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 (O2) diffusion rate (~10 000-fold less) during flooding is accompanied by a reduction in cellular O2 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 germlasm 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 SUB1B and SUB1C do not seem to be important in conferring this trait (Seiptiningsih 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 suppressor 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 intergeneric 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 intergeneric crosses is very low (Yan et al., 1997), the study of these species has 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, Adiurmi 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%
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...
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-82982 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

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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.cigar.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 OeSub1C-1-L1, 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,
identified by Fukao et al. (2009), together with the genes identified in this study showed that \( \text{SUB1A} \), \( \text{SUB1B} \) and \( \text{SUB1C} \) were resolved into two distinct clades with significant bootstrap values (Figure 5). \( \text{SUB1C} \) 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 \( \text{SUB1C-1-L} \) genes of the wild C-genome group accessions were all grouped together, suggesting a substantial difference when compared with the A-genome subgroup.

**Expression of anaerobic genes in \( O. \text{rhizomatis} \)**

The alignment between the genomic and the full-length mRNA sequences of the \( \text{OrhSUB1C-1-L1} \) allele revealed the presence of a full-length open reading frame corresponding to \( \text{O. sativa SUB1C-1} \) (Figure 6a). The expression of \( \text{OrhSUB1C-1-L} \) was increased by 3 days of submergence (Figure 6b) but, intriguingly, western-blot results indicated that the \( \text{OrhSUB1C-1-L} \) protein failed to accumulate either in air or submergence (Figure 6c). The transcript levels of hypoxia-inducible genes, such as alcohol dehydrogenase (\( \text{ADH2} \)) and pyruvate decarboxylase (\( \text{PDC2} \)), were induced in both \( O. \text{rhizomatis} \) (Figure 6b) and \( O. \text{eichingeri} \) (Figure S12) by the submergence treatment. Interestingly, although the level of ADH and PDC proteins was high in submerged \( N. \text{baret} \), and particularly in the \( \text{SUB1A} \)-harbouring variety \( \text{FR13A} \), these two proteins were barely detectable in \( O. \text{rhizomatis} \) samples (Figure 6c). The expression of \( \text{SLR1} \) and \( \text{SLRL1} \), repressors of GA-dependent elongation, was induced in submerged \( O. \text{rhizomatis} \) and \( O. \text{eichingeri} \), 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 Vooske, 2008), and possess the \( \text{SUB1A} \) gene of the \( \text{SUB1} \) locus. Genotypes with haplotypes other than \( \text{SUB1A-1/SUB1C-1} \) are intolerant to submergence (Singh et al., 2010).

We found that \( O. \text{rhizomatis} \) (IRGC-103421) and \( O. \text{eichingeri} \) (IRGC-101429), and other submergence-tolerant C-genome accessions, did not possess \( \text{SUB1A} \) gene orthologues (Figure 4). Despite the absence of \( \text{SUB1A} \), these
seems to be the major determinant of submergence (Figure 4). O. rhizomatis (IRGC-101429) 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., 2008). 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 over-expressing 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 qSUB1-1I 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 68% 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.

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

<table>
<thead>
<tr>
<th>Subspecies</th>
<th>OeSUB1C-1-L1</th>
<th>OeSUB1C-1-L2</th>
<th>OeSUB1C-1-L3</th>
<th>OeSUB1C-1-L4</th>
<th>OeSUB1C-1-L5</th>
</tr>
</thead>
<tbody>
<tr>
<td>OrhSUB1C-1-L</td>
<td>87.3</td>
<td>96.5</td>
<td>95.9</td>
<td>89.6</td>
<td>94.7</td>
</tr>
<tr>
<td>OeSUB1C-1-L1</td>
<td>88.6</td>
<td>85.6</td>
<td>93.7</td>
<td>90.2</td>
<td>97.8</td>
</tr>
<tr>
<td>OeSUB1C-1-L2</td>
<td></td>
<td>90.2</td>
<td>87.6</td>
<td>92.1</td>
<td></td>
</tr>
<tr>
<td>OeSUB1C-1-L3</td>
<td></td>
<td></td>
<td>89.6</td>
<td>85.6</td>
<td>97.6</td>
</tr>
<tr>
<td>OeSUB1C-1-L4</td>
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<td></td>
<td></td>
<td>96.7</td>
<td>91.7</td>
</tr>
<tr>
<td>OeSUB1C-1-L5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>98.5</td>
</tr>
</tbody>
</table>
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 harbour the SUB1A gene are preferentially located around the Brahmaputra and Ganges 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 ADH 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
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°C 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°C with a 12-h photoperiod (light intensity 2450 l mol m² 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 Mini Prep Kit (Sigma-Aldrich, http://www.sigmaaldrich.com), following the manufacturer’s protocol. The PCR reaction mixture was prepared in 20 µl 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 genic-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 transcription 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 891, 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 submersion.

**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 submersion.

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

**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. rhi-

zomatis* (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 submersion stress in whole shoot of the *O. eichingeri* (IRGC-101429) wild accession.

**Table S1.** List of the *Oryza* genotypes used for the screening of submersion 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 submersion-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.

**REFERENCES**


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