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ORIGINAL PAPER

Identifying novel QTLs for submergence tolerance in rice cultivars IR72 and Madabaru

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Abstract Short-term submergence is a recurring problem in many rice production areas. The *SUB1* gene, derived from the tolerant variety FR13A, has been transferred to a number of widely grown varieties, allowing them to withstand complete submergence for up to 2 weeks. However, in areas where longer-term submergence occurs, improved varieties having higher tolerance levels are needed. To search for novel quantitative trait loci (QTLs) from other donors, an $F_{2:3}$ population between IR72 and Madabaru, both moderately tolerant varieties, was investigated. After a repeated phenotyping of 466 families under submergence stress, a subset of 80 families selected from the two extreme phenotypic tails was used for the QTL analysis. Phenotypic data showed transgressive segregation, with several families having an even higher survival rate than the FR13A-derived tolerant check (IR40931). Four QTLs were identified on chromosomes 1, 2, 9, and 12; the largest QTL on chromosome 1 had a LOD score of 11.2 and R^2 of 52.3%. A QTL mapping to the *SUB1* region on chromosome 9, with a LOD score of 3.6 and R^2 of 18.6%, had the tolerant allele from Madabaru, while the other three QTLs had tolerant alleles from IR72. The identification of three non-*SUB1* QTLs from IR72 suggests that an alternative pathway may be present in this variety that is independent of the ethylene-dependent pathway mediated by the *SUB1A* gene. These novel QTLs can be combined with *SUB1* using marker assisted backcrossing in an effort to enhance the level of submergence tolerance for flood-prone areas.

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Introduction

Submergence is a widespread problem in rice-growing areas, especially in the flood-prone rainfed lowlands in South and Southeast Asia where at least 15 million ha of rice fields are affected. After a few days under complete submergence, most rice varieties will have depleted their energy reserves trying to reach the surface, leaving them unable to recover after the floods recede. Recently, this problem has escalated due to the impact of extreme weather events, such as unexpected heavy rains that inundate rice fields along the river banks and in low lying areas with poor drainage. In some areas, fields may remain flooded for a longer period of time leading to severe yield losses or total crop loss. More sustainable solutions are needed to overcome this increasing problem, and one of the best solutions is by providing tolerant varieties.

Several independent studies using the submergence tolerant *aus*-type variety FR13A have identified the major quantitative trait loci (QTL) *SUB1* and several small-effect loci (Nandi et al. 1997; Toojinda et al. 2003; Xu and Mackill 1996). *SUB1* has been fine mapped and cloned, and sequencing of the *SUB1* region in an FR13A-derived breeding line revealed a cluster of three ethylene

responsive factor (ERF) genes, i.e., *SUB1A*, *SUB1B*, and *SUB1C* (Xu et al. 2000; Xu et al. 2006). *SUB1B* and *SUB1C* were found in all indica and japonica accessions examined to date, while *SUB1A* has only been detected in a subset of indica and *O. rufipogon* accessions (Xu et al. 2006; Li et al. 2010). Furthermore, it has been confirmed that *SUB1A* is the key gene that confers a high degree of tolerance to complete submergence (Septiningsih et al. 2009; Singh et al. 2010; Xu et al. 2006). Under complete submergence, the presence of *SUB1A* in the submergence-tolerant genotype suppresses the perception and production of ethylene via its induction of the *Slender Rice-1* (*SLR1*) and *SLR Like-1* (*SLRL1*) genes (Fukao and Bailey-Serres 2008). In this scenario, high accumulation of SLR1 and SLRL1 transcripts and proteins lead to the inhibition of GA-mediated shoot elongation and conservation of energy. This conserved energy in turn helps the plant to survive and re-grow upon de-submergence. In the submergence-intolerant genotype, however, the accumulation of ethylene under complete submergence enhances GA responsiveness, shoot elongation, and carbohydrate consumption. Upon de-submergence, the plant dies running out of energy reserves (Fukao and Bailey-Serres 2008).

The *SUB1* locus has been introduced into six mega varieties through a marker-assisted backcrossing (MABC) strategy (Iftekharuddaula et al. 2011; Neeraja et al. 2007; Septiningsih et al. 2009). Multi-location field evaluations of these Sub1 varieties have shown that the *SUB1* locus is effective in different genetic backgrounds and environments (Sarkar et al. 2009; Septiningsih et al. 2009; Singh et al. 2009). Since the genetic background of the recipient variety is restored by repeated backcrossing, the *Sub1* varieties are nearly identical to the original parent without *SUB1* except that they are tolerant of submergence. All desirable agronomic traits and grain quality of the original variety therefore remain unchanged, which facilitates rapid adoption by farmers. Several of these lines, i.e., Swarna-Sub1, IR64-Sub1, and BR11-Sub1, have been released in Asian countries, such as India, Philippines, Indonesia, and Bangladesh, and the others are under evaluation. Recently, the *SUB1* locus has also been introgressed into popular varieties such as Ciherang from Indonesia and PSB Rc18 from the Philippines, at the International Rice Research Institute (IRRI) in close collaboration with partners in National Agricultural Research and Extension Systems (NARES). The MABC technology in conjunction with a reliable phenotyping system now enables NARES to develop additional local Sub1 varieties in their national breeding programs.

Although it was demonstrated that the Sub1 lines are significantly more tolerant compared to the original parents and have been an excellent solution for many submergence-prone regions, the level of tolerance varied among the improved lines (Septiningsih et al. 2009), indicating the effect of modifying genes that may interact with the *SUB1A*-mediated pathway. Enhanced tolerance is needed, especially in areas where prolonged or severe flooding is encountered. The current level of tolerance in the Sub1 lines is not sufficient in conditions where flooding lasts for more than 2 weeks. Likewise, submergence tolerance as conferred by *SUB1* is reduced when the water is turbid or warm. Under such conditions plants survive for less than 2 weeks.

Efforts have, therefore, been initiated to screen for novel sources of germplasm that are tolerant or moderately tolerant to submergence stress (IRRI unpublished; Singh et al. 2010). From the conducted submergence screening, the improved variety IR72 and Madabaru, a landrace from Sri Lanka, have been identified as moderately tolerant of submergence. Using these two parents, a QTL-mapping population was developed to facilitate the identification of novel QTLs that are non-allelic to the tolerant FR13A *SUB1* allele and can be used to develop rice varieties with higher tolerance of severe submergence stress.

Materials and methods

Plant material and population development

A cross was made between the indica rice cultivars IR72 and Madabaru (IRGC 15333), and the F_1 individual was confirmed to be true hybrid using SSR markers. F_2 seeds were collected from the SSR marker-validated F_1 plant, and a total of 466 F_2 plants were used to develop 466 $F_{2:3}$ families for screening under submergence stress. For molecular analysis of the *SUB1A* gene, Thadokkham 1 (TDK1), a susceptible check variety (IR42), and the FR13A-derived tolerant breeding line IR40931 were used in addition to IR72 and Madabaru.

Submergence screening for QTL mapping and RNA sampling

For the 466 $F_{2:3}$ families, two rounds of screenings were conducted. Fifty seeds per line were directly sown in the soil at the bottom of a submergence tank located inside a glasshouse. After 14 days, the tank was filled with 1.2–1.5 m tap water until susceptible IR42 plants showed severe stress symptoms (14 days of complete submergence). From this initial screening (data not shown), 80 families were selected representing the two tails of the phenotypic data, i.e., high and low survival under submergence, and were screened a second time for more reliable scores. The smaller number of families allows more precise screening inside a smaller outdoor concrete tank using seedling trays. Pre-germinated seeds of the 80

families were transferred to plastic trays (54 × 38.5 × 9.6 cm) filled with 5 cm of soil that was previously sifted using 0.3 cm mesh wire screen. Each tray accommodated 10 entries with 30 plants each. The parental lines IR72 and Madabaru, as well as the susceptible (IR42) and tolerant check (IR40931) were included as controls. An Alpha Plus design was used to enable the randomization of all entries, including the controls, and two out of the four controls were used in each tray. In total, 10 trays were used per replication, and this experiment was conducted with two replications. The plants were submerged 2 weeks after planting and de-submerged after a majority of the IR42 plants died (14 days of complete submergence). Plant survival data were collected at 21 days after de-submergence (Suppl. Fig. S1). Phenotype data collected from these 80 families were then used in the QTL analysis.

For *SUB1A* gene expression analysis by RT-PCR, pre-germinated seedlings from the five varieties (IR72, Madabaru, TDK1, IR42, and IR40931) were grown for 14 days in a seedling tray, and then completely submerged for 30 h in a cylindrical tank filled with tap water. From each variety, four plants were cut at the leaf base and samples were immediately frozen in liquid nitrogen and stored in a –80°C freezer until extraction of the RNA. Non-submerged control plants were sampled in parallel.

Linkage map construction and QTL mapping

Leaf samples were collected from a bulk of 20 seedlings per family from the 80 $F_{2:3}$ families, and genomic DNA was extracted according to Zheng et al. (1995). Genotyping was performed using SSR and insertion–deletion (indel) markers and 1,093 markers were tested for parental survey. Primer3 was used to design indel primers (http://frodo.wi.mit.edu/primer3/), BLAST search was used to find regions of similarity (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi), and sequence alignments were performed using CLUSTALW (http://www.genome.jp/tools/clustalw/). Nineteen new indel markers were developed. The PCR reaction was performed in a G-Storm GS1 thermal cycler (G-Storm Ltd., UK) in a total volume of 10 µl with 25 ng genomic DNA, 5 µM of each SSR primer, 200 µM dNTP mix, 1× PCR buffer (containing 50 mM KCl, 10 mM TRIS-Cl, pH 8.3, 3 mM MgCl), and 1 U of taq polymerase. The PCR profile was performed by initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 10 s, annealing at 55 or 60°C (depending on primer sequence) for 10 s, and extension at 72°C for 10 s, and final extension at 72°C for 2 min. PCR was performed on 96-well plates, the DNA fragments were separated on 8% acrylamide gels (C.B.S. Scientific, USA), and stained with SYBR-Safe (Invitrogen) for manual allele scoring. A parental survey was conducted to identify polymorphic SSR markers between the two parents. The markers that are polymorphic and relatively easy to score were used in the construction of the linkage map to cover the entire genome. The linkage map was constructed using Map Manager QTX, vQTXb20 (Manly et al. 2001).

The phenotypic and genotypic data were analyzed by interval mapping (IM) and composite interval mapping (CIM) using QGene v4.3.6 (Nelson 1997). Permutations of 10,000 iterations were used to determine the threshold of the QTLs in Qgene. Subsequently, the LOD values at $p \leq 0.05$ were used as the threshold to declare the significance of the QTLs. For comparison, data were also analyzed with QTL Cartographer v2.5 (Wang et al. 2010) using both methods and 1,000 permutations. Digenic interaction was analyzed using QTLNetwork-v2.1 (Yang and Zhu 2005; Yang et al. 2007; Yang et al. 2008). QTL names were designated following the standard rice QTL nomenclature (McCouch 2008).

SUB1A allele identification and sequencing analysis

The *SUB1A*-specific CAPS marker GnS2 (Neeraja et al. 2007) was used to determine the different *SUB1A* alleles in the varieties included in this study. DNA sequencing was performed to identify polymorphisms in the Madabaru *SUB1A* gene, including its promoter region. DNA from 1-week-old seedlings was extracted using the protocol followed by Pallotta et al. (2000). PCR was performed in a total volume of 25 µl with 100 ng genomic DNA, 1 U Pfx polymerase (Invitrogen, USA), 1× Pfx buffer, 50 mM $MgCl_2$, 5% DMSO, 2.5 mM each dNTP, and 10 µM of each, forward and reverse primers. Genomic DNA was denaturated at 94°C for 3 min, followed by 40 cycles with 94°C for 30 s, 58°C for 30 s, and 72°C for 3 min, and a final extension at 72°C for 10 min. Primers used for amplification of the 3 kb *SUB1A* promoter region were: OutRepFor388 5′-GAACGTATTGTTGTGCCTATTGC-3′ and Sub1APRev1 5′-TTCTGTCAACCCATGTCTCG-3′ and for the *SUB1A* genic region: Sub1A_NS_F 5′-CCCAGCC ATCAAGAAAAATCACATT-3′ and Sub1A_NS_R 5′-AT TATGCCTGCAACAACACGAAGTG-3′. For sequencing Madabaru and IR42, PCR amplicons were excised from 1% agarose gels and purified using the QIAquick gel extraction kit (Qiagen). Sequencing was done at Macrogen (Seoul, South Korea) and sequences were analyzed using CLUSTALW (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The sequences for IR40931 and Teqing were available from Xu et al. (2006).

RNA isolation and expression analysis

Total RNA was extracted from leaves derived from submerged and non-submerged seedlings (see above) using

Trizol according to the manufacturer's instructions (Sigma, Germany). DNA contaminations were removed with RNase-free DNase (Promega) and quality of the RNA dissolved in 100 µl DEPC-treated water was assessed by agarose gel electrophoresis and spectrophotometry. First strand cDNA was synthesized using SuperScript-II Reverse Transcriptase (Invitrogen) in a 20 µl reaction volume containing 50 µM Oligo$(dT)_{15}$ primers, 10 mM each dNTP, and 4 µg of total RNA, according to the manufacturer's instructions. The cDNA was diluted by adding 100 µl of water and 5 µl were used as a template for PCR amplification in 20 µl containing 1× PCR buffer, 2.0 mM $MgCl_2$, 10 mM dNTPs, 5% DMSO, 2 U iMAX Taq DNA polymerase (iNtRON BIOTECHNOLOGY, Korea), and 0.2 µM each forward and reverse primers. PCR was carried out in a G-Storm thermal cycler (94°C for 3 min; 25–30 cycles: 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, followed by a final extension at 72°C for 10 min). GnS2 primers were used for the expression analysis of *SUB1A* (Neeraja et al. 2007). Actin was used as control (for 5′-acaggtattgtgttggactc-3′; rev 5′-gcttagcat tcttgggtcc-3′).

Results

Phenotyping of the mapping population

Among the selected 80 $F_{2:3}$ families that were rescreened under submergence stress, the lowest rate of plant survival was 1.7%, while the highest was 94.2% (Fig. 1). The survival rates of the susceptible check IR42 and the tolerant check IR40931 were 2.7 and 82.9%, respectively. For the two parents of the mapping population, survival rates were 53.6% for IR72 and 33.9% for Madabaru (Fig. 1). The data showed that 29 out of the 80 families (36.3%) had a lower survival rate than Madabaru, while 26 (32.5%) had a survival rate that was higher than IR72. Four families (5%) had an even higher percentage of survival than the tolerant check IR40931 (Fig. 1). These data indicate transgressive segregation within the mapping population.

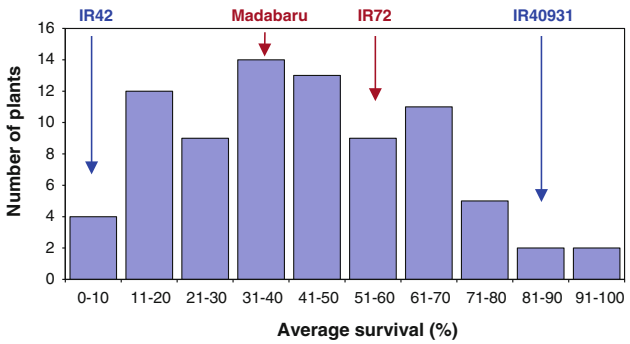

Fig. 1 Phenotypic evaluation of the mapping population under submergence stress. The phenotypic distribution of the 80 $F_{2:3}$ families shown with the average survival of the parents, i.e., Madabaru and IR72, and the check varieties, i.e., IR42 and IR40931, as the susceptible and the tolerant controls, respectively

Construction of the linkage map and QTL identification

A parental survey of IR72 and Madabaru was performed using 1,074 SSR and 19 indel markers, and out of these only 115 (10.5%) were polymorphic and reliable, and in this case only one indel marker was polymorphic, i.e., MDC17 (F: GTGCCTATTGCCTACTCACTC; R: CAAT TACAGTGATAGTGTAAGGCTG). These selected markers were then used to genotype the mapping population for construction of the linkage map. The map was constructed using MapManager QTX vQTXb20 (Manly et al. 2001) and had a total length of 1,617.3 cM with an average of 15.7 cM distance between markers (Fig. 2).

Based on 10,000 permutations in Qgene (Nelson 1997), LOD scores of 3.66 and 4.45 for IM and 3.91 and 4.86 for CIM were defined at the significance level $p \leq 0.05$ and $p \leq 0.01$. Likewise, in QTL Cartographer v2.5 (Wang et al. 2010) with 1,000 permutations, the LOD scores were 3.68 ($p \leq 0.05$) and 4.32 ($p \leq 0.01$) for IM, and 3.62 ($p \leq 0.05$) and 4.45 ($p \leq 0.01$) for CIM. From this analysis, four significant QTLs were identified. The region with the largest effect is located on chromosome 1 and was detected by both QGene and QTL Cartographer analysis. This QTL was denoted as *qSUB1.1* and is derived from IR72. The maximum LOD score of *qSUB1.1* was 11.2 with an R^2 of 52.3% (Table 1). QTLNetwork also showed high degree of heritability for this major QTL, e.g., 0.43 and this QTL was mostly additive. Two additional QTLs derived from IR72 were identified that are located on chromosome 2 and 12. The QTL *qSUB2.1* was detected by QTL Cartographer and QGene IM with a maximum LOD score of 4.8 and an R^2 of 19.4%. The QTL *qSUB12.1* was detected by QGene with a LOD score of 4.2 and an R^2 of 21.5% (Table 1). One significant QTL derived from Madabaru, *qSUB9.1*, was identified by QTL Cartographer CIM only, with LOD score of 3.6 and an R^2 of 7.3%. This QTL was also detected by QGene IM with LOD score of 3.6 and an R^2 of 18.6%, but just slightly below the threshold ($p \leq 0.05$; LOD of 3.66). This QTL mapped to the same region as the FR13A-derived *SUB1* locus (Fig. 2). There were no digenic interactions detected by QTLNetwork.

Allele assessment, expression, and sequencing analysis

To assess if the tolerant *SUB1A* allele is present in Madabaru, the gene was amplified with a gene-specific CAPS marker (GnS2) and digested with the *Alu*I restriction

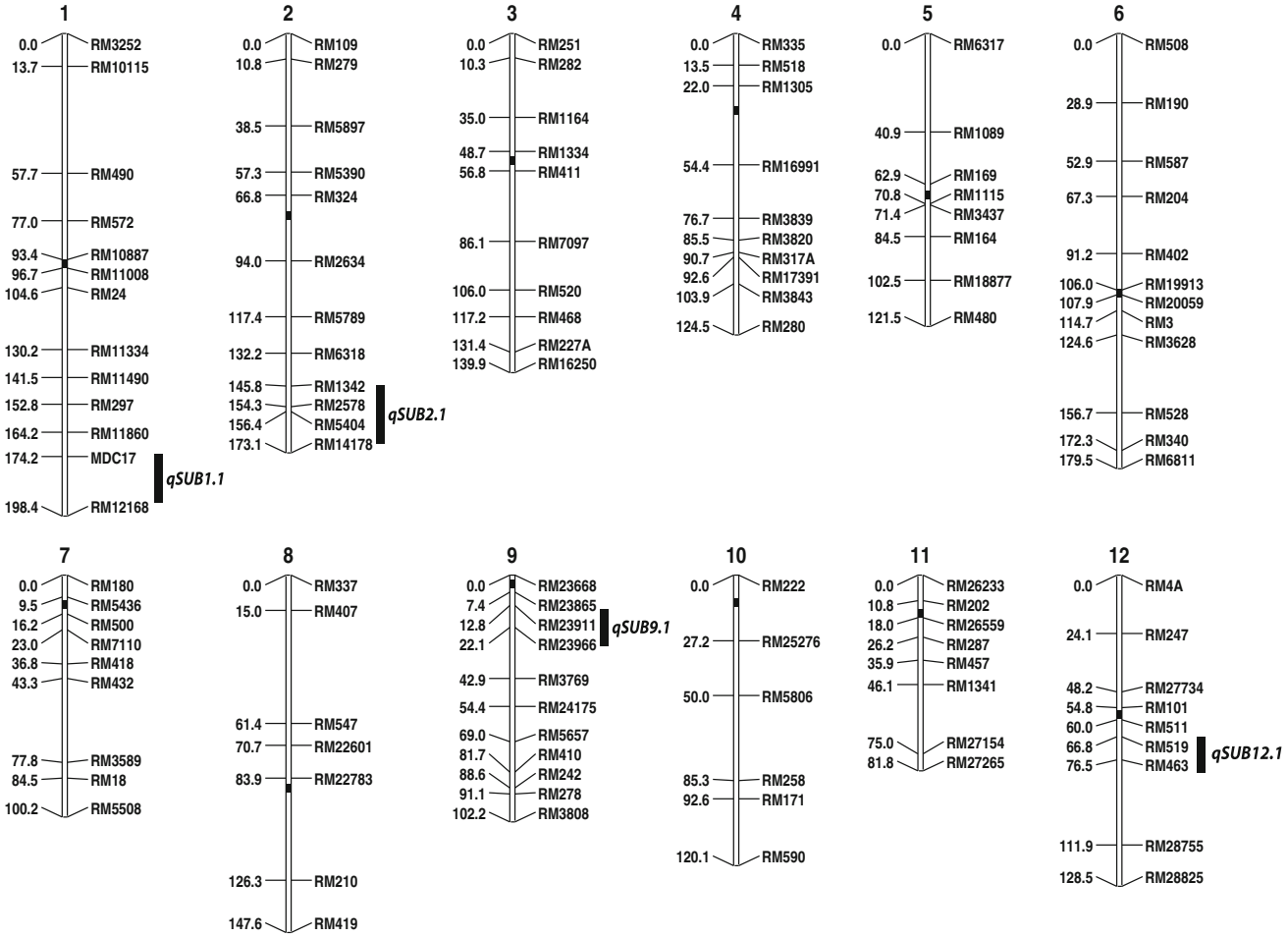

Fig. 2 Mapping of submergence-tolerant QTLs derived from IR72 and Madabaru. Molecular linkage map of an IR72 × Madabaru mapping population constructed with 115 SSR markers. The position of four significant submergence-tolerance QTLs on chromosomes 1, 2, 9, and 12 are illustrated by *black bars* next to the chromosomes. Centromeres are shown as *black boxes*

Table 1 QTLs for submergence tolerance identified from the IR72/Madabaru population

QTL	Chr.	Flanking markers	Source	QGene IM			QGene CIM			QTL Cart. IM			QTL Cart. CIM		
				LOD	R^2 (%)	Add	LOD	R^2 (%)	Add	LOD	R^2 (%)	Add	LOD	R^2 (%)	Add
qSUB1.1	1	MDC17-RM12168	IR72	**9.4**[a]	**41.9**	**30.5**	**9.2**	**40.9**	**28.5**	**11.2**	**52.3**	**24.5**	**11.1**	**37.7**	**21.2**
qSUB2.1	2	RM6318-RM2578	IR72	3.8[b]	19.6	1.2	*3.2*[c]	*16.6*	*2.1*	4.1	36.4	19.3	**4.8**	**19.4**	**15.8**
qSUB9.1	9	RM23911-RM23966	Madabaru	*3.6*	*18.6*	*13.7*	*3.3*	*17.4*	*12.7*	*3.4*	*17.1*	*12.2*	3.6	7.3	8.1
qSUB12.1	12	RM511-RM463	IR72	4.2	21.5	0.0	4.2	21.4	5.3	*3.5*	*16.3*	*13.0*	–	–	–

[a] QTLs in bold face were identified above the $p = 0.01$ threshold using permutation analysis

[b] QTLs in regular type were identified above the $p = 0.05$ threshold

[c] QTLs in italics were identified above LOD = 3.0, but below the $p = 0.05$ threshold

enzyme to differentiate between the tolerant *SUB1A-1* allele present in FR13A and the *SUB1A-2* allele, which is generally found in intolerant accessions (Neeraja et al. 2007; Xu et al. 2006). Our amplification data showed that the *SUB1A* gene is present in all analyzed genotypes. Digestion of the PCR products with *Alu*I revealed that both IR72 and Madabaru possess the *SUB1A-2* allele, which is also present in the susceptible check IR42 (Fig. 3).

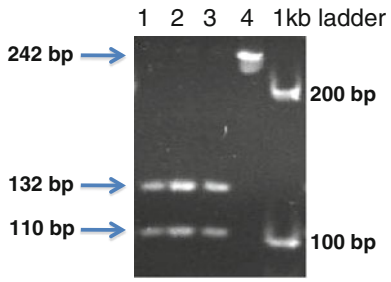

Fig. 3 Assessment of the *SUB1A* allele in IR72 and Madabaru. Four varieties were run with the CAPS marker GnS2: *Lane 1* = IR72, *Lane 2* = Madabaru, *Lane 3* = IR42, and *Lane 4* = IR40931. IR40931 shows the "tolerant" *SUB1A-1* allele (undigested), while the other three show the digested "susceptible" *SUB1A-2* allele, as previously defined (Xu et al. 2006; Neeraja et al. 2007)

Recent data published by Singh et al. (2010) had suggested that high expression of the *SUB1A-2* allele might confer some submergence tolerance. To address if this might be the case for Madabaru, we have conducted an RT-PCR gene-expression analysis. Under non-submerged conditions, the data showed little or no expression of the *SUB1A* gene in any of the analyzed accessions, including the tolerant check IR40931 (*SUB1A-1* allele) and intolerant check IR42 (*SUB1A-2* allele) (Fig. 4, left panel). After plants were submerged for 30 h, *SUB1A* expression was highest in IR40931 and lowest in IR42 (Fig. 4, right panel). In IR72, no *SUB1A-2* expression was detected. In contrast, the *SUB1A-2* allele was highly expressed in Madabaru (Fig. 4).

To assess if a specific *SUB1A-2* allele is present in Madabaru, we have sequenced the *SUB1A* gene and the corresponding upstream region (−1,984 bp) and compared it to the published sequences of IR40931, IR42, and Teqing. The data revealed a total of 12 single nucleotide polymorphism (SNPs) within the analyzed upstream region, as well as 5 SNPs in the coding and non-coding region of *SUB1A*. The *SUB1A-2* alleles of Madabaru and the intolerant variety IR42 were identical, while Teqing contained a SNP at position −1,006 bp upstream of the start codon (SNP^{-1006}) compared to the other varieties, i.e.,

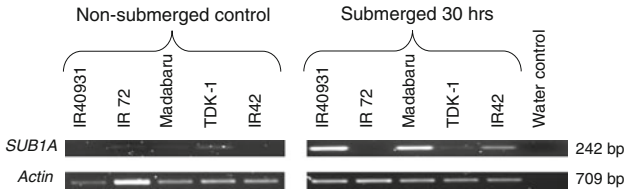

Fig. 4 Assessment of *SUB1A* gene expression in IR72 and Madabaru. RT-PCR analysis of *SUB1A* gene expression was conducted with the varieties indicated (*top panel*). RNA was extracted from leaf samples derived from 15-day-old seedlings submerged for 30 h and non-submerged controls. *Actin* was used as a control (*bottom panel*)

A versus G, respectively (Suppl. Fig. S2). However, both the tolerant variety IR40931 and the susceptible check IR42 have the same nucleotide as Madabaru in this position. The fact that IR42 and Madabaru share identical sequences at this locus suggests that the functional nucleotide polymorphism (FNP) controlling the expression of the *SUB1A* gene in Madabaru is outside of the gene itself.

Discussion

The availability of molecular markers specific for the *SUB1* locus and the *SUB1A* submergence-tolerance gene has facilitated the marker-assisted development of a range of mega varieties tolerant of submergence (Iftekharuddaula et al. 2011; Neeraja et al. 2007; Septiningsih et al. 2009). These Sub1 varieties have been extensively tested and some, such as Swarna-Sub1, IR64-Sub1, and BR11-Sub1, have already been released in several countries in South Asia and Southeast Asia. The deployment of these varieties has become an excellent solution for a large proportion of the submergence-prone target regions. However, further improvement of the Sub1 lines is still needed, in this instance especially for areas where prolonged or severe submergence stress is encountered due to heavy rain fall or vulnerable geographical positions (Septiningsih et al. 2009). In our study, we have therefore tried to identify novel QTLs that have substantial contribution to submergence tolerance and act additively with *SUB1* that will be desirable to enhance the level of tolerance provided by the *SUB1* gene.

After screening 466 $F_{2:3}$ families derived from IR72 and Madabaru, we selected 80 with extreme phenotypes to rescreen for more precise phenotyping for this QTL study. The results of the rescreening show that the data from both tails are consistent compared to the screening performed in the original population; however, the phenotyping performed in the smaller set produced a broader range of data within each category compared to the screening of the original population. This phenomenon could be explained by the different type of submergence tanks that were used in those two populations. The big tank used for the original population was located inside a glasshouse and the seeds were directly sown in the soil and then submerged after plants were 2 weeks old. The microclimate in the glasshouse, such as hotter temperatures and variable shade conditions, could potentially influence the expression of the phenotype. In contrast, the subsequent phenotyping on the smaller set was performed in a concrete tank located outdoors. Seeds were pre-germinated in petridishes and then transplanted into trays filled with 5 cm of sifted soil. This allowed for more uniform seedling development for all families before submergence, producing more accurate results for the QTL study.

The phenotyping of the mapping population revealed transgressive segregation since about one-third of the population in each tail showed a higher percent survival than IR72 or lower than Madabaru and intolerant check IR42, respectively. Interestingly, several families exhibited a higher submergence tolerance than the tolerant check IR40931 (Fig. 1). Several other studies have reported transgressive segregation, for instance, QTL studies on yield and yield components using populations derived from *O. sativa* and *O. rufipogon* (McCouch et al. 2007; Septiningsih et al. 2003; Thomson et al. 2003). This suggests that beneficial QTLs can come from the less tolerance parent Madabaru although it is expected that submergence-tolerant QTLs will be mostly detected from the more tolerant parent, in our case IR72.

The results of the QTL analysis revealed four QTLs located on the long arm of chromosome 1, 2, 9, and 12 (Fig. 2). The tolerant alleles in three out of the four QTLs were derived from IR72. The origin of these QTLs is currently not known since there are approximately 20 landraces in the pedigree of IR72 that may have contributed to submergence tolerance (Khush and Virk 2005). The only QTL derived from Madabaru mapped to the vicinity of the *Sub1* region on chromosome 9 (Table 1). It is, therefore, possible that this region represents the *SUB1A* major tolerance gene. In this context, it is important to note that both IR72 and Madabaru possess the same allele for the *SUB1A* gene, i.e., the intolerant *SUB1A-2* allele (Fig. 3), but there was no significant QTL mapped within this region in IR72.

To further address whether *SUB1A* plays a role in the submergence tolerance QTL detected from Madabaru, a gene expression analysis was performed. As expected, the *SUB1A* gene was highly expressed in the tolerant check IR40931 (*SUB1A-1* allele) after 30 h submergence and expressed at a low level in the intolerant check IR42 (*SUB1A-2* allele). In contrast, expression of the *SUB1A-2* allele was high in Madabaru with transcript abundance only slightly lower than in IR40931 (Fig. 4). This expression data suggests that, indeed *SUB1A* was the gene that contributes to the tolerance coming from Madabaru, despite its "susceptible allele." Likewise, recent data published by Singh et al. (2010) had shown that the susceptible *SUB1A-2* allele in the variety James Wee was highly expressed under submergence. This variety was also moderately tolerant to submergence. The RT-PCR results also showed that, even though IR72 was more tolerant to complete submergence than Madabaru, we were unable to detect *SUB1A-2* expression in this variety. This finding corresponds well with our data that failed to detect a QTL in the *Sub1* region in IR72. These findings support conclusions drawn from a study by Singh et al. (2010) that it is important to assess the level of *SUB1A* expression in addition to the determination of allelic differences.

To address whether FNPs may explain the effect of the QTL in Madabaru, the *SUB1A* gene was sequenced. The *SUB1A-2* alleles of Madabaru and the intolerant variety IR42 were identical, while Teqing contained a SNP at position −1,006 bp (SNP^{-1006}) compared to the other varieties (Suppl. Fig. S2). However, both the tolerant variety IR40931 and the susceptible check IR42 have the same nucleotide as Madabaru in this position. Therefore, it seems that the functional region underlying the Madabaru QTL was not positioned in this sequenced-*SUB1A* region. One potential explanation for this finding is that there could be an upstream enhancer regulating the *SUB1A* gene as found for the maize domestication gene, *tb1* (Clark et al. 2006). Further investigation is needed to identify the real FNPs underlying the QTL.

In contrast to the Madabaru QTL, none of the QTLs derived from IR72 have previously been reported and thus can be categorized as novel QTLs. It has been well documented that submergence tolerance in lowland rice is a *SUB1A*-dependent pathway mainly through its suppression of ethylene production and perception (Fukao and Bailey-Serres 2008). The three identified IR72 QTLs are located on different chromosomes and therefore clearly distinct from the *SUB1* locus on chromosome 9. A *Sub1*-independent tolerance mechanism is further indicated by the absence of *SUB1A-2* gene expression in IR72 under submergence. It will be interesting to investigate if distinct or *Sub1*-complementary pathways exist in IR72. For breeding applications, it will be critically important to determine if these QTLs act additively to *SUB1* and therefore have the potential to enhance submergence tolerance beyond what is currently achieved with *SUB1*. The observed transgressive segregation provides first evidence that a certain combination of QTLs can confer a level of submergence tolerance exceeding that of the tolerant check IR40931. Our study also showed that there were no QTL or gene interactions detected by QTLNetwork software; therefore, these QTLs can act independently. Moreover, the major QTL *qSUB1.1* has high heritability (h^2 of 0.43) and can act additively, which offers a valuable target for marker assisted breeding to be pyramided with the *SUB1* gene.

Despite more than a thousand markers covering the whole genome being tested, only a small number of markers were polymorphic between the two parents. Efforts were made to add markers in the QTL regions, however, these regions were highly monomorphic. For example, in the QTL region of *qSUB1.1*, 56 SSR and 19 new indel markers positioned between the two flanking markers, RM11860 and RM12168 were tested, but only one indel marker was polymorphic, i.e., MDC17. The distance between MDC17 and RM12168, where the *qSUB1.1* QTL is located, is still 24.2 cM, however, based on the Nipponbare sequence this region is only 2.68 Mb.

Development of near isogenic lines for fine mapping of the largest effect QTL *qSUB1.1* has now been initiated, and SNP markers will be explored to saturate the QTL region. Flanking markers of the delineated region can then be used to pyramid this QTL into *Sub1*-varieties. Once the gene(s) underlying the IR72 QTLs are identified, further investigations can be pursued to unravel the molecular mechanisms of submergence tolerance in rice, and gene-based markers can be developed to facilitate more precise marker-assisted breeding. The development of rice varieties with a higher level of submergence tolerance will provide improved solutions to farmers in regions affected by long-duration and severe floods and thus will help increase rice production in flood-prone regions.

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