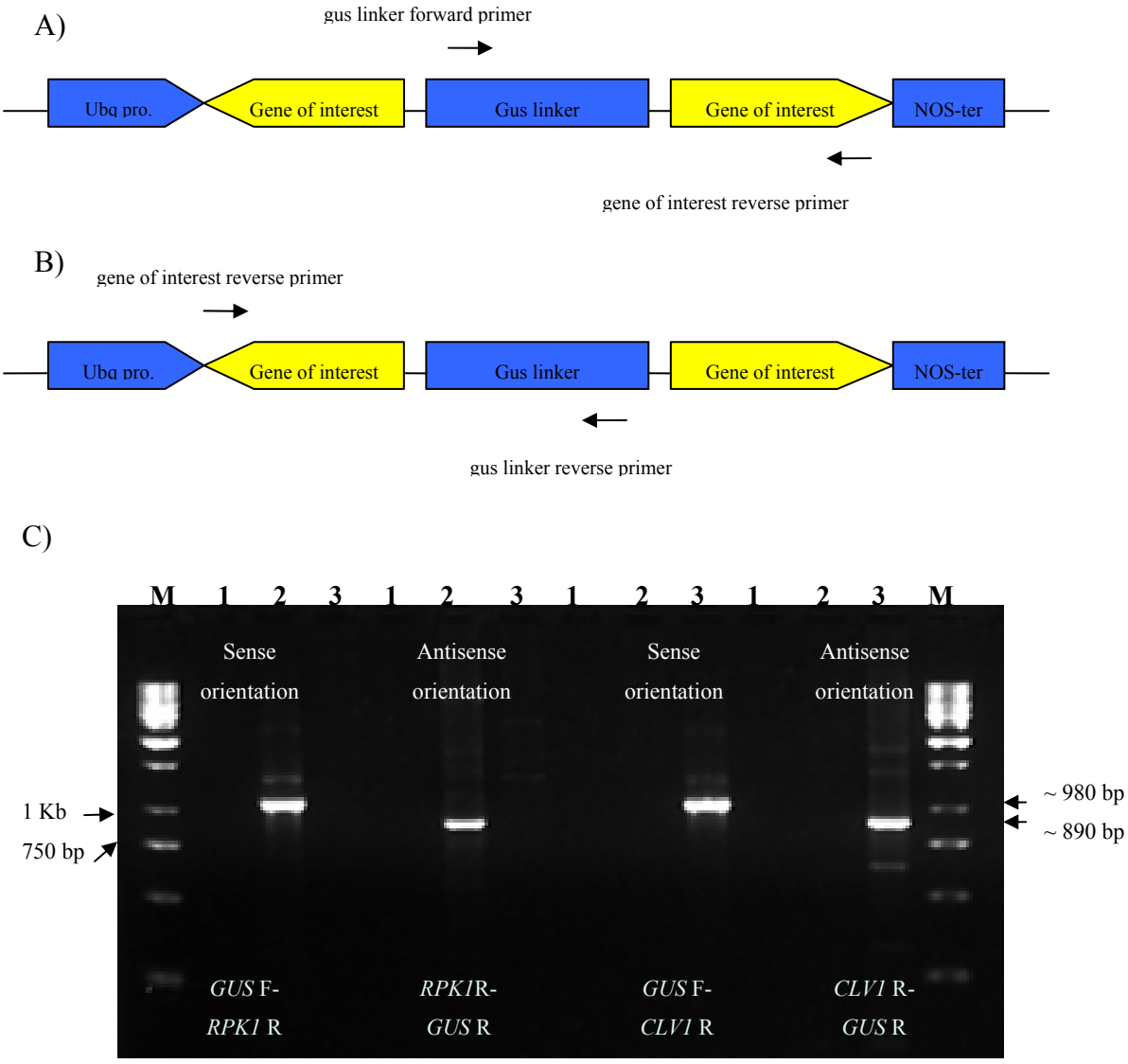



Figure 4.26: The sense orientation and antisense orientation of genes of interest for pANDA construct. (A) Gus linker forward primer and gene of interest reverse primer were used to check the sense orientation. (B) Gene of interest reverse primer and Gus linker reverse primer were used to check the antisense orientation. (C) PCR confirmation of vector construction orientation. Lane M: 1 kb marker; Lane 1: $H_2O$ template; Lane 2: positive clone of pANDA containing putative *RPK1* sequences; Lane 3: positive clone of pANDA containing putative *CLV1* sequences.

### 4.7.2 Plant Transformation

pANDA vectors containing the sequences of putative *RPK1* (*pANDA_RPK1)* and putative *CLV1* (*pANDA_CLV1*) were transformed into *Agrobacterium tumefaciens* strain EHA105 through a freeze and thaw method (Jyothishwaran *et al*., 2007). Next, positive *Agrobacterium* colonies were confirmed by PCR with a Gus linker primer (Figure 4.27). A 636 bp long fragment was successfully amplified from positive *Agrobacterium* colonies. Following this, *Agrobacterium tumefaciens* strain EHA105 containing *pANDA_CLV1* used to transform callus of *Oryza rufipogon* IRGC105491 (Sivakumar, unpublished data). A 636 bp long Gus linker fragment was successfully amplified from leaves of hygromycin resistant $T_0$ plants of *Oryza rufipogon* IRGC105491 by PCR with Gus linker primer (Figure 4.28).

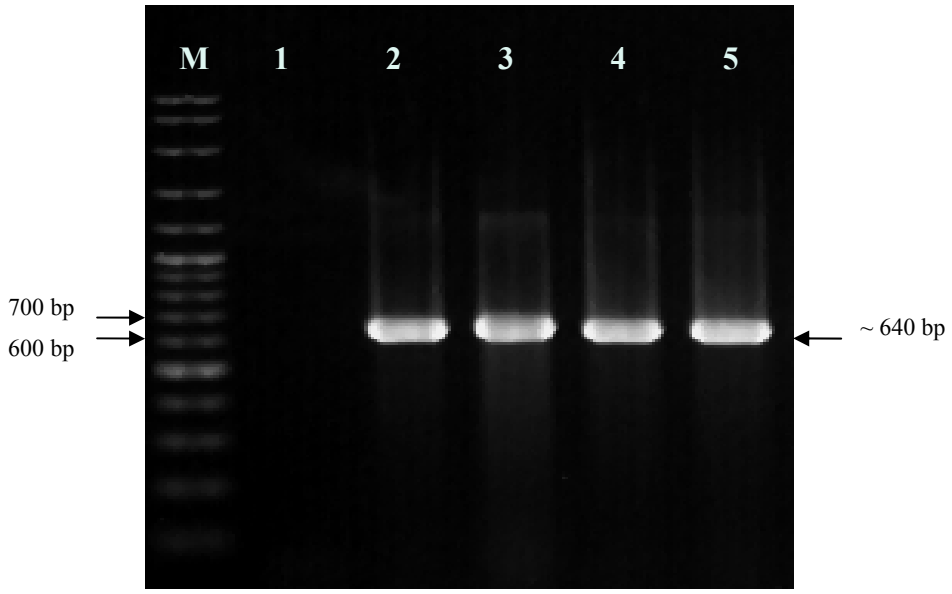



Figure 4.27: PCR screening of *Agrobacterium* colonies with Gus linker primer. Lane M: 100 bp DNA marker; Lane 1: $H_2O$ template; Lane 2 and 3: positive clone of *pANDA_RPK1*; Lane 4 and 5: positive clone of *pANDA_CLV1*.

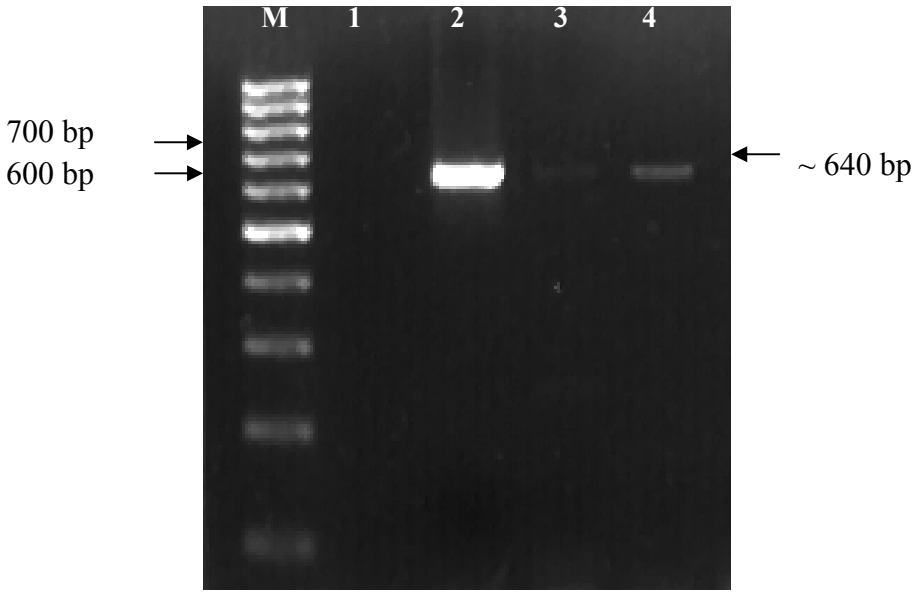



Figure 4.28: PCR screening of leaves hygromycin resistant $T_0$ plants at flowering stage with Gus linker primers. Lane M: 100 bp DNA marker; Lane 1: $H_2O$ template; Lanes 2, 3 and 4: DNA from leaves of hygromycin resistant plants.