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ORIGINAL PAPER

Molecular marker survey and expression analyses of the rice submergence-tolerance gene *SUB1A*

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Abstract The major rice quantitative-trait locus *Submergence 1* (*Sub1*) confers tolerance of submergence for about 2 weeks. To identify novel sources of tolerance, we have conducted a germplasm survey with allele-specific markers targeting *SUB1A* and *SUB1C*, two of the three transcription-factor genes within the *Sub1* locus. The objective was to identify tolerant genotypes without the *SUB1A* gene or with the intolerant *SUB1A-2* allele. The survey revealed that all tolerant genotypes possessed the tolerant *Sub1* haplotype (*SUB1A-1*/*SUB1C-1*), whereas all accessions without the *SUB1A* gene were intolerant. Only the variety James Wee with the *SUB1A-2* allele was moderately tolerant. However, some intolerant genotypes with the *SUB1A-1* allele were identified and RT–PCR analyses were conducted to compare gene expression in tolerant and intolerant accessions. Initial analyses of leaf samples failed to reveal a clear association of *SUB1A* transcript abundance and tolerance. Temporal and spatial gene expression analyses subsequently showed that *SUB1A* expression in nodes and internodes associated best with tolerance across representative genotypes. In James Wee, transcript abundance was high in all tissues, suggesting that some level of tolerance might be conferred by high expression of the *SUB1A-2* allele. To further assess tissue-specific expression, we have expressed the GUS reporter gene under the control of the *SUB1A-1* promoter. The data revealed highly specific GUS expression at the base of the leaf sheath and in the leaf collar region. Specific expression in the growing part of rice leaves is well in agreement with the role of *SUB1A* in suppressing leaf elongation under submergence.

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Introduction

Rice is the most important food crop and the primary source of calories for more than half of the world's population. Approximately one-third of the rice-growing area is classified as rainfed lowland, and a large proportion of it is prone to seasonal flooding (FAO 2003). Floods caused by heavy rains can occur at any growth stage and affect an estimated 10–15 million ha each year in South and Southeast Asia, causing an annual economic loss of approximately US$1 billion (Dey and Upadhyaya 1996). In eastern India, submergence was identified as the third most important limitation to rice production in rainfed lowlands (Widawsky and O'Toole 1990). Global climate change will further

aggravate the situation due to more extreme weather events and rising sea level (Bates et al. 2008).

Rice varieties with tolerance of submergence, for example, Flood Resistance 13A (FR13A), were identified more than 25 years ago (Mazaredo and Vergara 1982) and conventional breeding approaches have since been used to develop submergence-tolerant varieties with improved agronomic performance. However, these varieties were never widely adopted by farmers and rice consumers because of the presence of undesirable agronomic traits and poor grain quality (Septiningsih et al. 2009). A recent breakthrough in the development of submergence-tolerant varieties was facilitated by the application of a novel breeding approach (Collard and Mackill 2008) based on the fine mapping and sequencing of *Submergence 1* (*Sub1*), a major quantitative trait locus (QTL) located on rice chromosome 9 that confers tolerance of complete submergence for about 14 d (Xu and Mackill 1996; Nandi et al. 1997; Toojinda et al. 2003; Septiningsih et al. 2009). Within the sequenced *Sub1* locus of an FR13A-derived tolerant breeding line, three putative ethylene-responsive transcription factor (ERF) genes were identified and designated *SUB1A-1*, *SUB1B-1,* and *SUB1C-1* (Xu et al. 2006). The *SUB1A* gene is absent from the Nipponbare reference genome and all other analyzed *Oryza sativa japonica* varieties due to an inversion and deletion. In intolerant *O. sativa indica* varieties, the *SUB1A* gene is either absent or present as the allelic variant *SUB1A-2*. Single nucleotide polymorphisms (SNPs) were also identified for the *SUB1C* gene, distinguishing the tolerant *SUB1C-1* allele from those found in intolerant varieties (*SUB1C-2*–*SUB1C*-7). In contrast, no tolerant-specific allele for *SUB1B* was identified (Xu et al. 2006). Gene expression analyses showed that all three ERF genes were induced under submergence and revealed a specifically high *SUB1A* and low *SUB1C* expression in tolerant varieties. *SUB1A* had been subsequently identified as the major determinant of tolerance by a transgenic approach showing that constitutive expression of the *SUB1A-1* allele conferred tolerance of submergence to an intolerant variety (Liaogeng) that naturally lacks the *SUB1A* gene (Xu et al. 2006). Recent data derived from breeding lines with genetic recombination within the *Sub1* locus furthermore showed that plants carrying the *SUB1A-1* allele were submergence tolerant whereas plants with the *SUB1A-2* allele in combination with both, tolerant and intolerant *SUB1C* alleles were intolerant (Septiningsih et al. 2009).

Whereas intolerant rice varieties and deepwater rice show rapid growth under submerged conditions (escape response), the *Sub1* locus confers tolerance by suppressing plant elongation under submergence. In contrast to intolerant plants that deplete their starch reserves within about 3 days, *Sub1*-mediated suppression of growth preserves energy and carbohydrates that remain available for recovery once the water recedes (Fukao et al. 2006). In addition, higher activities of enzymes of the ethanolic-fermentation pathway (PDC and ADH) were detected in rice plants that possess the tolerant *Sub1* locus, which might further improve the energy status (Fukao et al. 2006; Ella et al. 2003; Das et al. 2005; Saika et al. 2007; for review see Fukao and Bailey-Serres 2007). Recent data by Fukao and Bailey-Serres 2008 revealed insight into the hormonal control of submergence tolerance. According to their model, the concentration of ethylene increases under submerged conditions, whereas the concentration of abscisic acid (ABA) decreases. In intolerant plants, this triggers an increase in gibberellic acid (GA) and the induction of cell elongation. In tolerant plants, the ethylene-induced increase in *SUB1A* expression decreases GA responsiveness inhibits the accumulation of GA by increasing the accumulation of the GA-signaling suppressors repressors *Slender Rice-1* (*SLR1*) and *SLR1 Like 1* (*SLRL1*).

Breeders are now using the *Sub1* locus to develop tolerant rice varieties for submergence-prone areas in Asia and Africa (Septiningsih et al. 2009). Based on detailed sequence information, allele-specific molecular markers for *SUB1A* and *SUB1C* were developed that are used for marker-assisted backcrossing (MABC) of the *Sub1* locus into widely grown, well-adapted rice varieties (Neeraja et al. 2007; Septiningsih et al. 2009). Analysis of different *Sub1*-introgression lines showed that *Sub1* acts largely independent of the genetic background and environment (Singh et al. 2009). Importantly, *Sub1*-introgression lines do not show the stunted growth observed in transgenic plants overexpressing the *SUB1A-1* gene (Xu et al. 2006).

Despite the large effect of *Sub1*, surveys in countries affected by floods showed the need to further enhance tolerance since rice fields often remain flooded for more than 14 days. We, therefore, conducted a germplasm screening with *SUB1A* and *SUB1C* allele-specific molecular markers in order to identify novel sources of submergence tolerance, i.e., tolerant rice varieties that lack the *SUB1A* gene or possess the intolerant *Sub1* haplotype.

Whereas most tolerant accessions possessed the tolerant *Sub1* haplotype, across all genotypes several exceptions were found which prompted us to conduct detailed gene-expression analyses. These analyses revealed interesting details on the tissue-specific expression of *SUB1A*.

Materials and methods

Plant material and submergence screening

Seeds of the 76 rice accessions used for the germplasm survey were derived from the International Rice Germplasm Collection (IRGC) at IRRI. Details on the accessions are

Table 1 *Sub1* haplotype and submergence tolerance of representative rice varieties

Variety name	IRGC acc. no.	Country of origin	Plant survival (SE) (%)	*SUB1A* SNP1	*SUB1A* SNP2	*SUB1C*
Bentobala	26948	Africa	0.0	–	–	C6/7
Besewar	26921	Philippines	3.3 (6.7)	–	–	C2/5
Daily	73193	Vietnam	9.5 (8.7)	–	–	C6/7
Maesawo	75040	Thailand	5.9 (6.8)	–	–	C6/7
Swarna	2027429	India	3.5 (2.4)	–	–	C6/7
FR43B	6143	India	55.0 (7.5)	A1	A1	C1
Gadar Uzarka	58975	Nepal	2.9 (3.7)	A1	A1	C1
Goda Heenati	15419	Sri Lanka	50.9 (12.3)	A1	A1	C1
Heenkarayal	36437	Sri Lanka	54.7 (7.3)	A1	A1	C1
Hindik Wee	36253	Sri Lanka	62.8 (10.9)	A1	A1	C1
IR40931-33-1-3-2	IR07F102	Philippines	65.4 (7.9)	A1	A1	C1
IR64-Sub1	IR8419422-139	Philippines	62.5 (12.5)	A1	A1	C1
Kaharamana	15379	Sri Lanka	42.4 (11.1)	A1	A1	C1
Kurkaruppan	15449	Sri Lanka	56.0 (10.5)	A1	A1	C1
MTU1	650	India	45.0 (8.5)	A1	A1	C1
Pokkali	8948	Sri Lanka	3.5 (4.4)	A1	A1	C1
Swarna-Sub1	IR82810-410	Philippines	51.5 (8.8)	A1	A1	C1
Urarkaruppan	28616	Sri Lanka	36.8 (7.2)	A1	A1	C1
Faro27	72915	Nigeria	6.2 (3.2)	A2	A2	C3/4
IR42	73193	Philippines	4.3 (5.5)	A2	A2	C3/4
IR64	66970	Philippines	22.0 (13.3)	A2	A2	C3/4
James Wee	15294	Sri Lanka	43.6 (3.0)	A2	A2	C3/4
Kalukanda	15430	Sri Lanka	32.6 (5.7)	A2	A2	C3/4

given in Supplementary Table S1 and Table 1. The intolerant variety IR42 and the FR13A-derived breeding line IR40931-33-1-3-2 were included as controls in all experiments. Of the initially screened set, representative accessions (Table 1) were selected for repeated submergence screenings and molecular analyses. Goda Heenati and Kurkaruppan were included as additional tolerant controls, as well as the *Sub1* introgression lines Swarna-Sub1 and IR64-Sub1 with their respective intolerant parental lines Swarna and IR64 (Table 1). Seeds are available at IRRI upon request. Seeds were incubated at 55°C for 5 days to break the dormancy before pre-germination in petri dishes at 37°C in an incubator for 3 days. Seedlings were transferred into trays filled with soil supplemented with 3 g of ammonium sulfate fertilizer in rows of 10–30 plants each entry with two to three replicates depending on the experiment. IR42 and the tolerant check IR40931-33-1-3-2 were included in each tray. After about 14 days, trays were completely submerged in a concrete tank filled with 120–200 cm of tap water for 2 weeks or until the intolerant check IR42 showed leaf damage. Shoot elongation was measured after de-submergence and plant survival was scored at 14 days after de-submergence as a measure for tolerance. Seedlings for non-submerged control treatments were transplanted into pots after applying 6 g of ammonium sulfate fertilizer and grown until maturity in a greenhouse under natural conditions.

Plants used for temporal and spatial gene expression analyses were grown until booting/heading stage when nodes and internodes could be sampled separately. The different accessions were grown in three pots with three plants each, and two pots were completely submerged as described above. Non-submerged control plants were kept under natural conditions in a screenhouse and were sampled in parallel.

Statistical analyses

Standard statistical analyses were conducted using SAS (REML algorithm of PROC MIXED) and Excel software.

RNA sampling and RT–PCR analyses

For *SUB1A* and *SUB1C* gene expression analyses, plants were submerged for 30 h according to the protocol described above and RNA was extracted from leaf samples of 2–3-week-old seedlings. For temporal and spatial expression analyses, plants at heading stage were submerged for 30 h and panicles of different developmental

stages (panicle length 4–6, 12.5–14.5, and 22–25 cm), stems, and leaves of different maturity (immature yellow enclosed by older leaves, young light green, and fully developed dark green leaves) were collected from three plants. For RT–PCR analyses of IR64 and IR64-Sub1, stems were divided into internodes, nodes (leaf base was not entirely removed), and the region immediately on top of the node (including shoot elongation zone). The leaf collar region was sampled separately. For RT–PCR analyses of representative tolerant and intolerant rice accessions, nodes with about 1 cm of adjacent stem region and internodes were separated. Samples were pooled for each of the two replicates and immediately frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted with TRIzol® according to the protocol provided by the manufacturer (Invitrogen). RNA samples were quantified using a Nanodrop ND-1000 spectrophotometer and by electrophoresis in a 1% non-denaturing agarose gel stained with ethidium bromide (Sigma). DNA contamination was removed by treatment with RNase-free DNaseI according to the protocol provided (Promega).

Semi-quantitative RT–PCR analyses were carried out using the SuperScript™ One Step RT–PCR with Platinum® Taq kit (Invitrogen) in a total volume of 25 µl (12.5 µl of 2× reaction mix, 0.5 µl of *Taq* mix, 60 ng of total RNA, 5% DMSO, and 0.2 µM of forward and reverse primers). Gene-specific primers were used to amplify *SUB1A* (Sub1A203: for: 5′-cttcttgctcaacgacaacg-3′; rev: 5′-aggctc cagatgtccatgtc-3′) and *SUB1C* (Sub1C173: for: 5′-aacgccaa gaccaacttcc-3′; rev: 5′-aggaggctgtccatcaggt-3′) (Septiningsih et al. 2009). Actin was amplified as a control in all experiments (for: 5′-acaggtattgtgttggactc-3′; rev: 5′-gcttagcattct-tgggtcc-3′). Reverse transcription was carried out at 50°C for 30 min, followed by 35 cycles (*SUB1A* and *SUB1C*) or 28 cycles (*actin*) of amplification by PCR (94°C for 2 min; 28–35 cycles of 15 s 94°C, 30 s 53°C, 60 s 70°C, followed by 10 min final extension at 70°C). RT–PCR products were separated on 1.2% agarose gels and stained with ethidium bromide (Sigma) or SYBR Safe (Invitrogen). Images were taken using an Alpha Imager 1220 (Alpha Innotech, CA, USA).

DNA analyses and *SUB1A* and *SUB1C* allele-specific markers

Genomic DNA was extracted from leaf samples following the protocol published by Pallotta et al. (2000). DNA was quantified using Nanodrop (ND-1000 spectrophotometer) and by agarose gel electrophoresis. To determine the *SUB1A* haplotype, a region flanking a restriction site specific to the intolerant *SUB1A-2* allele (*Alu*I: AGCT or *Pvu*II: CAGCTG) was amplified using GnS2 primers (for: 5′-cttcttgctcaacgacaacg-3′; rev: 5′-tcgatggggtcttgatctct-3′) with subsequent digestion of DNA fragments according to Neeraja et al. (2007). A second SNP that generates a *SUB1A-1*-specific MAPK phosphorylation site was targeted with the SNP primer pair AEXF (5′-aggcggagctacgagtac ca-3′) and AEX1R (5′-gcagagcggctgcga-3′) (Septiningsih et al. 2009). The specificity of the PCR product in Kalukanda was confirmed by amplifying a DNA fragment of 203 bp flanking the MAPK site using the primer pair Sub1A203 (see above). The fragment was sequenced using a GenomeLab GeXP sequencer following the provided protocol for Dye Terminator Cycle sequencing (Beckman Coulter®).

For accessions that did not show amplification with the GnS2 primers, absence of the *SUB1A* gene was confirmed with the primer pair Sub1A203.

Sequencing of the *SUB1C* gene in different rice accessions revealed seven different alleles (Xu et al. 2006). The primer pair Sub1C_173 (for: 5′-aacgccaagaccaacttcc-3′; rev: 5′-aggaggctgtccatcaggt-3′; Septiningsih et al. 2009) was developed to amplify a polymorphic region within the *SUB1C* coding region and it facilitates size differentiation of four allelic groups. Digestion with *Cac*8I (GCNNGC) increased the resolution of the small size difference between alleles.

All PCR analyses were performed in a G-storm thermal cycler (Applied Biosystems, USA) with 100 ng DNA template (3 min 94°C, 35 cycles of 30 s 94°C, 30 s 56–60°C, 30 s 72°C, final extension 10 min 72°C) using Takara Ex Taq (Takara Bio Inc., Japan) and 5% DMSO. An aliquot of the amplified DNA fragments was digested for 4 h at 37°C with *Alu*I (*SUB1A*) or *Cac*8I (*SUB1C*) according to the protocols provided (New England Biolabs). Depending on the size, DNA fragments were analyzed in 2% agarose gels or 8% PAGE gels stained with SYBR Safe (Invitrogen) and documented using an Alpha Imager 1220 (Alpha Innotech, CA, USA).

Generation of transgenic plants and promoter–reporter gene study

Genomic DNA fragments upstream of the *SUB1A-1* start codon (−4 bp to −990 bp) of the tolerant variety FR13A were amplified using two primer pairs (PrC1for/rev: 5′-ttgc gagctagctgtctgaa-3′/5′-tagtccacgcgctaatgtga-3′; (986 bp); PrC3for/rev: 5′-caataagactcgggctgtgc-3′/5′-tagtccacgcgct aatgtga-3′ (716 bp)). The amplicons were cloned into the entry vector pCR8/GW/TOPO TA (Invitrogen) and sent for sequencing (Macrogen, Korea). The two *SUB1A-1*-upstream regions were sub-cloned into the binary vector pMDC164 with the beta glucuronidase (GUS) reporter gene (Curtis and Grossniklaus 2003) using LR clonase II enzyme mix according to the protocol provided by the manufacturer (Invitrogen). Transgenic plants were generated

by Agrobacterium-mediated transformation of immature Nipponbare embryos using strain LBA4404 (Hoekema et al. 1984) according to the protocol published by Wise et al. (2006). T0 plants were grown in soil in a temperature-controlled (29°C ± 5) greenhouse. Transgenic Nipponbare plants are photosensitive and were, therefore, kept under long-day conditions to enhance tillering before reproductive growth was induced under natural short-day conditions.

At maturity, panicles were removed from the tiller to obtain the seeds and plants were allowed to recover for 2 weeks before pots were completely submerged for 6 h in a drum filled with tab water. Non-submerged control plants were sampled in parallel. Individual tillers of different developmental stages were sampled by cutting at about 3 cm above the soil surface. Samples were transferred to 50 ml Falcon tubes filled with GUS-staining solution (100 mM sodium phosphate buffer (pH 7), 1 mM KFerricyanide, 1 mM KFerrocyanide, 10 mM Na_2EDTA, 0.1% Triton X100) containing 2 mM 5-bromo-4-chloro-3-indolyl-1-glucuronide (X-gluc) dissolved in DMSO. Samples were vacuum infiltrated for 3 h and incubated over night at 37°C. Chlorophyll was removed using 100% ethanol and samples were stored in 70% ethanol until GUS expression was documented using a DP71 microscope with attached digital camera (Olympus Corporation).

Results

Submergence screening and *SUB1A*- and *SUB1C*-marker survey

A set of 76 rice accessions was chosen for a submergence screening based on their origin from flood-prone areas or because they were classified as deepwater rice. These accessions mainly originate from South and Southeast Asia, with a few accessions from East Asia, Africa, and other countries (supplementary Table S1). The objective of this screening was to identify submergence-tolerant rice accessions that do not possess the *SUB1A* gene or that possess the intolerant *Sub1* haplotype (*SUB1A-2*/*SUB1C-2* to *SUB1C-7*). The *Sub1* haplotype was determined with allele-specific PCR-based cleaved amplified polymorphic sequence (CAPS) markers. The *SUB1A* marker (GnS2) targets an *Alu*I restriction site that is specific to the *SUB1A-2* allele (Fig. 1a). After PCR amplification and treatment with *Alu*I, one DNA fragment of 242 bp is specific to the *SUB1A-1* allele, whereas two DNA fragments (133 and 109 bp) are indicative of the *SUB1A-2* allele (Fig. 2b). A second SNP marker targets a MAPK phosphorylation site that is specific to the *SUB1A-1* allele and this marker is, therefore, dominant (Fig. 2c). The data showed that the allele classification with both markers was identical in all accessions analyzed. In the case of Kalukanda, the PCR data initially suggested a *SUB1A-2*/*SUB1A-1* haplotype since a faint band was repeatedly obtained with the primer pair that target the *SUB1A-1*-specific MAPK site whereas the GnS2 marker indicted a *SUB1A-2* allele (compare Fig. 2b, c). However, sequencing of the PCR amplicon subsequently showed that the band was unspecific and confirmed the *SUB1A-2*/*SUB1A-2* haplotype in Kalukanda (data not shown). In some varieties, no PCR product was obtained with the GnS2 marker, indicating absence of the *SUB1A* gene (data not shown). An additional primer pair (Sub1A_203) amplifying a DNA fragment within the *SUB1A* coding region was, therefore, used to confirm the absence of *SUB1A* from the genome of seven out of the 28 analyzed accessions (Fig. 2a). The *SUB1C* marker (Sub1C_173) targets a polymorphic, proline-rich region within the seven *SUB1C* alleles (Fig. 1b; Xu et al. 2006). After restriction with *Cac*8I, the tolerant *SUB1C-1* allele can be distinguished from other alleles (Fig. 2d). The DNA fragments of the *SUB1C-2* and *SUB1C-5*, *SUB1C-3* and *SUB1C-4* alleles, and the *SUB1C*-6 and *SUB1C*-7 alleles, respectively, are of the same size and can, therefore, not be differentiated with this marker (Figs. 1b, 2d).

To determine the level of submergence tolerance, rice seedlings were completely submerged for 14 days and plant survival was scored at 14 days after de-submergence. Under these conditions, intolerant plants showed extensive shoot elongation and died once removed from the water, whereas tolerant plants showed reduced elongation and fully recovered within 2 weeks after de-submergence (Fig. 3a). In agreement with the expectation, accessions with the *SUB1A-1* allele ($n = 15$) showed reduced average shoot elongation and a higher plant survival rate than accessions with the *SUB1A-2* allele ($n = 34$; intolerant check IR42) or those that lack the *SUB1A* gene (A0; $n = 27$) (Fig. 3b, c). However, average plant survival of the *SUB1A-1* group was only about 30% and was, therefore, much lower than that of the tolerant check IR40931 (Fig. 3c; supplementary Table S1).

With respect to the *SUB1C* gene, accessions with the *SUB1C-1* allele were the most tolerant (plant survival av. 30.3%; shoot elongation av. 68%). However, with one exception, all entries in this group also possessed the *SUB1A-1* allele, which prevents differentiation of gene effects (Table 2). Accessions with the *SUB1C-2/5* allele were the most intolerant (plant survival av. 9.9%; shoot elongation av. 103.8%), whereas plant survival was higher in accessions with the *SUB1C-3/4* allele (21%) or the *SUB1C-6/7* allele (19.8%) at similar shoot elongation rates (93.7–111%).

Based on this initial data set, representative accessions with contrasting *Sub1* haplotypes were selected for

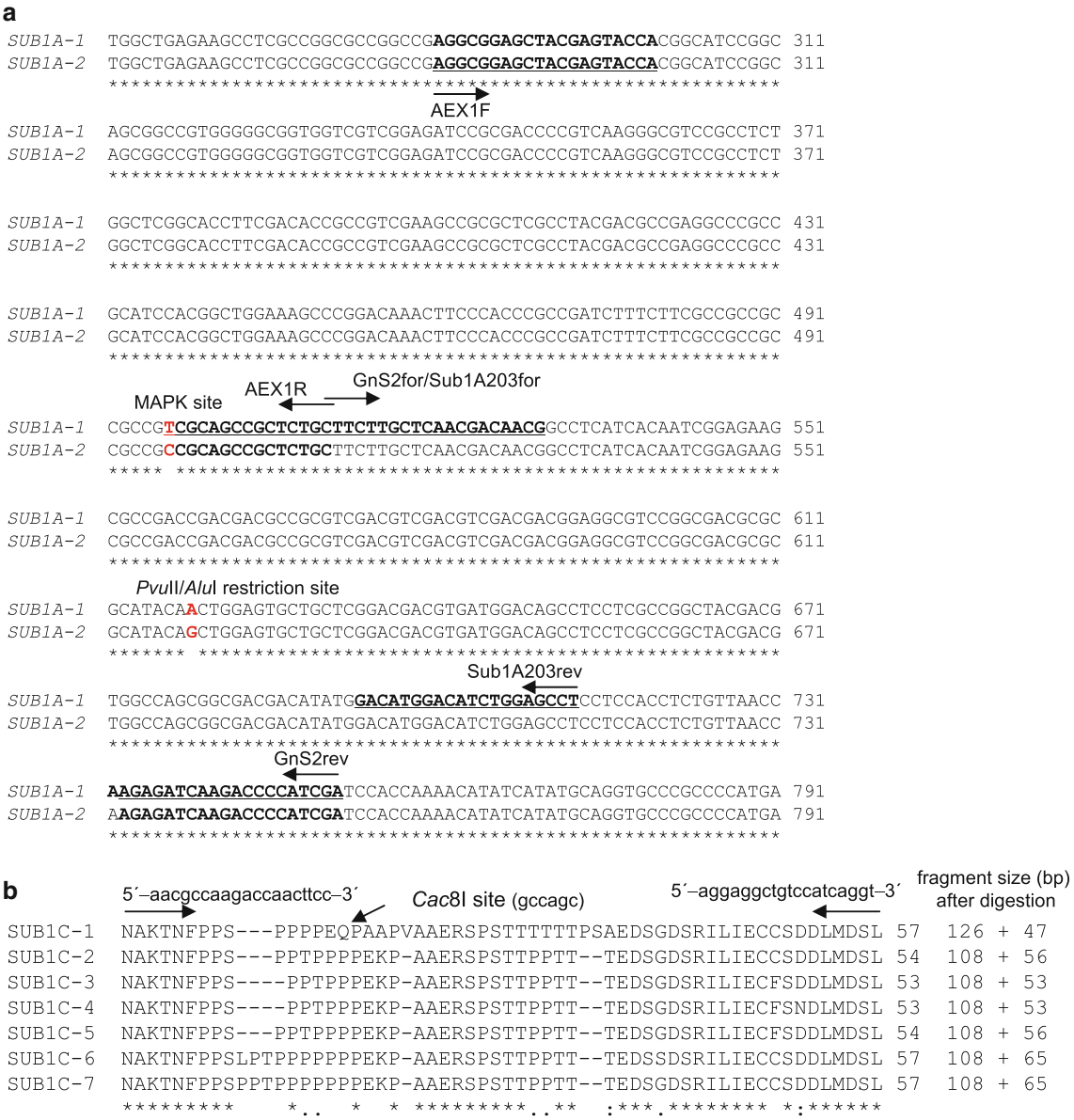

Fig. 1 *SUB1A* and *SUB1C* allele-specific molecular markers. The alleles *SUB1A-1* (tolerant) and *SUB1A-2* (intolerant) are distinct by two single nucleotide polymorphisms generating a mitogen-activated kinase (MAPK) site specific to the *SUB1A-1* allele and an *Alu*I/*Pvu*II restriction site specific to the *SUB1A-2* allele (**a**). The MAPK site is targeted by a SNP marker that specifically amplifies only the *SUB1A-1* allele. Other primers used for allele determination are indicated (see Fig. 2 for details). In **b**, an amino acid alignment of a polymorphic proline-rich region within the *SUB1C* gene is shown. The corresponding genomic region is amplified with the primer pair Sub1C_173 (sequence given on top of alignment). A *Cac*8I restriction site is present in all seven *SUB1C* alleles and allows size differentiation of four allelic groups (*SUB1C-1*, *SUB1C-2/5*, *SUB1C-3/4*, *SUB1C-6/7*) after digestion (see also Fig. 2). Sequence data were derived from Xu et al. (2006)

subsequent submergence screenings and molecular analyses (Table 1; see below). Among the selected *SUB1A-1* accessions, only three varieties (FR43B, Heenkarayal, Hindik Wee) were as tolerant (55–63% plant survival) as the tolerant checks IR40931, Goda Heenati, and Kurkaruppan (51–65% plant survival) (Table 1). Other *SUB1A-1* accessions were moderately tolerant to highly intolerant (e.g., Pokkali, Gadar Uzarka; 3–3.5% plant survival). Within the *SUB1A-2* group, plant survival after complete submergence varied widely, ranging from 43.6% (James Wee) to 4.3% (IR42). Accessions without the *SUB1A* gene (*SUB1A-0*) consistently showed a low (0–9.5%) plant survival independent of the *SUB1C* allele (Table 1). Likewise, differences in the degree of tolerance were not associated with *SUB1C* in the other haplotype groups since all except one *SUB1A-1* accession possessed the *SUB1C-1* allele and all *SUB1A-2* accessions possessed the *SUB1C-3/4* allele (Table 1).

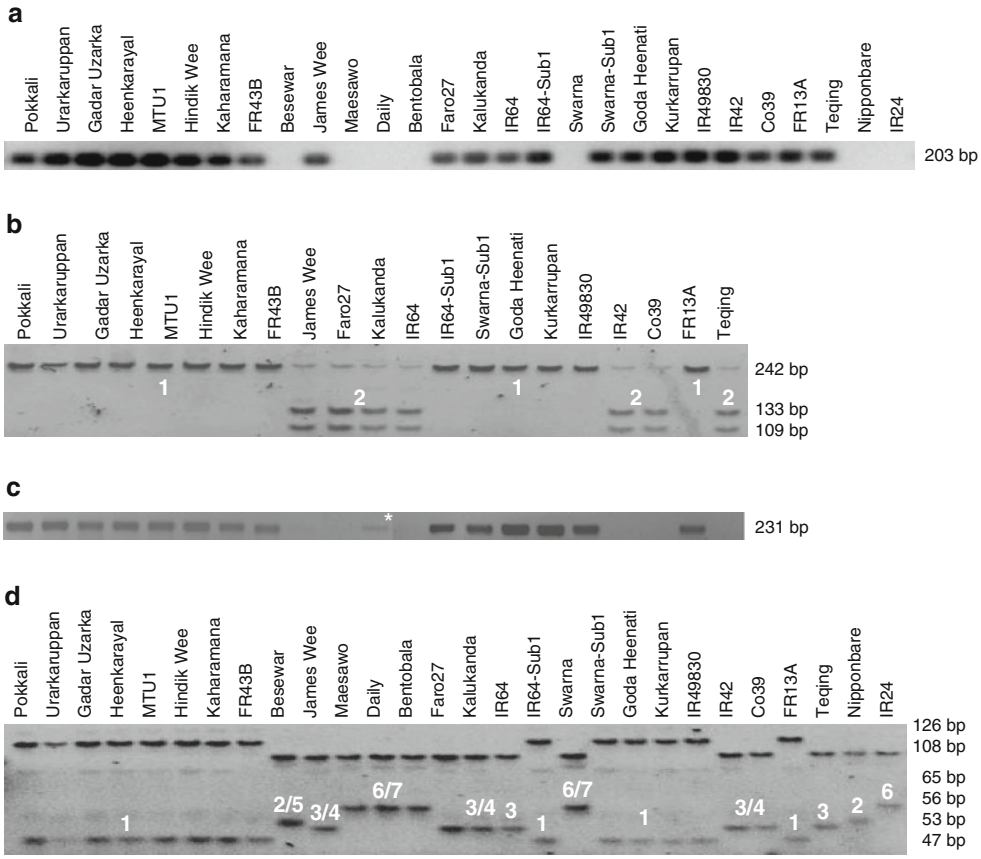

Fig. 2 Germplasm survey with *SUB1A*- and *SUB1C*-specific markers. Presence of the *SUB1A* gene in genomes of the rice accessions indicated was determined by PCR using the primer pair Sub1A203for/rev (**a**). In accessions that possessed the gene, a *SUB1A* fragment was amplified with the GnS2 primer pair and digested with *Alu*I generating two DNA fragments specific to the *SUB1A*-2 allele (**b** indicated with "2") due to the presence of the *Alu*I restriction site. The *SUB1A-1* allele is not cleaved (**b** indicated with "1"). The second *SUB1A*-specific single nucleotide polymorphism (SNP) located in a *SUB1A-1*-specific MAPK site (see Fig. 1) was targeted by a SNP marker that specifically amplifies alleles that possess the MAPK site (**c**). The PCR amplicon (*asterisk*) in Kalukanda was unspecific as was confirmed by sequencing. The *SUB1C* gene is present in all analyzed accessions and was amplified using the primer pair SUB1C_173 (**d**). Four allelic groups (*SUB1C-1*, *SUB1C-2/5*, *SUB1C-3/4*, *SUB1C-6/7*) can be distinguished based on size differences of DNA fragments. The *SUB1C* allele of IR64, Teqing, Nipponbare, and IR24 was determined by sequencing (Xu et al. 2006)

SUB1A and *SUB1C* gene expression analyses

The observation that the presence of the tolerant *SUB1A-1* allele does not confer tolerance in all accessions raised the question whether gene expression rather than allelic differences might be important for tolerance. We, therefore, selected 15 representative tolerant and intolerant accessions with different *SUB1A* alleles for gene expression analysis, including the *Sub1* breeding lines Swarna-Sub1 and IR64-Sub1, and the respective intolerant parental varieties Swarna and IR64 as additional controls. Details on these accessions are given in Table 1. In an initial experiment, submerged leaf samples of rice seedlings were analyzed by semi-quantitative RT–PCR using *SUB1A*- and *SUB1C*-specific primers. In this experiment, the highest *SUB1A* expression was detected in tolerant to moderately tolerant accessions (37–65% plant survival), including the *SUB1A-2* variety James Wee (Fig. 4). In intolerant accessions, expression was generally absent or very low for both the *SUB1A-1* (Pokkali) and the *SUB1A-2* (Faro 27, IR64, IR42) allele. However, *SUB1A-1* expression was comparably low in the tolerant check varieties Kurkaruppan and IR64-Sub1 (56 and 62.5% plant survival, respectively), and comparably high in the intolerant *SUB1A-1* accession Gadar Uzarka (2.9% survival).

Expression of *SUB1C* was generally highest in *SUB1A-0* and *SUB1A-2* accessions and very low or undetectable in accessions with the *SUB1A-1* allele. However, *SUB1C* expression did not associate with submergence tolerance since transcript abundance was similar in accessions with different tolerance levels (*SUB1A-1*/*SUB1C-1*: compare Hindik Wee and Kaharamana; *SUB1A-2*/*SUB1C-3/4*: compare James Wee and Faro 27; Fig. 4).

The absence of a consistent association of submergence tolerance, *Sub1* haplotype, and gene expression level raised the possibility that *SUB1A* and *SUB1C* expression in leaves

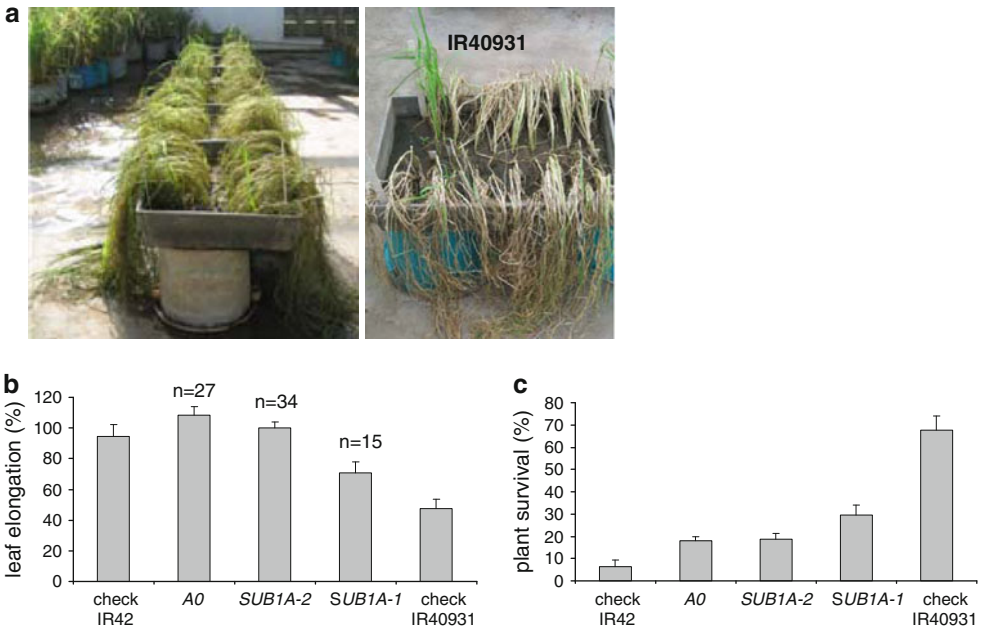

Fig. 3 Germplasm screening for submergence tolerance. Seedlings of 76 rice accessions were submerged for 2 weeks and scored for leaf elongation and survival. The tolerant check IR40931 and the intolerant check IR42 were additionally included in all experiments (**a**). Leaf elongation was higher in genotypes without the *SUB1A* gene (*A0*), and in IR42 and other genotypes with the *SUB1A-2* allele compared with genotypes with the *SUB1A-1* allele and IR40931 (**b**). Plant survival was higher in *SUB1A-1* accessions than in *A0* and *SUB1A-2* accessions, but lower than in the tolerant check (**c**)

Table 2 *SUB1A* and *SUB1C* alleles and plant survival under submergence

Allele	No. of entries *SUB1A*-1	Survival min–max (av) (%)	No. of entries *SUB1A*-2	Survival min–max (av) (%)	No. of entries *SUB1A*-0	Survival min–max (av) (%)	Total no. of entries	Av PE (SE)	Av PS (SE)
SUB1C-1	12	4.0–57.4 (31.4)	1	16.7	0	–	13	68.0 (5.4)	30.3 (5.3)
SUB1C-2/5	0	–	1	14.3	6	0–23.9 (9.1)	7	103.8 (14.4)	9.9 (3.1)
SUB1C-3/4	0	–	10	3.6–39.0 (19.9)	6	9.1–32.1 (22.8)	16	93.7 (4.2)	21.0 (3.0)
SUB1C-6/7	0	–	2	14.3–27.3 (20.8)	15	5.0–36.4 (19.7)	17	111.1 (7.1)	19.8 (2.0)

Av average, *PE* plant elongation, *PS* plant survival, *SE* standard error

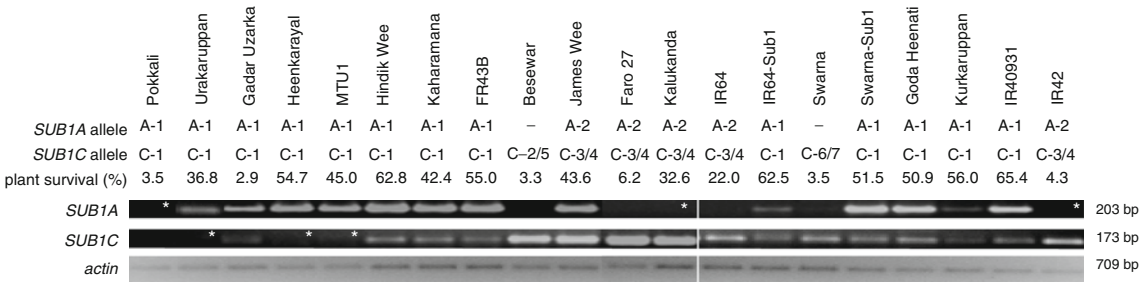

Fig. 4 *SUB1A* and *SUB1C* expression in submerged seedling leaves. Expression of the *SUB1A* and *SUB1C* genes was analyzed by semi-quantitative RT–PCR in rice seedlings after 30 h of submergence. The *Sub1* haplotype and % plant survival are indicated. *SUB1A* expression was absent from varieties that do not possess the gene (−) and was generally highest in tolerant and moderately tolerant accessions. In Pokkali, gene expression was either undetectable or very low despite the presence of the *SUB1A-1* allele. Among *SUB1A-2* varieties, gene expression and plant survival were highest in James Wee, whereas expression was low in Kalunkanda despite a similar tolerance level. *SUB1C* expression was not associated with *SUB1A* transcript abundance or plant survival. A representative *actin* control is shown. The size of PCR amplicons is indicated in base pairs (bp). *Asterisks* low expression was seen in some independent experiments

might not be causally related to submergence tolerance or that the RNA samples used for this analysis might not be representative of the tissue/cells in which the genes are expressed. We, therefore, conducted an initial temporal and spatial gene expression analysis to assess the level of gene expression and gene response to submergence in a tolerant (Goda Heenati) and intolerant (IR42) genotype (Fig. 5a). This experiment revealed an overall high *SUB1A-1* expression in the tolerant accession in all analyzed submerged tissues (panicles, stems, leaves) with high background expression in un-submerged controls in some tissues (12–23 cm panicles, immature/young leaves). In IR42, *SUB1A-2* expression was induced by submergence in all tissues, though at a much lower level than in Goda Heenati, and only very low background expression was detected in some un-submerged control samples. In contrast, expression of *SUB1C-1* was at the detection limit in the tolerant accession Goda Heenati and was constitutively high in most submerged and un-submerged tissues in IR42 (*SUB1C*-3/5) (Fig. 5a). To further assess gene expression, submerged and non-submerged tissues of IR64 (*SUB1A-2*/*SUB1C-3*) and the submergence tolerant breeding line IR64-Sub1 (*SUB1A-1*/*SUB1C-1*) were analyzed (Fig. 5b). The data showed that *SUB1A* expression was induced under submergence in both genotypes in all analyzed tissues (leaf collar, node, internode, shoot elongation zone) though transcript abundance was generally lower in IR64.

Subsequently, 20 genotypes contrasting in the *Sub1* haplotype and submergence tolerance were analyzed to determine whether *SUB1A* expression in nodes and internodes might associate with submergence tolerance. The data showed that tolerant to moderately tolerant accessions consistently showed higher *SUB1A* expression in nodes and internodes than intolerant genotypes (Fig. 6). Transcript abundance in internodes was lowest in MTU1 (plant survival, PS 45%) and Kaharamana (PS 42%), and highest in genotypes with plant survival rates >50% (Heenkarayal, Hindik Wee, FR43B, all check varieties). In James Wee (*SUB1A-2*; PS 44%), expression was high in nodes and internodes. In intolerant accessions that possess the *SUB1A* gene (Pokkali, Gadar Uzarka: *SUB1A-1*; Faro 27: *SUB1A-2*), gene expression was consistently very low or absent in both tissues. As expected, no gene expression was detected in genotypes without the *SUB1A* gene (Besewar, Swarna) (Fig. 6).

SUB1A-1 promoter studies

The spatial expression of the *SUB1A-1* gene was further analyzed by promoter::GUS reporter-gene studies in transgenic Nipponbare plants. For this study, we have cloned PCR fragments of different size (PrC-1, 986 bp; PrC-3, 716 bp) amplified from the genomic region upstream of the start ATG of the *SUB1A-1* gene from the tolerant

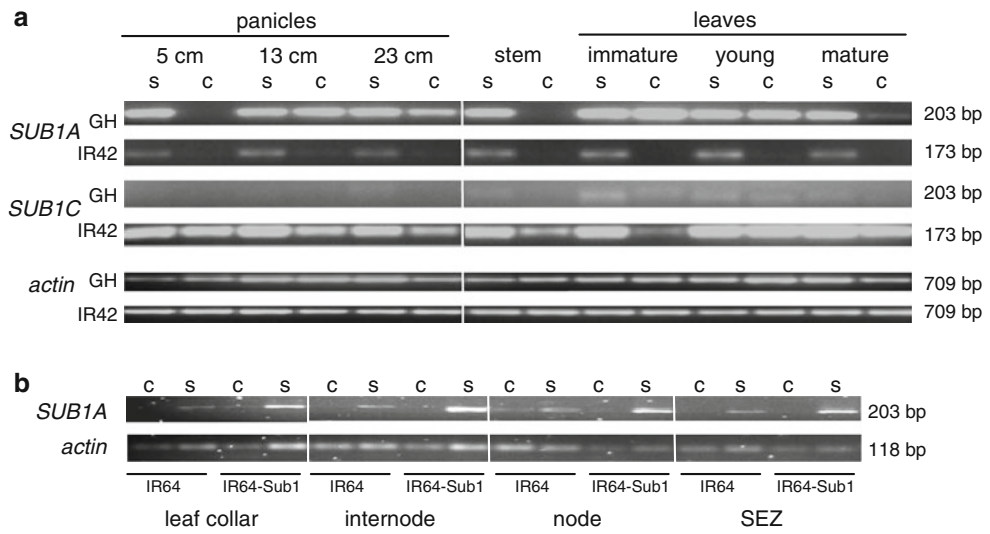

Fig. 5 Temporal and spatial expression analyses of *SUB1A* and *SUB1C*. Gene expression was analyzed in the tolerant variety Goda Heenati (GH) and the intolerant variety IR42 by semi-quantitative RT–PCR of the tissues indicated after 30 h of complete submergence (s). Non-submerged control (c) samples were analyzed in parallel. In Goda Heenati, submergence-induced expression of *SUB1A-1* was detected in all tissues analyzed (**a**, *upper panel*). In addition, gene expression was detected in most non-submerged tissues. *SUB1C* expression was barely detectable in any tissues under submerged and control conditions (**a**, *third panel*). In IR42, *SUB1A-2* expression was induced in all tissues upon submergence but at a much lower level than in Goda Heenati, and was mostly undetectable in non-submerged tissues (**a**, *second panel*), whereas *SUB1C* expression was constitutively high (**a**, *fourth panel*). A representative *actin* control is shown. Tissue-specific expression of *SUB1A* was additionally analyzed in IR64 (*SUB1A-2* allele) and IR64-Sub1 (*SUB1A-1* allele) (**b**). Gene expression was induced by submergence in both genotypes though it was lower in IR64. *SEZ* shoot elongation zone

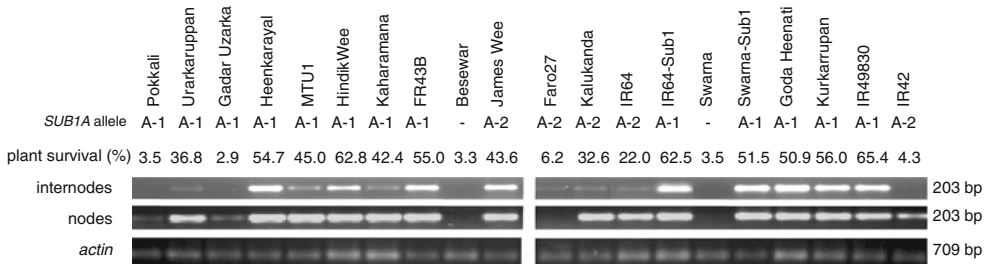

Fig. 6 Germplasm survey for tissue-specific *SUB1A* expression. Expression of *SUB1A* was analyzed in rice accessions with different submergence tolerance (% plant survival after 2 weeks of submergence). Two varieties without the *SUB1A* gene (−) were included as controls. Expression in internodes (*first panel*) and nodes (*second panel*) was highest in tolerant and moderately tolerant accessions and absent or very low in intolerant accessions. An actin control is shown

variety FR13A. The data revealed highly specific GUS expression in the node region (Fig. 7a, c) and the leaf collar region (Fig. 7g, h). In nodes, GUS staining was restricted to the base of the leaf sheath above the nodal septum (Fig. 7b, d). Faint and spotted GUS staining was further detected in the leaf sheath and leaf blade (Fig. 7h), as well as in internodes (data not shown). This expression pattern was observed in four independent transgenic plants with one (T0#192) or two (T0#39, T0#127, T0#170) copies of the transgene as was determined by Southern blot analysis (data not shown). The tissue-specific expression was independent of the promoter construct used (see legend Fig. 7 for details). In non-submerged control plants with any of the two promoter constructs, GUS expression was generally low or undetectable (Fig. 7e, f, i, j), however, wound-induced expression was sometimes observed in folded leaf samples and when samples were sectioned before GUS staining (Fig. 7k, l).

Discussion

Germplasm survey with *Sub1* allele-specific markers

The *Sub1* locus confers tolerance of complete submergence for about 2 weeks which is not always sufficient since rice fields might remain flooded for a longer period of time and it is, therefore, important to identify additional QTLs with an additive effect to *Sub1*. Because of the large effect of the *Sub1* locus, its presence might mask other QTLs with smaller effects and can therefore impair their mapping. The approach taken in our study was, therefore, to screen for tolerant rice genotypes that lack the *SUB1A* gene or carry the intolerant *SUB1A-2* allele and likely possess tolerance mechanisms distinct from *Sub1*. The molecular markers that were used for this germplasm survey were designed earlier based on detailed information available on *SUB1A* and *SUB1C* allelic variants and are now routinely used in breeding programs (Neeraja et al. 2007; Septiningsih et al. 2009; Singh et al. 2009). Our germplasm survey showed that these markers are diagnostic across a wide range of different genotypes and therefore of high value for breeders. Overall, the survey confirmed the large effect of the *Sub1* QTL since all accessions with the highest level of tolerance invariably possessed the tolerant *SUB1A-1/SUB1C-1* haplotype whereas all accessions without the *SUB1A* gene were intolerant. Only one variety (James Wee) with the intolerant haplotype (*SUB1A-2/SUB1C-3/4*) consistently showed moderate submergence tolerance and therefore met the objective of this study (see below).

Conclusions on the effect of *SUB1C* on submergence tolerance cannot be drawn from this survey since the tolerant *SUB1C-1* allele was, with one exception (Kalagyi: *SUB1A-2/SUB1C-1*), invariably associated with the tolerant *SUB1A-1* allele. However, Kalagyi showed an intolerant phenotype that would be in agreement with the general perception that *SUB1A* is the major determinant of submergence tolerance and that *SUB1C* does not contribute to tolerance. This is based on data showing the tolerant phenotype of Ubi::*SUB1A-1* overexpression lines (Xu et al. 2006) and more recent data from breeding lines with different recombinations within the *Sub1* locus (Septiningsih et al. 2009). The authors had shown that plants with the *SUB1A-2* allele in combination with the *SUB1C-1* or *SUB1C-3* allele were intolerant, whereas plants with the *SUB1A-1* allele in combination with the tolerant *SUB1C-1* or intolerant *SUB1C-3* allele were tolerant.

Earlier reports had suggested that *SUB1C* might be a direct downstream target of *SUB1A* (Xu et al. 2006; Fukao et al. 2006). Our data from the temporal and spatial expression analysis confirmed the generally contrasting expression pattern for *SUB1A* and *SUB1C* in IR42 (*SUB1A*: low; *SUB1C*: high) and Goda Heenati (*SUB1A*: high; *SUB1C*: low). However, low and high *SUB1C* expression was detected regardless of the *SUB1A* transcript abundance, suggesting that *SUB1C* is not directly regulated by either *SUB1A* allele.

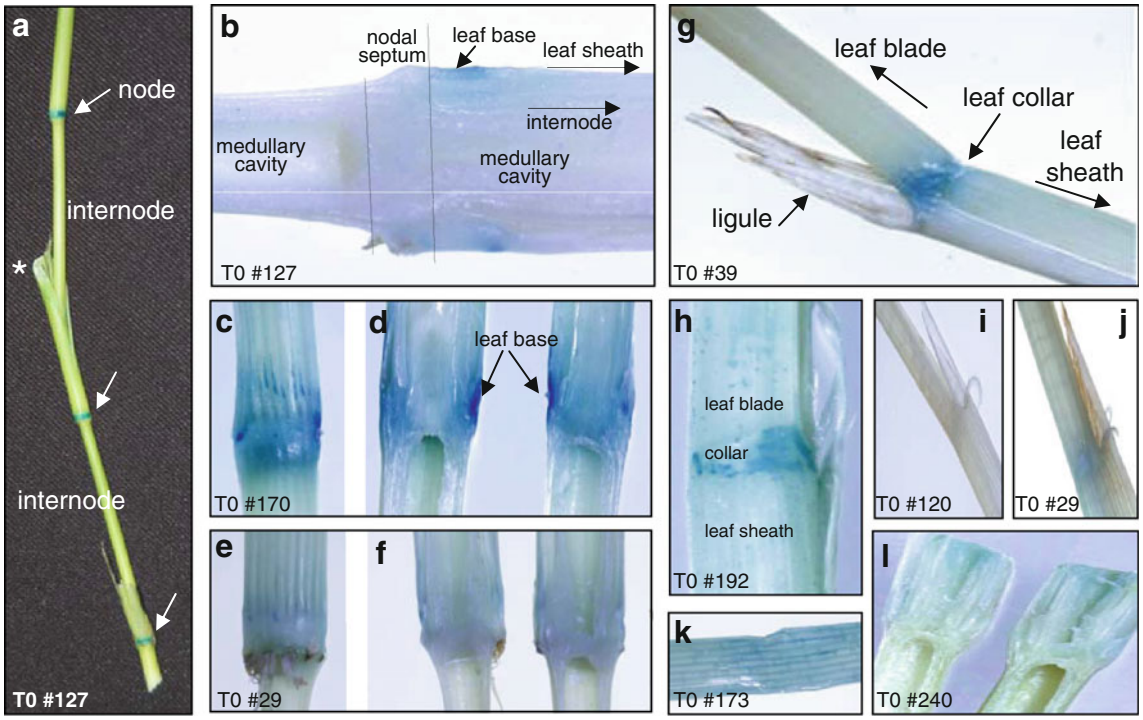

Fig. 7 *SUB1A-1* promoter::GUS expression analysis. Nipponbare plants were transformed with two *SUB1A-1*-promoter constructs (PrC-1, PrC-3; see Materials and methods) to drive the expression of the GUS-reporter gene. Independent transgenic plants (T0#n) with one or two introgressions of the transgene (see below) were completely submerged for 6 h and stained for GUS activity overnight. Specific GUS expression was detected in stem nodes (**a**, **c**) where expression was restricted to the base of the leaf sheath above the nodal septum (**b**, **d**). GUS expression was also detected in the leaf collar region that separates the leaf sheath and the leaf blade (**g**, **h**). In some leaves, spotted GUS staining was detected in the leaf sheath and blade (**h**). Non-submerged plants were used as controls and showed low or no GUS expression in nodes (**e**, **f**) and leaf collars (**i**, **j**). Wound-induced GUS staining was sometimes observed in folded leaves (**k**) and stems stained after sectioning (**l**). *T0#29* PrC-1 (2 copies), *T0 #39* PrC-3 (2 copies), *T0#120* PrC-1 (1 copy), *T0#127* PrC-1 (2 copies), *T0 #170* PrC-3 (2 copies), *T0#192* PrC-1 (1 copy), *T0 #240* PrC-3 (1 copy)

SUB1A RNA expression analyses

The finding that not all accessions with the tolerant *Sub1* haplotype were submergence tolerant prompted us to compare the expression of *SUB1A* in tolerant and intolerant genotypes. The data derived from leaf RNA samples indeed showed that accessions in which *SUB1A-1* expression was undetectable or very low were highly intolerant. Whether this is due to mutations in the promoter region or the absence of an *SUB1A*-upstream regulator remains to be analyzed. However, gene expression in leaves was variable and not clearly associated with submergence tolerance in those *SUB1A-1* accessions that expressed the gene. In addition, high transcript abundance was detected in James Wee, an *SUB1A-2* variety. These findings were unexpected and raised the question whether RNA from leaf blades, as used in our study, is representative and suitable for quantifying *SUB1A* expression. As was shown by Lee and Kende (2002), cell elongation in deepwater rice leaves is restricted to a short region (0–7 cm) above the collar, the part of the leaf that separates the leaf sheath and leaf blade, whereas leaf blades show no or little cell elongation. The authors also showed high and specific expression of several expansin genes in the elongation zone (2–7 cm above the collar). Expansin genes are cell wall-loosening enzymes that facilitate cell elongation and are therefore important for the escape response in deepwater rice. In agreement with this, it was shown that expansin genes are expressed at a lower level in a tolerant *Sub1*-introgression line (M202-Sub1) than in the intolerant M202 control (Fukao et al. 2006).

In a first attempt to further differentiate tissue-specific *SUB1A* expression, we conducted temporal and spatial expression analyses that indeed showed submergence-induced *SUB1A* expression in the leaf collar region, as well as in internodes and stem nodes. Furthermore, *SUB1A* expression was additionally detected in developing panicles and immature leaves even under non-submerged conditions, suggesting that *SUB1A* might have some role in plant developmental processes. However, since this was observed only in Goda Heenati but not in IR42, an essential role of *SUB1A* in cell division or cell speciation processes seems unlikely.

The RT–PCR analyses showed the largest difference in gene expression between submerged and non-submerged

controls in samples derived from nodes and internodes and these tissues were, therefore, chosen to compare *SUB1A* expression across tolerant and intolerant genotypes. This analysis indeed revealed clear quantitative differences in *SUB1A* transcript abundance, especially in internode samples, between tolerant and intolerant genotypes. The RT–PCR data are well in agreement with the fact that a submergence-escape response, which is generally observed in deepwater rice and other submergence-intolerant rice accessions, is mainly mediated by internode elongation (for review, see Bailey-Serres and Voesenek 2008). Two ERF-type transcription factor genes, *SNORKEL1* and *SNORKEL2*, that regulate internode elongation in deepwater rice have recently been identified (Hattori et al. 2009).

Submergence tolerance and *SUB1A-2* expression

In James Wee, *SUB1A-2* transcript abundance in nodes and internodes, and also in leaves was high and comparable with that of *SUB1A-1* expression in tolerant accessions. It is, therefore, possible that high expression of the *SUB1A-2* allele can confer at least some submergence tolerance. In agreement with this, two other varieties, Kalukanda and IR64, that showed high *SUB1A-2* expression in nodes and low expression in internodes, had some tolerance of submergence (32.6 and 22% PS, respectively). The main difference between the two *SUB1A* alleles is the specific presence of a mitogen-activated kinase (MAPK) site in the *SUB1A-1* allele (Xu et al. 2006). Absence of the MAPK site in the SUB1A-2 protein would accordingly render this allele insensitive to posttranslational regulation, which might affect the activity of the protein and thereby the regulation of downstream genes. This might explain why James Wee was only moderately tolerant despite a *SUB1A* expression level comparable to those observed in tolerant accessions. However, further experiments, e.g., overexpression of *SUB1A-2*, are needed to demonstrate that this allele can indeed confer tolerance. Phosphorylation of the MAPK site in the *SUB1A-1* allele has been demonstrated in vitro (Julia Bailey-Serres, personal communication) and it will be interesting to assess whether differences in protein-dimerization properties, nuclear localization, and/or DNA-binding properties exist between phosphorylated and un-phosphorylated *SUB1A* proteins.

Alternatively, it is possible that novel submergence-tolerance QTLs might be present in James Wee, Kalukanda, and IR64. In this context, it is noteworthy that IR64-Sub1 was more tolerant than, e.g., Sambha Mahsuri-Sub1 under field conditions (Singh et al. 2009) which would support this hypothesis. Smaller-effect QTLs have been reported (Nandi et al. 1997; Toojinda et al. 2003) and it will be interesting to analyze if these, or novel QTLs are present in the three varieties. Pyramiding of *Sub1* with novel QTLs would further enhance submergence tolerance for the benefit of rice farmers in flood-prone areas.

SUB1A-1 promoter::GUS analysis

The promoter studies conducted with T0 plants at the booting/heading stage are largely in agreement with the RT–PCR data since highly specific, submergence-induced GUS expression was detected in stem nodes and leaf collars. Within nodes, GUS expression was highest at the base of the leaf sheath but no staining was detected in the node itself and directly adjacent internode regions. Likewise, GUS expression in leaves was strongest in leaf collars but was faint and spotted in the leaf sheath and leaf blade. In rice, cell division and elongation of the leaf sheath take place at the nodes whereas leaf blades grow from a region directly above the collar (Lee and Kende 2002). Specific expression of S*UBA-1* in the leaf-sheath base and the leaf collar region is therefore in agreement with its predicted role in suppressing leaf elongation under submergence (e.g., Xu et al. 2006). The collar region is furthermore distinct from other parts of the leaf since it starts elongating only after the leaf sheath and leaf blade are fully elongated, thereby diverging the leaf blade from the center of the tiller (Dahl 1995). Expression of *SUB1A* in the collar region might therefore serve to suppress elongation until the leaf is fully elongated. As was observed in the leaf sheath and leaf blade, GUS staining in internodes appeared random and spotted without clearly defined regions of GUS expression. Whereas these findings are in agreement with the variability observed in RT–PCR analysis of leaf samples, we had expected a more defined GUS expression in internodes based on the RT–PCR that showed consistent association of submergence tolerance with *SUB1A* expression in this tissue. Temporal and spatial GUS expression analyses using T1 plants are now underway to determine the specific cell types in leaves, internodes, and other plant tissues in more detail. Analysis of T1 plants will also allow us to assess gene expression in nodes and internodes of young seedlings, which was not possible by RT–PCR since internodes are less than 1 mm apart until plants enter the booting/heading stage (Sato et al. 1999; Fornara et al. 2004). This will establish if the observed gene expression is causally related to submergence tolerance.

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