Identification of Aquaporin genes from pearl millet [*Pennisetum glaucum* (L.) R. Br.]

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Abstract

Aquaporins facilitate water transport and also several other small molecules through cell membranes, thereby showing a central role in plant growth and development. Although extensive research work has been carried out on aquaporin proteins and genes that encode them in a wide variety of plant species, very little is known about the aquaporins in pearl millet. Hence, the present study was focussed on identification of aquaporin genes in pearl millet. A set of 37 primers pairs were designed and PCR was carried out on the genomic DNA of four genotypes (PRLT2/89-33/ H77/833-2, ICMB 841 and 863B). Out of 37 primer pairs, twelve resulted in amplified fragments and among these, five good quality amplicon sequences were derived from four genotypes. Poor quality regions at the ends of the sequences of the four genotypes were trimmed and aligned to get the consensus sequence. Annotation of these consensus sequences showed similarity with the PIP1;2, TIP1;1 and TIP2;1 genes of *Setaria italica*. These sequences will be useful in further expression studies to explore various physiological roles of PIP and TIP genes. SNPs and INDEL identified in the consensus sequences also serve as potential sources of markers in further genetic studies.

Introduction

Water transport across the cell membranes is facilitated through aquaporins, member of a major intrinsic protein family. Ample evidence is available regarding aquaporin structure and properties of water transportation. A few aquaporins are purely water transporters while others carry a wide range of substrates such as urea or glycerol, carbon dioxide, ammonia, hydrogen peroxide, antimonite, arsenite and silicon (Maurel et al. 2008; Heinen et al. 2009). These aquaporins are ubiquitously distributed across the plant tissues and contribute to many plant processes that can have important agricultural implications for crops. For example, the over expression of transgenic *Os PIP2;7* in rice enhanced its transpiration and tolerance to chilling (Li et al. 2008). Aquaporins play a key role in plant water relations, facilitating passive exchange of water molecules and 95% of water permeability of cell membranes (Henzler and Steudle 2004). Along with the aquaporin mediated water movement (symplastic path), water transport also occurs through transcellular (traversing the cell membranes) and apoplastic paths (across cell walls) of plant tissues. Apoplastic pathway was believed to be the main route for water movement during transpiration while cell to cell pathway attributes to many

physiogical processes such as homeostasis, maintenance of turgor and opening and closing of stomata (Heinen et al. 2009). However, the impact of environmental conditions on the molecular and cellular processes of root aquaporins and in turn with the shoot growth is still unclear. With the aim of understanding the proportion of water transport controlled by aquaporins and their link with the water saving traits (by restricting transpiration rate under high VPD as discussed by Kholova et al. 2010) in drought contrast parental lines of two mapping populations, research was initiated with the first criteria of identifying aquaporin genes (PIPs and TIPs) in pearl millet.

Based on their sequence similarities, the plant aquaporins are classified into five different sub-families, namely PIPs (plasma membrane intrinsic proteins), TIPs (tonoplast intrinsic proteins), NIPs (nodulin26-like intrinsic proteins), SIPs (small basic intrinsic proteins), and XIPs (X intrinsic proteins) (Heinen et al. 2009). There are a number of studies focussed on identification of aquaporin genes in plants. For instance, 33 aquaporin genes were detected in *Oryza sativa L*. (Sakurai et al. 2005), and *Zea mays* (Chaumont et al. 2001) each respectively but very little data is available regarding aquaporins in pearl millet. Hence, aim of this study was to identify the aquaporin genes (PIPs and TIPs) from pearl millet.

Materials and methods

Plant materials and DNA isolation

Pot grown plants were kept in a glasshouse and maintained at approximately 35°C/25°C, under fully irrigated conditions. Young fully developed leaf tissues of four genotypes of pearl millet (PRLT2/89-33, H77/833-2, ICMB 841 and 863B) were harvested from 20 day-old-plants grown in glasshouse, frozen with liquid nitrogen and preserved at -80°C before grinding. DNA isolation was carried out using modified CTAB/β-mercaptoethanol method as described by Mace et al. (2003).

PCR amplification, sequencing and annotation

Aquaporin genes of all the poaceae members (rice, sorghum, maize, foxtail millet) were downloaded and BLAST searched against the pearl millet transcript assembly reported by Rajaram et al. 2013. The pearl millet transcripts with best hits (e-value ≤ 1E-10) were used to design forward and reverse primers. A set of 37 primers were designed (Table 1) and PCR was carried out on genomic DNA of four genotypes using a Gen-Amp PCR system 9700® thermocycler (Applied Biosystems, USA). PCR reactions were performed using 5 ng of genomic DNA template, 1 picomole of forward primer, 1 picomole of reverse primer, 0.5 ul of 2 mM dNTPs suspended in 0.5 ul of 10X PCR buffer and finally 0.1 units of Taq DNA polymerase was added in a 5 ul PCR reaction volume. PCR conditions include, initial denaturation at 94°C for 5 min followed by ten cycles of denaturation (94°C for 15s), annealing (61°C to 51°C, touch-down cycles for 30s), and extension (72°C for 30s), followed by 35 cycles of denaturation (94°C for 10s), annealing (54°C for 30s), and extension (72°C for 30s), followed by final extension (72°C for 20 min). Amplified PCR product was verified on 1.2% agarose gels. PCR product was treated with Exonuclease (10 U/ul) and Shrimp alkaline phosphatase (1 U/ul) and incubated at 37°C followed by 80°C for 15 min through a PCR program to clean up the PCR products before sequencing. PCR products were sequenced using Sanger method on ABI 3730 sequencing machine.

Sequences of four genotypes were retrieved through DNA baser, aligned through clustalX and the fragments were trimmed using Bioedit software to get the consensus sequence. Consensus sequences were queried through

BLASTn to identify the nearest similar aquaporin genes. The SNPs were identified based on sequence alignment.

Results and discussion

PCR amplification of 37 primer pairs (Table 1) on the genomic DNA of four parents (PRLT2/89-33/ H77/833-2, ICMB 841 and 863B) of mapping populations resulted in 12 primer pairs yielding amplification in all the four genotypes (Fig. 1). These 12 primer PCR products of four genotypes were sequenced using Sanger method. Out of 12 primer pairs, sequenced products from five primer pairs were of good quality (Table 2). Low quality regions at the ends of sequences of all the four genotypes were trimmed off (DNA baser), aligned through clustalX software and consensus sequences (Bioedit software) obtained were used for further blast analysis. Annotation of these five consensus sequences (Additional file) through BLASTn search showed top hits with the PIP and TIP of *Setaria italica* (Table 2). Among the five primer pairs, consensus sequences of three were found similar to PIP1;2 and the other hits correspond to TIP1;1 and TIP2;1 of *Setaria italica*. Consensus sequence of AQPM25, 35 and 37 showed highest identity (5e-51, 6e-51, 8e-147) of 99%, 96%, 97% with PIP1;2 of *Setaria italica*. Consensus sequences of AQPM17, 21 showed highest identity of (5e-25, 9e-72) 85% and 93% with TIP1;1 and TIP2;1 of *Setaria italica*. Most of the sequences showed top hits with foxtail millet (*Setaria italica*) than maize revealing that foxtail millet is the closest relative to pearl millet among all other cereals, which was also reported earlier by Rajaram et al. (2013) through their synteny relationships (Fig. 2).

Three SNPs were identified between four parents with AQPM25 consensus sequence (located at 141, 227, 390 positions on consensus sequence, Fig. 3). SNP variations resulted in nucleotide A in 863B parent, T in all the other three parents (141 position), variant G in 863B parent, T in all the other three parents (227 position) and variant G in 863B parent in spite of A (390 position) in the other three genotypes (Fig. 3). These variants were considered as SNPs after thoroughly verifying with the nucleotide peaks of sequences and the region was also devoid of any noise. An indel (3 bp) was observed in the parental pairs with the consensus sequence of AQPM17 (445 position, Fig. 4). Occurrence of indel in two sensitive genotypes (ICMB 841, H77/833-2) could be considered as markers of interest in further aquaporin studies. Source of these SNPs and Indel may be due to DNA synthesis, repair or recombination and finally remain unexplained as discussed by Batley et al. (2003), but these SNPs and indel are potential sources of genetic markers for studying various traits. Even though this is a small data set, PIP1;2, TIP1;1 and TIP2;1 sequences obtained through the candidate gene approach could serve as a better option for starting up further expression studies. SNPs and indel identified in the present study could be used as markers in future studies on aquaporins.

Table 1. Primer pairs designed for PCR amplification.

Primer Name	Forward Primer	Reverse Primer	Pearl millet Transcriptome sequence[1]	Transcriptome sequence similarity to
AQPM 1	GGCCAAGAACAGTTCCAAAA	ATCACCATCCTCACCGTCAT	Sample4_FL4RA0R02J34XR	PIP 1-2
AQPM 2	ATGGCCCTGTATTGATCGAC	TTACTGGCACTGGCATCAAC	Sample4_FL4RA0R02H1V8A	PIP 1-2
AQPM 3	AAGAAAACCCAAGACCAACG	TAGGTGCCGATGGTAGGAAC	Sample2_FL4RA0R01DV76C	SIP 1-1
AQPM 4	CGTACAGTGTTTGGCACCAC	CTGGTCCTTTGGAGGAATGA	SCL4Contig21	PIP 1-1
AQPM 5	AGGAATGATCTTCGCGCTC	TAACCCATCCAATAGTATTAACACGC	Sample1_FMD86AS01CJGJ5	PIP 1-2
AQPM 6	TCACCGTACCAGAGAAACCC	CAGGGACTCCCATGTTCCTA	Sample4_FL4RA0R02F8TNH	PIP 2.2
AQPM 7	TAGGAAAGGCTTTTGCTTGG	GGTCGTTGACTTTCTTCCTCC	CL16236Contig1	SIP 2-1
AQPM 8	GAGATCATTGGCACCTTCGT	CACCTGCGGAAGCATATTTT	Sample4_FL4RA0R02IZ9CQ	PIP 1-1
AQPM 9	ACATTAACAAAGGTAGGCGACG	GGCCATCCCGTTCAAGAG	Sample2_FL4RA0R01D0YV5	PIP 1-4
AQPM 10	AGGAGCAACGCTTGAAGCTA	GACAGGTTACGAGCGACACA	SCL89Contig31	PIP 2
AQPM 11	TCCAAGTCCAACACCAAGTG	TGCCAGATATGTCGTTCCTG	Sample4_FL4RA0R02GKWT1	PIP 1-2
AQPM 12	CCACCATCCCCATTTACTTG	ATCACCTGGTGGTAGATGGC	Sample2_FL4RA0R01BFW8X	PIP 1
AQPM 13	ATGGGATGACCACTGGATCT	AGCTGTGTAACCGTTGCCTT	Sample2_FL4RA0R01AFHX7	PIP 2
AQPM 14	GTTCTTCGGAACTGGCATGT	CTTGGTAGCGACCATTGGAT	SCL4Contig323	PIP 1
AQPM 15	CTACAACAGGGATCACGCCT	TTCTTCTTCGGAACTGGCAT	SCL4Contig136	PIP 1
AQPM 16	TCAGCAGTTAGTCCTGAAGGG	ATCCCGTTCAAGAGCAGGTC	Sample4_FL4RA0R02GEBK0	PIP 1-4
AQPM 17	TCGGGTTATCTACGAGGTGC	CTACATACGGTGATGCGTGG	CL282Contig1	TIP
AQPM 18	AGGTGATCATCAGGGCCA	CGAAACAGTGCTTTACCCCT	Sample1_FMD86AS01BOEU2	PIP 1-4
AQPM 19	GTACCGCACAGACACACACC	TGAAGGGAGACGAGGAATTG	SCL4Contig289	PIP 1
AQPM 20	CTTGAACGGGTTTTATTGGC	TCAACCCAGCTAGGAGCCTT	Sample4_FL4RA0R02FLESA	PIP 1
AQPM 21	ATGGACTGAAACCGATCGAC	CCTTGAAAGATGAGAACCGC	CL1006Contig1	TIP 2
AQPM 22	CACGTACGGTAACGATTCTGA	ATGGGATGACCACTGGATCT	Sample2_FL4RA0R01EV4PR	PIP 2
AQPM 23	TACCTCAATTGCCCCAACAT	ATGCCAGCGATGTAGACCTT	CL2054Contig1	SIP
AQPM 24	CCATTGCCCATGTAAAGTCC	GACGTACGGGCCATCTTCTA	Sample4_FL4RA0R02JZILN	PIP 1
AQPM 25	GAGATCATTGGCACCTTCGT	AGGCGTGATCCCTGTTGTAG	SCL4Contig232	PIP 1
AQPM 26	CTGAAGACCCATCCAGCAAG	CATTTGGCTGGGCATATGTA	Sample2_FL4RA0R01AX4C5	SIP 1
AQPM 27	GGCGAGGATAATTCACTGCT	AGATGCCTAATGCACCAACC	SCL32Contig23	PIP 1-3
AQPM 28	ATACCAAATGGTCGCTCCAG	GGGGGCAACAACAACTACTG	Sample4_FL4RA0R02G9W0Q	PIP 1
AQPM 29	ATTGGATCTTCTGGGTCGG	AAGACGCTCGTTCTTCTTCG	Sample2_FL4RA0R01CR2X8	PIP 1-4
AQPM 30	CTGGGATGACCACTGGATCT	AAATGGAACAGCTCCACAGC	SCL4Contig123	PIP
AQPM 31	TGGATCACGAGCATATGGAA	TCAGTTTCTTTCCTGATCTCCA	Sample4_FL4RA0R02FK7KP	SIP 2
AQPM 32	CGTTTTCGGGTTCTTACCTT	AAATGGAACAGCTCCACAGC	Sample2_FL4RA0R01DFP11	PIP 1
AQPM 33	CCCTTCATCGGAACGAACTA	AAATGGAACAGCTCCACAGC	Sample1_FMD86AS01C5T8A	PIP 1-3
AQPM 34	ATAGAACCCAGCCTAACGCA	TAGGTGCCGATGGTAGGAAC	Sample1_FMD86AS01BM9VQ	PIP 1-3
AQPM 35	ACTACAACAGGGATCACGCC	TACGCTCGTTCTTCGGAACT	SCL4Contig279	PIP 1
AQPM 36	TCTACAACAGGAGCCAAGCC	AGCACAGGACACAGCAGCTA	Sample2_FL4RA0R01AQU73	PIP 1
AQPM 37	GCGAAGATCATTCCTCCAAA	ACAGACACACACCGCTGTTC	Sample4_FL4RA0R02FQ6QU	PIP 1

[1]Rajaram et al. 2013.

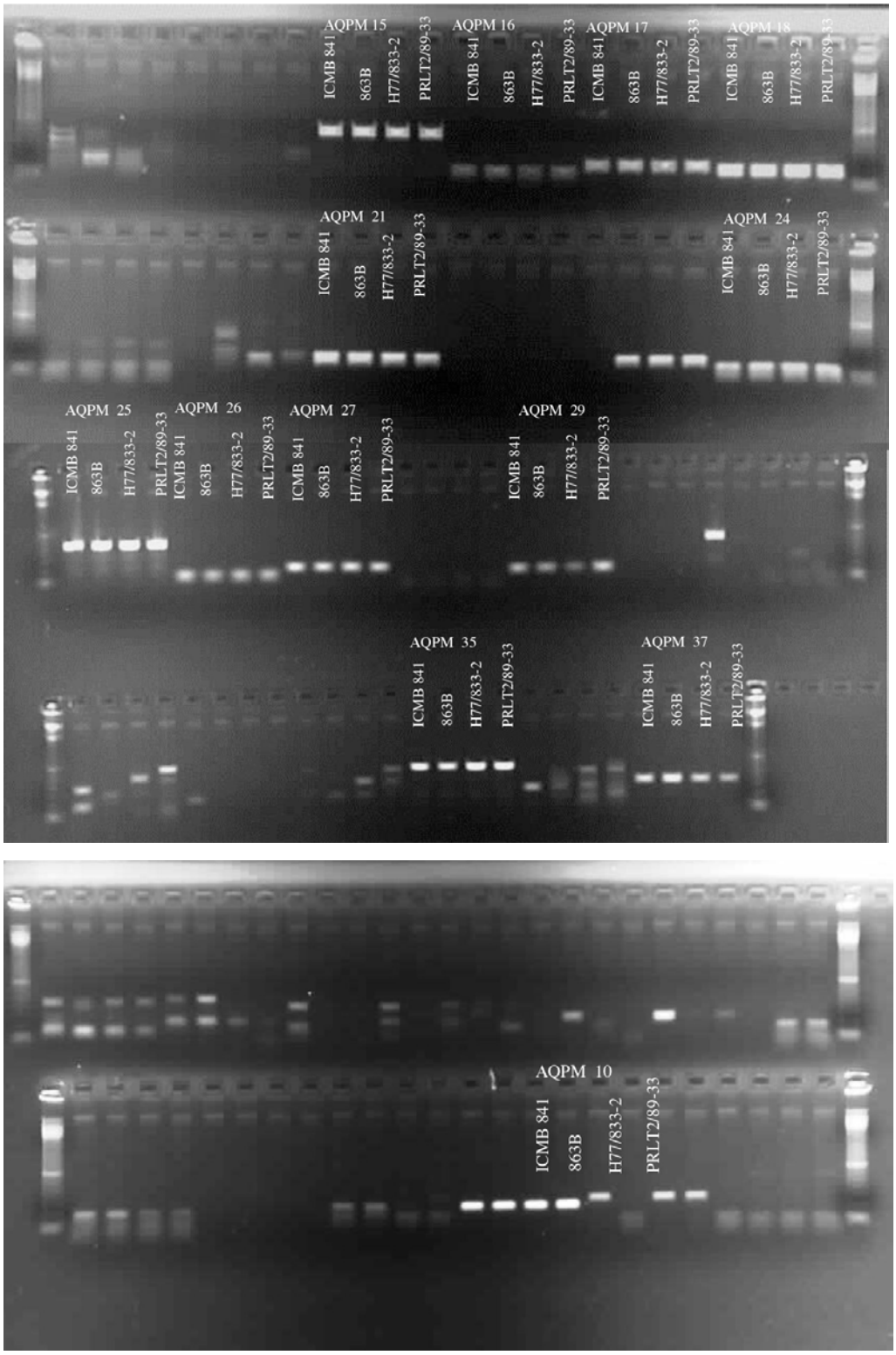

Figure 1. PCR amplification of the 37 primer pairs in the four genotypes of genomic DNA. Primer pairs which yielded amplification with four genotypes were labelled in the figure.

Table 2. Similarity of PCR amplicons based on best BLASTn[2]hit (*evalue*≤ 1E-10) on NCBI database.

Primer Name	Similarity of PCR amplicon	SNP/INDELs detected
AQPM25	PIP1;2 (from *Setaria italica*)	3 SNPs
AQPM35	PIP1;2 (from *Setaria italica*)	Nil
AQPM37	PIP1;2 (from *Setaria italica*)	Nil
AQPM17	TIP1;1 (from *Setaria italica*)	1 INDEL
AQPM21	TIP2;1 (from *Setaria italica*)	Nil

[2]http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome

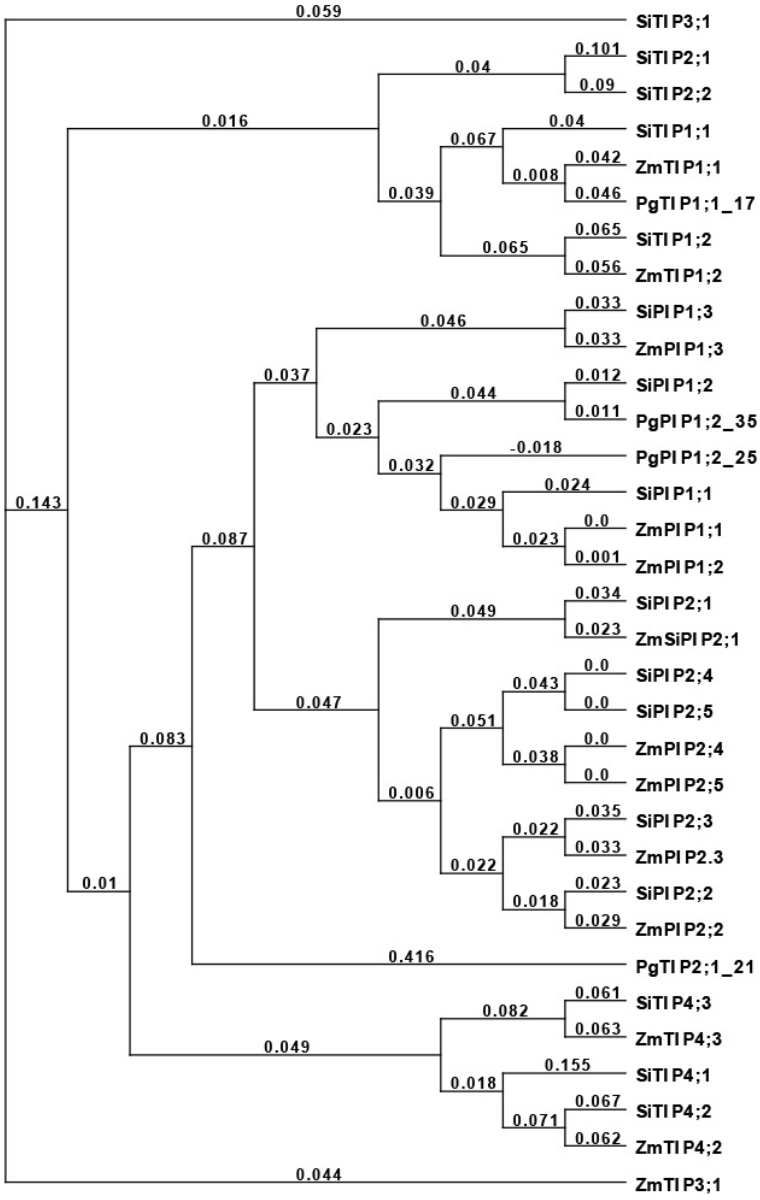

Figure 2. Phylogenetic analysis showing the relative closeness of PgAquaporin genes to Foxtail millet and Maize Aquaporin genes. This phylogenetic tree was drawn using the Clustal W program of MacVector. The numbers above the horizontal lines are proportional to the difference between the sequences.

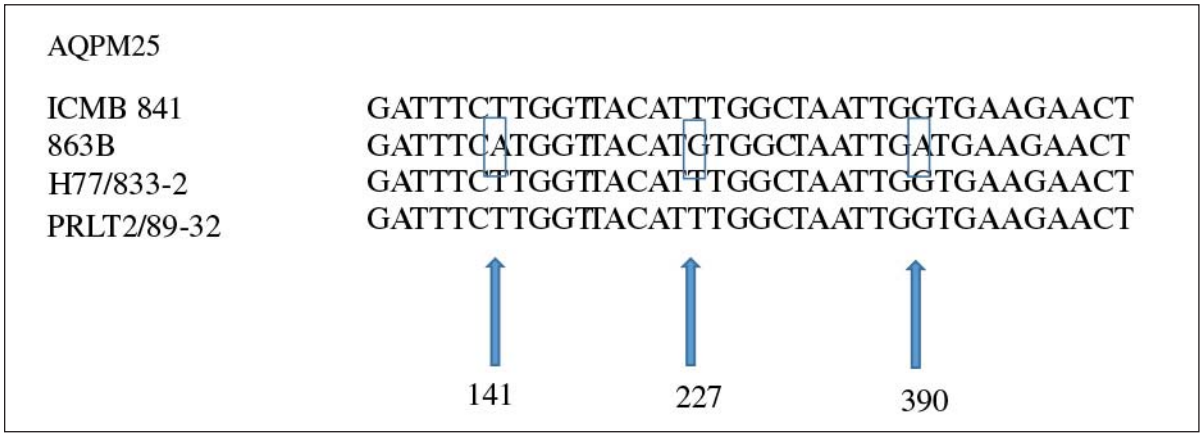

Figure 3. SNP sites identified with AQPM25 primer pair among four parents of mapping populations. Arrow indicates the position of the variant observed in the consensus sequence of 863B parent (tolerant).

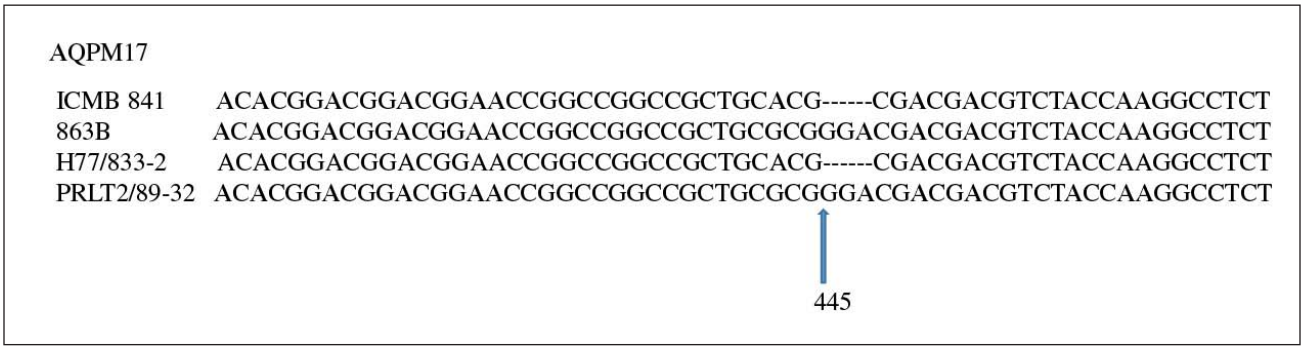

Figure 4. INDEL identified with AQPM17 primer pair among four parents of mapping populations. Arrow indicates the position of the indel (3 bp) in the consensus sequence observed in two sensitive genotypes, ie, ICMB 841 and H77/833-2.

Additional file: Sequences obtained from each primer PCR product of four genotypes and their consensus sequence derived after aligning the sequences of four genotypes

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