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SUB1A-dependent and -independent mechanisms are involved in the flooding tolerance of wild rice species

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SUMMARY

Crop tolerance to flooding is an important agronomic trait. Although rice (*Oryza sativa*) is considered a flood-tolerant crop, only limited cultivars display tolerance to prolonged submergence, which is largely attributed to the presence of the *SUB1A* gene. Wild *Oryza* species have the potential to unveil adaptive mechanisms and shed light on the basis of submergence tolerance traits. In this study, we screened 109 *Oryza* genotypes belonging to different rice genome groups for flooding tolerance. *Oryza nivara* and *Oryza rufipogon* accessions, belonging to the A-genome group, together with *Oryza sativa*, showed a wide range of submergence responses, and the tolerance-related *SUB1A-1* and the intolerance-related *SUB1A-2* alleles were found in tolerant and sensitive accessions, respectively. Flooding-tolerant accessions of *Oryza rhizomatis* and *Oryza eichingeri*, belonging to the C-genome group, were also identified. Interestingly, *SUB1A* was absent in these species, which possess a *SUB1* orthologue with high similarity to *O. sativa SUB1C*. The expression patterns of submergence-induced genes in these rice genotypes indicated limited induction of anaerobic genes, with classical anaerobic proteins poorly induced in *O. rhizomatis* under submergence. The results indicated that *SUB1A-1* is not essential to confer submergence tolerance in the wild rice genotypes belonging to the C-genome group, which show instead a SUB1A-independent response to submergence.

Keywords: flooding tolerance, wild rice, *SUB1* genes, *Oryza eichingeri*, *Oryza rhizomatis*, *Oryza sativa*.

INTRODUCTION

Flooding is a widespread environmental stress, particularly dramatic in the lowlands of South, Southeast and East Asia, where rice (*Oryza sativa*) is predominantly cultivated. The rapid decline in the oxygen (O_2) diffusion rate (~10 000-fold less) during flooding is accompanied by a reduction in cellular O_2 levels and an energy crisis, which are particularly severe when photosynthesis is limited or absent (Bailey-Serres and Voesenek, 2008; Licausi and Perata, 2009). In fact, most rice varieties die within 14 days of complete submergence, thus causing serious famine in various regions of Asia (Xu *et al.*, 2006).

Rice ecotypes vary considerably in their responses to flooding. Deep-water rice and most lowland rice genotypes generally adopt an 'escape' strategy, characterized by the ethylene-mediated rapid elongation growth promoted by gibberellins (GA), associated with carbohydrate consumption (Bailey-Serres and Voesenek, 2008; Bailey-Serres *et al.*, 2010). In deep-water rice the 'escape strategy' is regulated by two ethylene-responsive factors (ERFs), *SNORKEL1* (*SK1*) and *SNORKEL2* (*SK2*), that trigger considerable internode elongation via GA during flooding (Hattori *et al.*, 2009; Nagai *et al.*, 2010). *SK1* and *SK2* are absent in the non-deep-water rice varieties evaluated to date, but are present in some wild rice genotypes that show a deep-water response (Hattori *et al.*, 2009).

Lowland rice genotypes also show this adaptive response, but it is only advantageous if floodwaters are shallow or rise gradually (Voesenek *et al.*, 2004). This is because shoot

elongation is a favourable trait only when the associated costs are outweighed by being able to reach the water surface before carbohydrate starvation intervenes.

Only a few rice varieties can withstand more than 2 weeks of complete submergence (Xu *et al.*, 2006). These varieties restrict elongation growth when submerged, thus preserving carbohydrates for recovery when desubmerged (quiescence strategy). This kind of response is conditioned by the presence of the major quantitative trait locus (QTL) *SUBMERGENCE 1* (*SUB1*) that encodes a variable cluster of two or three ERF genes: *SUB1A*, *SUB1B* and *SUB1C* (Fukao *et al.*, 2006; Xu *et al.*, 2006). *SUB1B* and *SUB1C* are present in all of the *indica* and *japonica* accessions that have so far been examined, whereas *SUB1A* is restricted to a part of *indica* accessions (Fukao *et al.*, 2006, 2009; Xu *et al.*, 2006). Only submergence-tolerant genotypes possess the *SUB1A-1* allele, whereas genotypes containing the *SUB1A-2* or lacking the *SUB1A* gene are intolerant to flooding. A recent germplasm survey also revealed that all of the tolerant genotypes analysed to date possess the tolerant *SUB1* haplotype *SUB1A-1*/*SUB1C-1* (Singh *et al.*, 2010). The allele *SUB1C-1* is invariably associated with the allele *SUB1A-1*, with the exception of the variety Kalagyi, where it is associated with the *SUB1A-2* allele (Singh *et al.*, 2010). In addition to the presence of *SUB1A-1*, a high expression level of the *SUB1A-2* allele appears to be part of the submergence tolerance mechanism (Singh *et al.*, 2010). An evaluation of the backcross *SUB1* recombinant lines indicated that *SUB1A-1* is the primary contributor to submergence tolerance, and that *SUB1*B and *SUB1*C do not seem to be important in conferring this trait (Septiningsih *et al.*, 2009).

Fukao and Bailey-Serres (2008) used transgenic lines ectopically expressing *SUB1A* to demonstrate that this gene significantly limits underwater elongation, through the accumulation of the GA response suppressors DELLA protein SLENDER RICE 1 (SLR1) and non-DELLA protein SLR-LIKE-1 (SLRL1), thus increasing the submergence tolerance. Jung *et al.* (2010) recently identified that *SUB1A-1* upregulates the accumulation of transcripts associated with anaerobic respiration, hormone responses and antioxidant system pathways under submergence.

The wild relatives of rice offer a largely untapped resource of agriculturally important genes that have the potential to mitigate the environmental adversity aggravated by climate change (Brar and Khush, 1997). Moreover, the *Oryza* species may not only provide useful genes for breeding, but could also shed light on the evolution and domestication of cultivated rice (Kim *et al.*, 2008). The genus *Oryza* consists of 23 species, the genomes of which are classified into nine groups (A, B, C, BC, CD, E, F, G and JH) on the basis of morphological, physiological and biochemical differences, crossing relationships, chromosome number and chromosome pairing in interspecific hybrids (Aggarwal *et al.*, 1997; Vaughan *et al.*, 2003; Miyabayashi *et al.*, 2007). The cultivated *O. sativa* belongs to the A-genome group together with the wild rice *Oryza rufipogon* and *Oryza nivara*, from which *O. sativa* is believed to have been domesticated (Fukao *et al.*, 2009). Although major reproductive barriers exist between plants belonging to different genome groups, and the rate of success for intergenomic crosses is very low (Yan *et al.*, 1997), the study of these species have an interesting potential to unveil genetic traits associated with different stress resistance. Indeed, some reports have been recently published on this topic (Mahmoud *et al.*, 2008; Fukao *et al.*, 2009; Koseki *et al.*, 2010; Li *et al.*, 2010; Philippe *et al.*, 2010).

In this work we screened for flooding tolerance in 109 rice genotypes belonging to 12 *Oryza* species, including wild ones, representing four different genomes. The *O. nivara* and *O. rufipogon* accessions showed a wide range of tolerance to submergence. The presence of the *SUB1A-1* allele was always associated with flooding-tolerant *O. nivara* and *O. rufipogon* accessions.

We also identified some wild rice accessions belonging to the C-genome group, which were highly tolerant to submergence, showing quiescence traits related to survival. These species do not posses the *SUB1A-1* gene, and display limited induction of anaerobic genes, suggesting a different mechanism of tolerance.

RESULTS

Screening of rice genotypes for submergence tolerance

One hundred and nine rice genotypes (Table S1) were screened for flooding tolerance. Rice cultivars and wild species growth responses to submergence varied significantly (Figure 1; Table S2). Based on the elongation index, L102-8 and *O. nivara* (IRGC-105725) had the highest and lowest elongation values, respectively: L102-8 elongated almost eight times more under flooding than in air, whereas *O. nivara* (IRGC-105725) elongated almost 10 times less, exceeding the performance of the FR13A variety that is well known for activating the 'quiescence strategy' under flooding conditions (Xu *et al.*, 2006) in the repression of growth when submerged (Figure 1). Twenty-four genotypes displayed flooding-enhanced elongation (elongation index >1; Figure 1). Whereas in some genotypes growth mostly resulted from enhanced leaf elongation, in others enhanced stem growth was predominant (Figure S1). The genotypes showing the highest elongation index (i.e. L102-8, Adiourmi 2 and *O. nivara* IRGC-80717) showed stem growth as a major contribution to plant elongation (Figure S1).

Flooding tolerance also varied significantly across the genotypes studied (Figure 2; Table S3). The wild rice species *Oryza eichingeri* (IRGC-101429), *Oryza alta* (IRGC-100161) and *Oryza rhizomatis* (IRGC-103421) displayed a survival rate similar to the tolerant *indica* variety FR13A (Figure 2). These three wild species showed, together with a near 100%

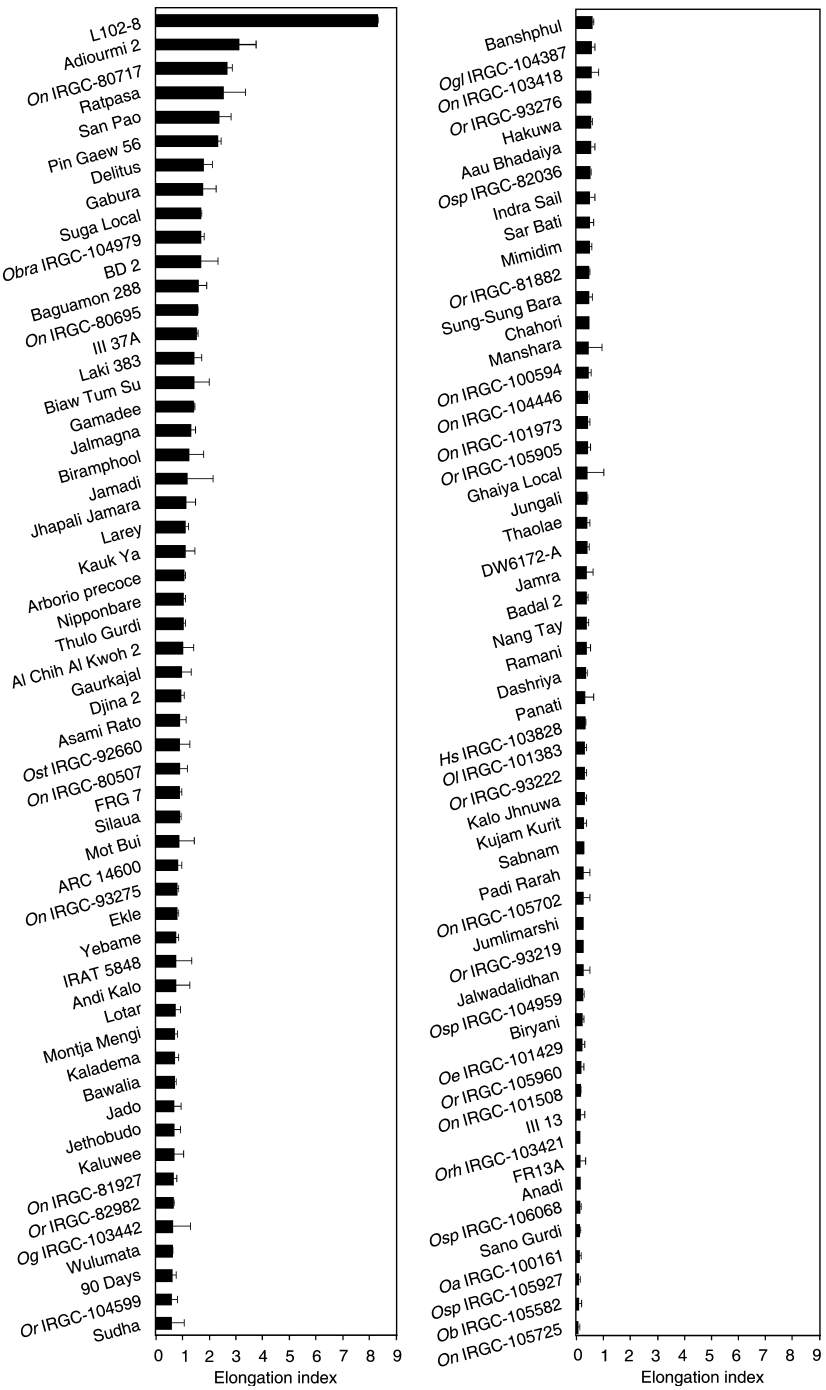

Figure 1. Elongation index of the 109 rice genotypes used for the submergence tolerance screening. FR13A and Nipponbare were used as tolerant and sensitive internal controls, respectively. Data were collected after 14 days of submergence (means ± SDs). Three biological replications were used, each including 18 seedlings. The abbreviations used are as follows: *Hs*, *hybrid swarm*; *Oa*, *Oryza alta*; *Ob*, *Oryza barathii*; *Obra*, *Oryza brachyantha*; *Oe*, *Oryza eichingeri*; *Og*, *Oryza glaberrima*; *Ogl*, *Oryza glumepatula*; *Ol*, *Oryza longistaminata*; *On*, *Oryza nivara*; *Orh*, *Oryza rhizomatis*; *Or*, *Oryza rufipogon*; *Osp*, *Oryza spontanea*; *Ost*, *Oryza stapfii*.

survival rate (Figure 2), an elongation index almost identical to that of FR13A (Figure 1). The two accessions of common wild rice *O. nivara* (IRGC-80507) and *O. rufipogon* (IRGC-81882), which are considered immediate ancestors of domesticated rice (Vaughan *et al.*, 2008), also possessed a survival ability comparable with that of FR13A (Figure 2). None of them elongated under flooding compared with air (elongation index <1). Although a large number of accessions of domesticated and wild rice genotypes apparently followed a quiescence strategy (low elongation index), their survival rate was very poor. This was demonstrated by a low correlation between the elongation index and the percentage of survival (Figure S2).

The *SUB1A* gene is present in some *O. nivara, O. rufipogon* and other A-genome group wild rice accessions

Different accessions of *O. nivara* and *O. rufipogon* showed a wide range of flooding-tolerance responses, from highly

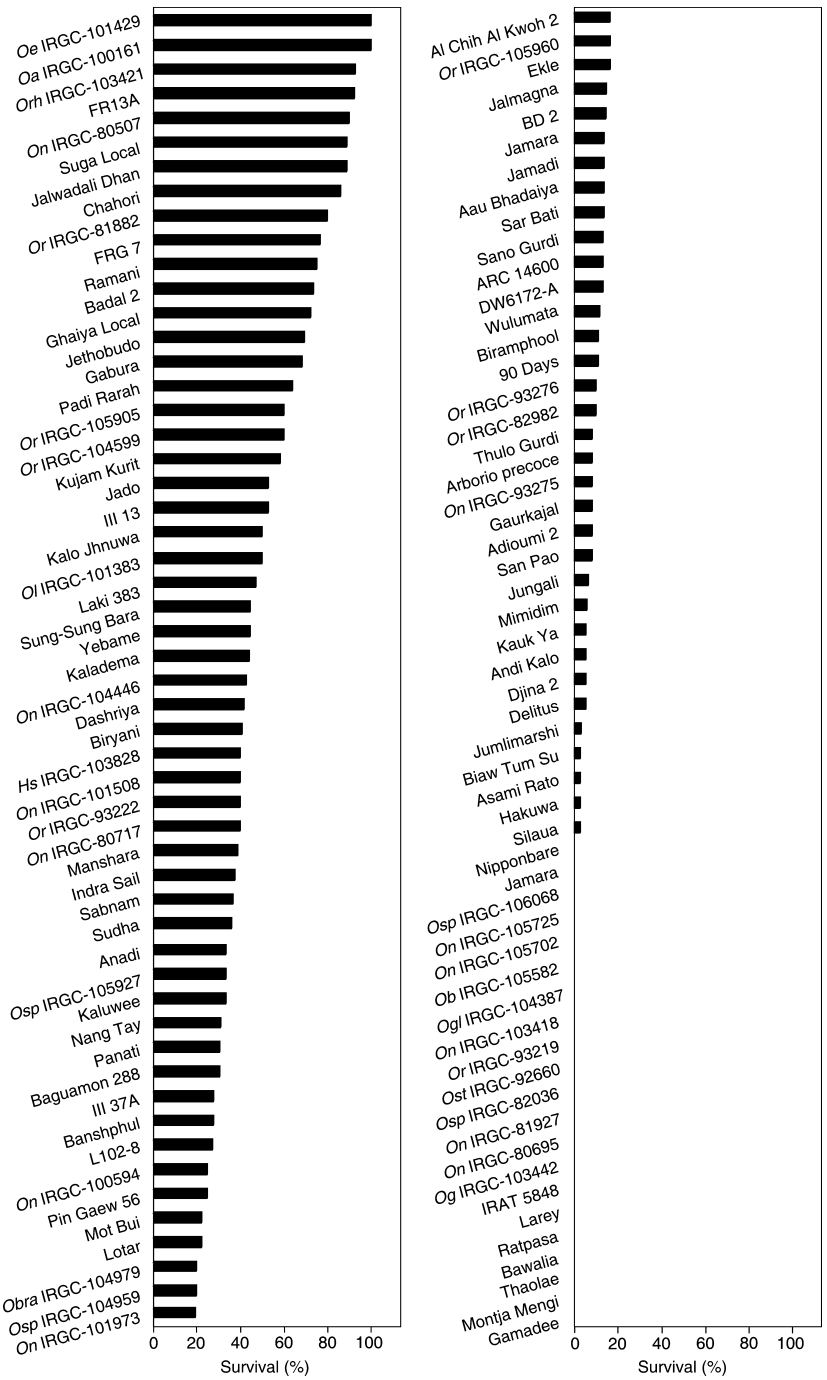

Figure 2. Percentage survival rates of the 109 rice genotypes used for the submergence tolerance screening. FR13A and Nipponbare were used as tolerant and sensitive internal controls, respectively. Three biological replications were used, each including 18 seedlings. The ±SD for each value was lower than 11% of the reported data (n = 3). The plant survival was estimated after 14 days of submergence followed by 7 days of recovery. See the legend for Figure 1 for the list of abbreviations.

tolerant accessions (e.g. *O. nivara* IRGC-80507) to very sensitive ones (e.g. *O. nivara* IRGC-80695) (Figure 2). They belong, together with *O. sativa*, to the A-genome group of the genus *Oryza* (Khush, 1997; Wing *et al.*, 2005; Vaughan *et al.*, 2008). To further investigate the relation between submergence tolerance and the presence of the *SUB1A* gene, we selected 22 rice accessions including *O. nivara*, *O. rufipogon* and other A-genome varieties for subsequent analysis (Figure 3).

Several wild rice accessions of *O. rufipogon*, some accessions of *O. nivara* as well as rice species such as the natural hybrids *Oryza spontanea* (IRGC-93300) and hybrid swarm (IRGC-103828) showed the presence of a *SUB1A*-like gene (Figure 3). *Oryza nivara* IRGC-80507 and IRGC-101508, *O. rufipogon* IRGC-81882 and hybrid swarm (IRGC-103828), which were highly tolerant to submergence (Figure 2) and showed reduced elongation when flooded (Figure 1), possessed the tolerance-specific *SUB1A-1* allele. Moreover, *O. rufipogon* IRGC-82982 and IRGC-105960, intolerant to submergence (Figure 2) but with reduced elongation under these conditions (Figure 1), showed the presence of the

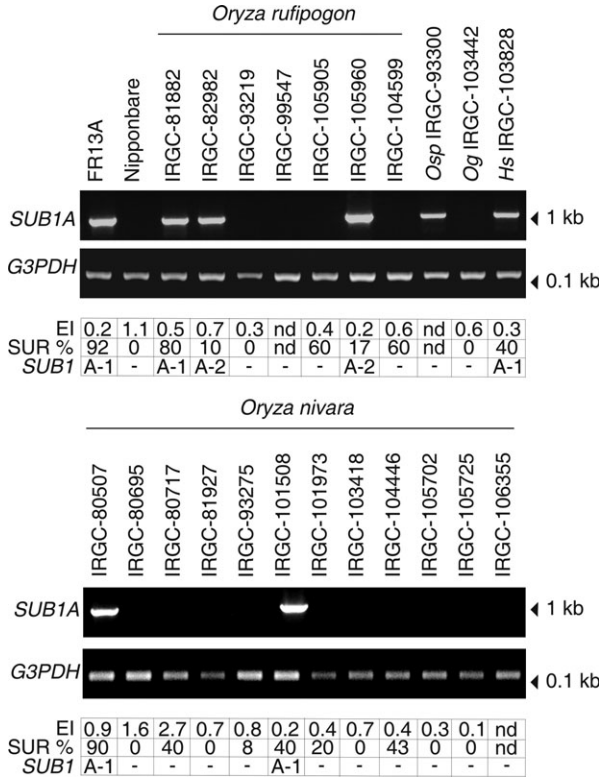

Figure 3. *SUB1A* gene presence screening with specific primers (see Table S5) on *Oryza nivara*, *Oryza rufipogon* and other A-genome group *Oryza* spp. accessions with genomic PCR. The two rice varieties FR13A and Nipponbare were used as internal controls, harbouring and not harbouring the *SUB1A* gene, respectively. The abbreviations used are as follows: EI, elongation index; SUR, survival (%); SUB1, *Sub1A* allele. See the legend for Figure 1 for a list of abbreviations.

intolerance-specific *SUB1A-2* allele (Figure 3). The alignment among the representative *SUB1A* sequences from the wild genotypes and *O. sativa* showed a very high level of amino acid sequence identity, and the P → S substitution in position 184 was conserved in the *SUB1A-1* wild rice allele (Figure S3). This single nucleotide polymorphism in the putative mitogen-activated protein kinase site of *SUB1A* distinguishes the tolerant from the intolerant allele (Xu *et al.*, 2006). The silent substitution in position 678 of the coding sequence was also conserved (Figure S4).

A survey of the tolerant genotype *O. nivara* (IRGC-80507) revealed the presence of the *O. sativa* tolerant *Sub1* haplotype *SUB1A-1*/*SUB1C-1* (Singh *et al.*, 2010) (Figure S5), associated with the *SUB1B-1* allele (Figure S6), previously identified as one of the *SUB1B* alleles belonging to tolerant varieties (Xu *et al.*, 2006).

The source country and collection site of the A-genome group wild rice accessions was studied using the System-wide Information Network for Genetic Resources (SINGER, http://singer.cgiar.org). The map localization showed the preferential presence of genotypes harbouring the *SUB1A* gene around the valleys of the Ganges and Brahmaputra rivers (Figure S7). The genotypes not harbouring the *SUB1A* gene showed a broader diffusion in all the southern regions of Asia (Figure S7).

SUB1A is absent in some submergence-tolerant Oryza species

Tolerant genotypes, other than *O. nivara* and *O. rufipogon*, showing a high flooding survival rate were screened for the presence of *SUB1A* (Table S4). The two flooding-tolerant species *O. rhizomatis* (IRGC-103421) and *O. eichingeri* (IRGC-101429) belonging to the C-genome group, which also showed a reduced elongation under submergence (Figures 1 and 4a), were found to lack the *SUB1A* gene (Figure 4b). No amplification was observed with any of the seven primer pairs of *SUB1A* (Xu *et al.*, 2006) used for the screening. Other accessions belonging to the C-genome group were then screened for the presence of *Sub1A* as well as for submergence tolerance and elongation under submergence. Results showed that all the accessions belonging to the C-genome group investigated lack the *SUB1A* gene (Figures 4b and S8), have reduced elongation under flooding and are tolerant to flooding stress (Figure 4c). A search for other *SUB1* orthologues in *O. rhizomatis* (IRGC-103421) and *O. eichingeri* (IRGC-101429) using degenerate primers in a PCR reaction resulted in a distinct band of an expected size (~600 bp) from genomic DNA (Figure S9). Another band of approximately 730 bp was also amplified in *O. rhizomatis* (Figure S9), and we found that it was an *ERF*-like gene, characterized by being similar to *SUB1A-2* (Xu *et al.*, 2006), but with significant differences from the *SUB1A* genes (Figure S10), as was also highlighted by the absence of an amplification with the *SUB1A*-specific primers (Figure 4b). This sequence also showed a premature stop codon, suggesting the presence of a truncated gene product.

Genomic sequences of the *SUB1* orthologues (600-bp band) isolated from the two submergence-tolerant species *O. eichingeri* and *O. rhizomatis* were compared with the *SUB1A*, *SUB1B* and *SUB1C* alleles of *O. sativa*. Sequence analysis revealed that *O. eichingeri* and *O. rhizomatis* possess a single *SUB1* gene orthologue with a high sequence similarity to the *SUB1C-1* allele found in cultivated rice, which we named *OeSub1C-1-L* and *OrhSub1C-1-L*, respectively (*SUB1C-1–Like*). In a pool of *O. eichingeri plants*, the *SUB1C-1-L* gene was found in five allelic forms, characterized by a high degree of single nucleotide polymorphism (Figure S11). These were named *OeSUB1C-1-L1*, *OeSUB1C-1-L2*, *OeSUB1C-1-L3*, *OeSUB1C-1-L4* and *OeSUB1C-1-L5*. All of these *SUB1C-1*-like genes shared more than 85% similarity in genomic sequences with the *SUB1C-1* allele found in *O. sativa* (Table 1). Nucleotide comparison of the wild *SUB1C-1-L* genes showed a strong similarity between the genes of different species (Table 2).

Phylogenetic analysis of *SUB1* gene orthologues

Multiple sequence analysis and the phylogenetic tree of *SUB1* genes found in *O. sativa*, *O. nivara* and *O. rufipogon*,

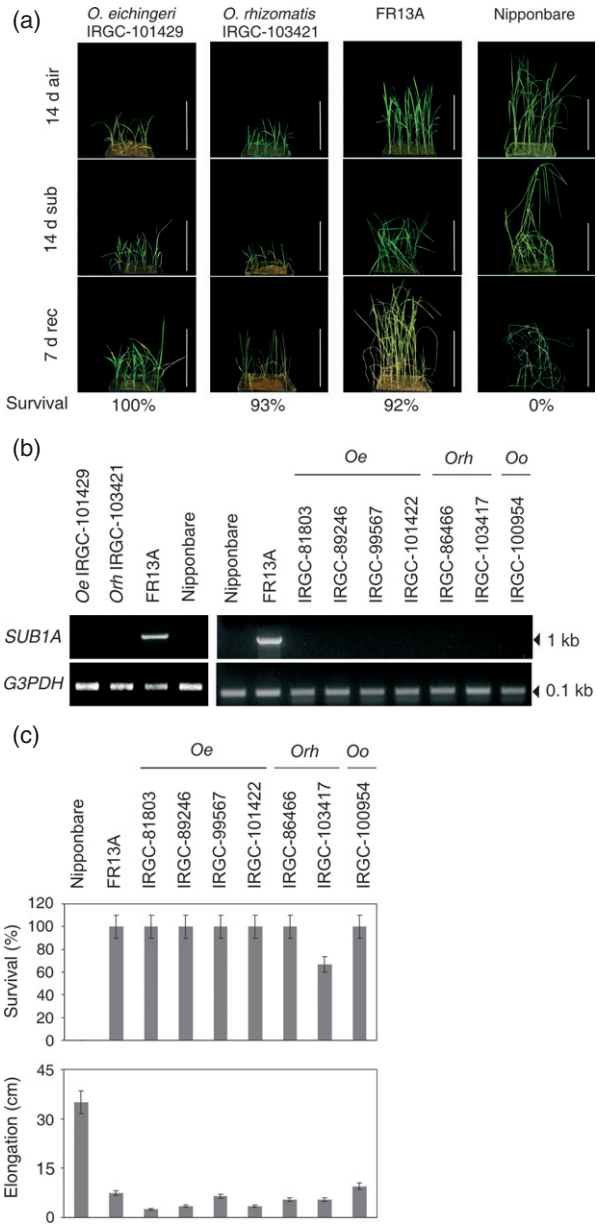

Figure 4. Comparative submergence response of tolerant wild rice genotypes belonging to the C-genome group. The two rice varieties FR13A and Nipponbare (Nip) were used as tolerant and sensitive internal controls, respectively.
(a) Plant phenotype and survival percentages of the rice genotypes *Oryza eichingeri* (IRGC-101429) and *Oryza rhizomatis* (IRGC-103421) after 14 days of complete submergence followed by 7 days of recovery.
(b) *SUB1A* genomic PCR amplification for gene screening in wild rice genotypes belonging to the C-genome group.
(c) Survival percentage and elongation under submergence of wild rice genotypes belonging to the C-genome group.
The abbreviations used are as follows: 14 d sub, 14 days of submergence; 7 d rec, 7 days of recovery. See the legend for Figure 1 for the other abbreviations. Scale bars: 15 cm.

identified by Fukao *et al.* (2009), together with the genes identified in this study showed that *SUB1A, SUB1B* and *SUB1C* were resolved into two distinct clades with significant bootstrap values (Figure 5). *SUB1C* alleles in species from the A- and C-genome groups were resolved into a distinct clade, showing that the genes were derived from a common ancestor, and that they diverged significantly during species differentiation. Moreover, the *SUB1C-1-L* genes of the wild C-genome group accessions were all grouped together, suggesting a substantial difference when compared with the A-genome subgroup.

Table 1 Percentage identity of partial genomic sequences of the *Oryza sativa SUB1C-1* gene and the orthologues in *Oryza eichingeri* (IRGC-101429) and *Oryza rhizomatis* (IRGC-103421)

Species and genome	Allele	*O. sativa* allele	Nucleotide identity (%)
O. eichingeri (C)	*OeSUB1C-1-L1*	*SUB1C-1*	88.6 (526/594)
	OeSUB1C-1-L2	*SUB1C-1*	87.6 (507/579)
	OeSUB1C-1-L3	*SUB1C-1*	85.2 (506/594)
	OeSUB1C-1-L4	*SUB1C-1*	88.1 (526/597)
	OeSUB1C-1-L5	*SUB1C-1*	87.7 (508/597)
O. rhizomatis (C)	*OrhSUB1C-1-L*	*SUB1C-1*	87.2 (506/591)

Expression of anaerobic genes in *O. rhizomatis*

The alignment between the genomic and the full-length mRNA sequences of the *OrhSUB1C-1-L1* allele revealed the presence of a full-length open reading frame corresponding to *O. sativa SUB1C-1* (Figure 6a). The expression of *OrhSUB1C-1-L* was increased by 3 days of submergence (Figure 6b) but, intriguingly, western-blot results indicated that the OrhSUB1C-1-L protein failed to accumulate either in air or submergence (Figure 6c). The transcript levels of hypoxia-inducible genes, such as alcohol dehydrogenase (*ADH2*) and pyruvate decarboxylase (*PDC2*), were induced in both *O. rhizomatis* (Figure 6b) and *O. eichingeri* (Figure S12) by the submergence treatment. Interestingly, although the level of ADH and PDC proteins was high in submerged Nipponbare, and particularly in the *SUB1A*-harbouring variety FR13A, these two proteins were barely detectable in *O. rhizomatis* samples (Figure 6c). The expression of *SLR1* and *SLRL1*, repressors of GA-dependent elongation, was induced in submerged *O. rhizomatis* and *O. eichingeri*, respectively (Figures 6b and S12).

DISCUSSION

The submergence-tolerant rice genotypes that have been examined so far (Xu *et al.*, 2006; Singh *et al.*, 2010) show a low oxygen quiescence syndrome (LOQS)-related growth mechanism in response to flooding (Bailey-Serres and Voesenek, 2008), and possess the *SUB1A* gene of the *SUB1* locus. Genotypes with haplotypes other than *SUB1A-1/SUB1C-1* are intolerant to submergence (Singh *et al.*, 2010).

We found that *O. rhizomatis* (IRGC-103421) and *O. eichingeri* (IRGC-101429), and other submergence-tolerant C-genome accessions, did not possess *SUB1A* gene orthologues (Figure 4). Despite the absence of *SUB1A*, these

Table 2 Percentage identity between *Oryza eichingeri* (IRGC-101429) and *Oryza rhizomatis* (IRGC-103421) partial genomic sequences of *SUB1C* orthologues

	OrhSUB1C-1-L	*OeSUB1C-1-L1*	*OeSUB1C-1-L2*	*OeSUB1C-1-L3*	*OeSUB1C-1-L4*	*OeSUB1C-1-L5*
OrhSUB1C-1-L		87.3	96.5	95.9	89.6	94.7
OeSUB1C-1-L1			89.6	85.6	96.7	91.7
OeSUB1C-1-L2				93.7	90.2	97.8
OeSUB1C-1-L3					87.6	92.1
OeSUB1C-1-L4						88.5
OeSUB1C-1-L5						

accessions did not elongate when submerged (Figure 4). *O. rhizomatis* (IRGC-103421) and *O. eichingeri* (IRGC-101429) possessed instead a *SUB1* gene similar to the *SUB1C-1* of domesticated submergence-tolerant *indica* rice (Figure S11; Table 1). The C-genome group is a defined monophyletic clade (Ge *et al.*, 1999), and phylogenetic and population genetic studies showed that the three closely related species belonging to this group, *O. rhizomatis*, *O. eichingeri* and *Oryza officinalis*, have diverged recently with a low level of species differentiation (Ge *et al.*, 1999; Bao and Ge, 2003; Bao *et al.*, 2006; Bautista *et al.*, 2006). The absence of *SUB1A* in C-genome rice species is not surprising. This gene has only two allelic variations in domesticated rice in comparison with the multiple alleles found for *SUB1B* and *SUB1C* genes, suggesting that *SUB1A* was created more recently than the other *SUB1* genes (Fukao *et al.*, 2009).

Underwater elongation in rice is triggered by ethylene and GA (Fukao *et al.*, 2006; Fukao and Bailey-Serres, 2008). Previous studies have shown that *SUB1C* is responsive to both ethylene and GA (Fukao *et al.*, 2006). In rice, it is suggested that, in the absence of *SUB1A-1*, *SUB1C* facilitates shoot elongation during drowning, through a GA-dependent mechanism (Fukao and Bailey-Serres, 2008). *SUB1A-1* reverses the ethylene-dependent increase in GA responsiveness and consequent *SUB1C* mRNA accumulation. This is achieved via a mechanism mediated by *SLR1* and *SLRL1*, which are both suppressors of GA responses, thereby repressing the GA-induced growth and carbohydrate breakdown (Fukao and Bailey-Serres, 2008). This underwater suppression of *SUB1C* mRNA accumulation in the presence of *SUB1A-1* has been observed in the introgressed line M202(*SUB1*) and in *SUB1A-1* overexpressing transgenic lines (Fukao *et al.*, 2006; Xu *et al.*, 2006). However, based on recombinant genetic studies, *SUB1A* seems to be the major determinant of submergence tolerance, as *SUB1C* allele expression level does not significantly affect the tolerance (Septiningsih *et al.*, 2009). *SUB1A* represses *SUB1C*, which might be negatively involved in rice tolerance to submergence (Fukao *et al.*, 2006). It is thus tempting to speculate that the absence of SUB1C protein observed in *O. rhizomatis* under submergence (Figure 6c) might enable *SUB1A*-less plants to avoid the otherwise inevitable enhanced growth when submerged.

Our discovery of flooding-tolerant rice accessions not containing the *SUB1A* gene suggests the presence of a yet unidentified *ERF* transcription factor with a similar function to *SUB1A-1*, or the presence of a different submergence tolerance mechanism. Indeed, several QTLs associated with submergence tolerance have already been described (Nandi *et al.*, 1997; Toojinda *et al.*, 2003), and recently non-*SUB1* QTLs for submergence tolerance were identified in the IR72 cultivar (Septiningsih *et al.*, 2012). The major *qSUB1.1* QTL on chromosome 1 of IR72 explains around 40% of the phenotypic variance (Septiningsih *et al.*, 2012), whereas the *SUB1A* QTL on chromosome 9 accounts for up to 69% of phenotypic variance in the IR40931-26 tolerant background (Xu and Mackill, 1996).

Our data provide evidence of intraspecific variations at the *SUB1* locus associated with distinction in submergence tolerance across rice species. We also found that *SUB1A* is not only confined to a few accessions of *indica* rice of *O. sativa*, but is also present in some accessions of the closely related A-genome species *O. nivara* and *O. rufipogon* (Figure 3). Fukao *et al.* (2009) reported the absence of *SUB1A* in two accessions of *O. nivara* and *O. rufipogon*, which were different from those used in our study, further highlighting intraspecific biodiversity. Intraspecific biodiversity in deep-water elongation capacity was also demonstrated by the variability of the *SNORKEL* regions in cultivars and wild rice species genome structure (Hattori *et al.*, 2009). The *O. nivara* and *O. rufipogon* genotypes described here with a high degree of survival under submergence and a reduced elongation showed the presence of the tolerance-specific *SUB1A-1* allele, whereas the other accessions of these species that were intolerant to submergence harboured the intolerance-specific *SUB1A-2* allele (Figure S4). This result was in agreement with the haplotype survey of the *SUB1A* locus determined by Xu *et al.* (2006). However, Singh *et al.* (2010) suggested that some level of tolerance might be conferred by high expression of the *SUB1A-2* allele.

Several wild rice species showed reduced growth under submergence and displayed an enhanced survival rate (Figures 1 and 2). This 'quiescence' response eventually leads to a higher survival rate through minimal shoot elongation and restriction of carbohydrate consumption

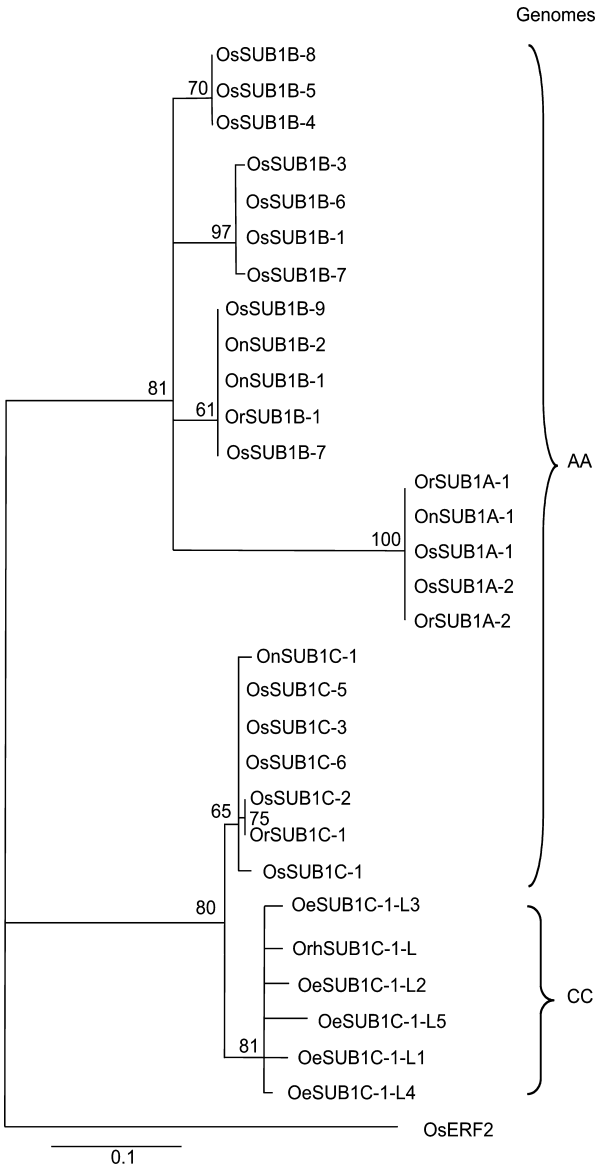

Figure 5. Phylogenetic tree of *SUB1* genes of *Oryza sativa*, *Oryza nivara* and *Oryza rufipogon* together with the orthologues found in *Oryza eichingeri* (IRGC-101429) and *Oryza rhizomatis* (IRGC-103421). The phylogenetic tree was constructed using the central part of the sequences for the maximum likelihood method implemented in PhyML 3.0 (aLRT). *ERF2* (LOC_Os01g21120) was used as an out-group. The length of each branch is proportional to the sequence divergence. The bootstrap values from 100 replicates are shown above the branching nodes. See the legend for Figure 1 for the abbreviations used.

for anaerobic energy production, which is a beneficial adaptive trait for deep and prolonged submergence conditions (Kende *et al.*, 1998). This strategy is also beneficial under flash flooding conditions, as when submerged the plant does not consume energy that can be used after the water recedes (Nagai *et al.*, 2010). However, we did not find any correlation between elongation and survival ability when considering all the genotypes screened (Figure S2), indicating that a reduced growth rate is not sufficient for sustaining survival during prolonged submergence. Rice accessions in which reduced growth under submergence is not associated with improved viability might have a less successful energy management, and/or cellular homeostasis, than the *SUB1A*-harbouring accessions. Exploration of the metabolome of various *SUB1* haplotypes could shed light on this interesting aspect.

The A-genome group wild rice accessions investigated that harboured the *SUB1A* gene are preferentially located around the Brahmaputra and Gages Delta (Figure S7). Yet the original site of collection of the *O. rhizomatis* and *O. eichingeri* accessions without a *SUB1A*-like gene corresponds instead to Sri Lanka and Uganda, respectively. This supports the presence of distinct evolutionary paths leading to two distinct mechanisms of growth suppression and survival. In this context, *O. eichingeri* is the only wild rice isolated in both Asia (e.g. Sri Lanka) and Africa (e.g. Uganda and Cote d'Ivoire), suggesting a large ancestral population that was then subdivided, or a long-distance dispersal between two continents (Zhang and Ge, 2007). Intriguingly, the *O. eichingeri* and *O. rhizomatis* populations overlap in both the northern and the southern regions of Sri Lanka (Bautista *et al.*, 2006). It is interesting to note that the neighbour-joining tree of the *ADH1* sequence in C-genome species showed *O. rhizomatis* (IRGC-103421) to be associated with the *O. eichingeri* accession coming from Uganda, rather than other *O. rhizomatis* accessions (Zhang and Ge, 2007).

In conclusion, this large-scale rice accession screening produced two findings. Firstly, *SUB1A* is not restricted to the *O. sativa* species, but can be found in some submergence-tolerant A-genome wild rice accessions. The tolerant *SUB1* haplotype is also conserved among tolerant cultivars and A-genome wild rice genotypes. Secondly, the presence of *SUB1A-1* is not essential to confer submergence tolerance in secondary gene pools of rice. *O. rhizomatis* (IRGC-103421) and *O. eichingeri* (IRGC-101429) accessions are submergence tolerant, despite being devoid of *SUB1A-1*. The presence of a *SUB1C*-like variant in wild relatives of rice belonging to the C-genome group is intriguing, and deserves further study. The mechanism of tolerance in *O. rhizomatis* and *O. eichingeri* involves reduced elongation, but is independent from the enhanced expression of AHD and PDC, further highlighting the existence of a mechanism of submergence tolerance that is distinct from the one controlled by SUB1A.

EXPERIMENTAL PROCEDURES

Plant material and submergence treatments

The rice accessions analysed in this study were selected using the International Rice Gene Bank Collection Information System (IRGCIS) (http://irfgc.irri.org). One hundred and nine rice genotypes were chosen to represent different genomic groups and to

(a)

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OrSUB1C-1-L1    MRRGVSSS-SSSSSSSPARDHKRRRSRRKLVVDEDWEAAFREFAARDDED
OsSUB1C-1       MRRRVSSSPSSSSSSSPARHHKARRSRRKLVADEDWEAAFREFLSRDDDD
                *** **** **********.** ********.*********** :***:*

OrSUB1C-1-L1    DDGGLDDHHHHVVVAPLIRS-NKCIHGHEV--TTIGGGAPPSRSRRRADD
OsSUB1C-1       DDD--DDDGHHVVVAPLIRSSNKCVHGHEVVASTVGGGASGGRRRADDD-
                **.  **. ********** ***:*****  :*:****. .* *   *

OrSUB1C-1-L1    GGER--RRRREKRSYPYRGIRHRPWGRWASEIRDPVKGIRVWLGTFDTAE
OsSUB1C-1       DGERRRRRRRERRSYPYRGIRQRPWGRWASEIRDPVKGIRVWLGTFDTAE
                .***  *****:*********:**************************
                                      ERF domain
OrSUB1C-1-L1    GAARAYDDEVRLIYGRNAKTNFPPAPPPPPEQPAPVAAESSPST---TTP
OsSUB1C-1       GAARAYDDEVRRIYGGNAKTNFPPSPPPPEQPAAPVAAERSPSTTTTTTP
                ********** *** ********:**** : .****** ****   ***

OrSUB1C-1-L1    TAEDSGNSHILIECCSDDLMDSLLAAFDMTAGDLDRRIWN
OsSUB1C-1       SAEDSGDSRILIECCSDDLMDSLLAAFDMTTGDM--RFWS
                :*****:*:**********************:**:  *:*.
```

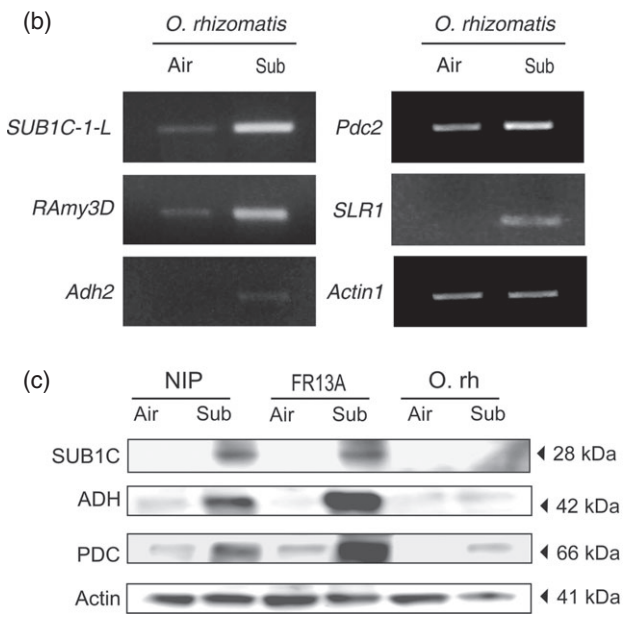

Figure 6. Anaerobic genes in *Oryza rhizomatis*.
(a) Amino acid alignment of the *SUB1C-1* allele of *Oryza sativa* and the *SUB1C-1* orthologue *Orh-SUB1C-1-L1* found in the *Oryza rhizomatis* (IRGC-103421) wild accession. The highly conserved DNA binding ERF domain is underlined. Amino acid identity, and semi-conserved and conserved substitutions are indicated with an asterisk, a dot and two dots, respectively. The nucleotide alignment of the *SUB1C-1* orthologues found in *Oryza rhizomatis* (IRGC-103421) is available in Figure S11.
(b) Gene expression pattern analysed by semi-quantitative RT-PCR of anaerobic gene transcripts induced after 3 days of submergence stress in the whole shoot of the *Oryza rhizomatis* (IRGC-103421) wild accession. *Actin1* was used as a loading control.
(c) Immunoblotting of anaerobic proteins extracted from *Oryza sativa* cv. Nipponbare (NIP) and FR13A and *Oryza rhizomatis* whole shoot under submergence. The antibody for actin was used as the loading and transfer control.
See the legend for Figure 1 for a list of abbreviations.

guarantee coverage of various geographic areas of origin and habitat (Table S1). Seeds were supplied by the International Rice Research Institute (IRRI, http://www.irri.org) and the Nepal Agricultural Research Council (NARC, http://narc.gov.np). For the screening of submergence-tolerant plants, seeds of 109 rice genotypes (Table S1) were soaked in Petri dishes with water-wetted filter paper kept at 28 ± 2°C in the dark for 2–3 days. Approximately 60 pre-germinated caryopses were sown in plastic pots filled with 2 kg of topsoil. The seedlings were grown for 14 days at 28 ± 2°C with a 12-h photoperiod (light intensity ~50 μmol m^{-2} s^{-2}). Twelve days after sowing, plants were thinned to 18 per pot in a completely randomized experimental design (CRD), with three replications. To evaluate the submergence response, 14-day-old seedlings were completely submerged (leaves below the water level) with tap water for a further 14 days in 300-L plastic tanks (of 88 cm in depth). The two rice varieties FR13A and Nipponbare were used as tolerant and sensitive internal controls, respectively (Xu *et al.*, 2006). Plant height was recorded after 14 days of submergence; plant survival was estimated after 14 days of submergence followed by 7 days of recovery. Tolerant genotypes are those actively re-growing after de-submergence (>40% of survival; Lee *et al.*, 2009). To select genotypes with reduced growth, the elongation index was calculated as follows: (length of shoots submerged for 14 days – length of shoots at the beginning of submergence)/(length of aerobic-grown shoots after 14 days – length of shoots at the beginning of submergence). In this way, when the index is >1 elongation is a result of the submergence treatment.

For the gene expression analysis, dehulled seeds of *O. rhizomatis* (IRGC-103421, collected in Sri Lanka) and *O. eichingeri* (IRGC-101429, collected in Uganda) were sterilized in 70% (v/v) ethanol for 2 min and then in 3% (v/v) sodium hypochlorite for 20 min. After sterilization, seeds were rinsed thoroughly with sterilized distilled water. Fifteen seeds of each rice species were sown in 5-L glass bottles containing 250 ml of half-strength Murashige and Skoog medium (pH 5.8), and grown at 25°C for 9 days with a 12-h photoperiod (light intensity ~50 μmol m^{-2} s^{-2}). For the submergence treatment, the glass bottles were filled with 5 L of sterilized distilled water and incubated for 3 days at 25°C under fluorescent light and a 12-h photoperiod (light intensity ~50 μmol m^{-2} s^{-2}). A pool of aerial parts of submerged and aerobic-grown seedlings was

harvested after 3 days of treatment, immediately frozen in liquid nitrogen and stored at −80°C until use. Single plants were also collected for a more detailed analysis.

Confirmation of the rice genotype accession *O. eichingeri* IRGC-101429, previously classified as *Oryza punctata* (Miyabayashi *et al.*, 2007) was obtained on the basis of rice chloroplast microsatellite marker screening (Ishii and McCouch, 2000). The primers and PCR conditions used are listed in Table S5.

Monitoring of the SUB1 haplotype

The genomic DNA of 33 rice accessions was prepared using the GenElute™ Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, http://www.sigmaaldrich.com), following the manufacturer's protocol. The PCR reaction mixture was prepared in 20 μl of total volume using Red Taq Master Mix (Invitrogen, http://www.invitrogen.com), 0.25 μM primers and 100 ng DNA. PCR was performed using *SUB1A* genomic-specific primers on all the accessions, and a *SUB1B* and *SUB1C* genomic survey was performed on *O. nivara* (IRGC-80507), in accordance with Xu *et al.* (2006) (Table S5). PCR products of positive amplifications were gel purified, cloned into pGEM®-T Easy Vector (Promega, http://www.promega.com) and sequenced on both strands using standard procedures. The screening between the *SUB1A-1* and the *SUB1A-2* alleles in the other genotypes was obtained by digesting the amplicons with the *BsrI* enzyme, cutting only the *SUB1A-1* gene in position 681–683.

Screening of *SUB1* gene orthologues by PCR and cloning

Total DNA from the rice genotypes *O. eichingeri* (IRGC-101429) and *O. rhizomatis* (IRGC-103421) was isolated as described above. The genomic PCR was performed in a 50-μl reaction volume containing 250 ng of DNA template, 1 U Phusion DNA Polymerase (Finnzymes, http://www.finnzymes.com), 0.2 mM deoxynucleotide triphosphates and 0.5 μM primers. Primer pairs and PCR conditions used for the amplification of *SUB1* genes were in agreement with Fukao *et al.* (2009) (Table S5). Amplified PCR products were purified using Wizard® SV Gel and the PCR Clean-Up System (Promega). Gel-purified amplicons were cloned into pGEM®-T Easy Vector (Promega), as described in the user manual, and sequenced on two strands following the standard procedures. The PCR products of the 600-bp band from the selected genotypes together with the 730-bp band of *O. rhizomatis* were extracted from the gel, purified and cloned (22 independent clones for each band).

Isolation of *O. rhizomatis* and *O. eichingeri SUB1* orthologues

Total RNA was isolated from the whole shoot of submerged *O. rhizomatis* (IRGC-103421) and *O. eichingeri* (IRGC-101429) plants using the RNAqueous Plant Mini Kit (Ambion, now Invitrogen, http://www.invitrogen.com), according to the manufacturer's instructions. Isolated RNA was treated with DNase using the Turbo DNA-free™ Kit (Applied Biosystems, http://www.appliedbiosystems.com). Amplification of the *O. rhizomatis* full-length sequence and the *O. eichingeri* 3′ untranslated region (UTR) was performed following the 5′–3′ RACE Kit manufacturer's protocol (Roche Applied Science, http://www.roche-applied-science.com). PCR products were gel purified, cloned into a pGEM®-T Easy Vector (Promega) and sequenced on both strands using standard procedures. The presence of introns/exons was evaluated by designing primers in the 5′ and 3′ UTRs, and amplifying the DNA and cDNA to obtain the whole genomic and coding sequences, respectively. All the primers used for 5′–3′ rapid amplification of cDNA ends (RACE) PCR are listed in Table S5.

Gene expression experiments

Total RNA extraction and DNAse treatment were performed as described above. One microgram of RNA was reverse-transcribed using SuperScript® III Reverse Transcriptase (Invitrogen), according to the manufacturer's protocol. Additional reverse transcriptions were performed with AMV (Promega) reverse transcriptase to avoid possible experimental artefacts (Houseley and Tollervey, 2010). RT-PCR was performed using the GoTaq® Green Master mix (Promega) in a reaction mixture of 25 μl, containing 100 ng cDNA and 1 μM primers. The number of cycles and the annealing temperature for each primer pair were optimized. The level of the *Actin1* transcript was used as an internal loading control. Gene-specific primers for *O. rhizomatis* (IRGC-103421) and *O. eichingeri* (IRGC-101429) *SUB1C* orthologues were designed on the basis of the sequence obtained by the 5′–3′ RACE PCR. Primers for the flooding stress-inducible genes were in accordance with those used by Fukao *et al.* (2006) and Fukao and Bailey-Serres (2008). All the primers used for the semi-quantitative RT-PCR are listed in Table S5.

Sequence data analysis

The nucleotide and amino acid sequences obtained in this study were aligned using EMBOSS (Labarga *et al.*, 2007) and CLASTALW2 (Larkin *et al.*, 2007). For the phylogenetic analyses, partial sequences of the *SUB1* genes from *O. sativa* and their wild orthologues were aligned with the out-group sequence *ERF2* (LOC_Os01g21120), belonging to subgroup VII of the ERF rice gene family (Fukao *et al.*, 2009), using the T-COFFEE 6.85 alignment tool. The phylogenetic tree was derived from this multiple alignment, using the maximum likelihood method (http://www.phylogeny.fr; Dereeper *et al.*, 2008). The precision and significance of the phylogenetic tree were assessed using a bootstrap analysis with 100 replicates.

Immunoblotting

Proteins were extracted as described by Banti *et al.* (2010) from the whole shoot of control and submerged plants. Total protein content was quantified with a BCA Protein Assay (Pierce, http://www.piercenet.com). SDS-PAGE was performed on a 10% Criterion polyacrylamide gel (Bio-Rad Laboratories, http://www.bio-rad.com). Blotting on an Amersham Hybond-P polyvinylidene difluoride membrane was performed with a Novablot electrophoretic transfer system (Amersham Pharmacia Biotech, now GE Healthcare, http://www.gelifesciences.com). Immunoblotting was performed using Immun-Star HRP Chemiluminescent Detection Kits (Bio-Rad Laboratories). The antibodies against Sub1C, ADH and PDC were purchased from Agrisera (product code AS11 1770, AS10 685 and AS10 691, respectively; Agrisera, http://www.agrisera.com). Immunoblotting using the antibody against Actin (AS10 702; Agrisera) was used to confirm correct loading and transfer.

Accession numbers

Sequences were submitted to the GenBank EMBL data library under accession number HM117839, FR720457-FR720461 and FR720463-720467.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Stem and leaf elongation of rice genotypes under submergence.

Figure S2. Scatter plot showing the degree of association between the percentage survival and the elongation index of rice genotypes.

Figure S3. Aminoacid alignment of the *SUB1A* alleles of *O. sativa*, *SUB1A-1* orthologues of *O. nivara* (IRGC-80507 and IRGC-101508) and *O. rufipogon* (IRGC-81882), and the *SUB1A-2* orthologues of *O. rufipogon* (IRGC-82982 and IRGC-105960).

Figure S4. Nucleotide alignment of *SUB1A* alleles of *O. sativa*, *SUB1A-1* orthologues of *O. nivara* (IRGC-80507 and IRGC-101508) and *O. rufipogon* (IRGC-81882), and *SUB1A-2* orthologues of *O. rufipogon* (IRGC-82982 and IRGC-105960).

Figure S5. Nucleotide alignment of *SUB1C* alleles of *O. sativa* and the *SUB1C* orthologue found in *O. nivara* (IRGC-80507), tolerant to submergence.

Figure S6. Nucleotide alignment of *SUB1B* alleles of *O. sativa* and the *SUB1B* orthologue found in *O. nivara* (IRGC-80507), tolerant to submergence.

Figure S7. Location of the original collection sites of the A-genome group wild genotypes, with or without *SUB1A* genes.

Figure S8. Additional C-genome rice accessions showing no amplification of the *Sub1A* gene.

Figure S9. *SUB1* orthologues amplified by genomic PCR in *O. rhizomatis* (IRGC-103421) and *O. eichingeri* (IRGC-101429).

Figure S10. Alignment of truncated genomic sequences of *SUB1A-1* and *SUB1A-2* from *O. sativa* and the AP2 domain-containing protein obtained from the *O. rhizomatis* (*Orh*) 730-bp amplicon.

Figure S11. Alignment of truncated genomic sequences of *SUB1* gene orthologues from *O. sativa*, *O. eichingeri* (*Oe*) and *O. rhizomatis* (*Orh*).

Figure S12. Gene expression pattern analyzed by semi-quantitative RT-PCR of anaerobic gene transcripts induced after 3 days of submergence stress in whole shoot of the *O. eichingeri* (IRGC-101429) wild accession.

Table S1. List of the *Oryza* genotypes used for the screening of submergence tolerance.

Table S2. Statistical analysis of differences in elongation among rice accessions.

Table S3. Statistical analysis of differences in survival among rice accessions.

Table S4. List of submergence-tolerant C-genome wild rice accessions used to monitor the presence of *SUB1A* genes.

Table S5. List of primers used for *SUB1A* monitoring, cloning, 5′–3′ RACE PCR, semi-quantitative RT-PCR and species validation in rice genotypes.

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