

## Evolutionary Analysis of the *Sub1* Gene Cluster that Confers Submergence Tolerance to Domesticated Rice

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• **Background and Aims** Tolerance of complete submergence is recognized in a small number of accessions of domesticated Asian rice (*Oryza sativa*) and can be conferred by the *Sub1A-1* gene of the polygenic *Submergence-1* (*Sub1*) locus. In all *O. sativa* varieties, the *Sub1* locus encodes the *ethylene-responsive factor* (*ERF*) genes *Sub1B* and *Sub1C*. A third paralogous *ERF* gene, *Sub1A*, is limited to a subset of *indica* accessions. It is thought that *O. sativa* was domesticated from the gene pools of the wild perennial species *O. rufipogon* Griff. and/or the annual species *O. nivara* Sharma et Shastry. The aim of this study was to evaluate the orthologues of the *Sub1* locus in the closest relatives of *O. sativa* to provide insight into the origin of the gene and allelic variation of the *Sub1* locus.

• **Methods** Orthologues of the *Sub1* genes were isolated from *O. rufipogon* and *O. nivara* by use of oligonucleotide primers corresponding to the most highly conserved regions of the *Sub1* genes of domesticated rice. The phylogenetic relatedness of *Sub1* genes of *O. sativa* and its wild relatives was evaluated.

• **Key Results and Conclusions** Both *O. rufipogon* and *O. nivara* possess two *Sub1* gene orthologues with strong sequence identity to the *Sub1B* and *Sub1C* alleles of cultivated rice. The phylogeny of the *Sub1* genes of the domesticated and wild rice suggests that *Sub1A* arose from duplication of *Sub1B*. Variation in *Sub1B* alleles is correlated with the absence or presence of *Sub1A*. Together, the results indicate that genetic variation at the *Sub1* locus is due to gene duplication and divergence that have occurred both prior to and after rice domestication.

**Key words:** *Oryza sativa*, *Oryza nivara*, *Oryza rufipogon*, submergence tolerance, *Sub1* genes, gene duplication.

### INTRODUCTION

Rice (*Oryza sativa*) is cultivated in flooded paddy fields since its culture requires a large amount of water. However, excessive rainfall and poor regulation of paddy water level can result in partial and complete inundation of aerial tissue, which compromises crop productivity. Indeed, most rice cultivars die within 2 weeks of complete submergence, causing serious loss of rice production in South and South-east Asia (Xu *et al.*, 2006).

Rice varies dramatically in its response to flooding and submergence. Deep-water and most lowland rice accessions accelerate carbohydrate consumption and gibberellic acid-promoted elongation growth when submerged to escape the inundation (Fukao and Bailey-Serres, 2008). This response strategy is effective if floodwaters are shallow or rise gradually. Only a few exceptional rice cultivars, including Flood Resistant 13A (FR13A) can endure complete submergence for 2 weeks or longer (Setter and Laureles, 1996; Fukao *et al.*, 2006; Xu *et al.*, 2006). The submergence response in this as well as other rice accessions is regulated by the *Submergence-1* (*Sub1*) locus, which encodes a variable cluster of up to three *ethylene-responsive factor* (*ERF*) genes: *Sub1A*, *Sub1B* and *Sub1C* (Fig. 1; Fukao *et al.*, 2006; Xu *et al.*, 2006). The *Sub1* genes are members of group VII in the *ERF* gene family (Nakano *et al.*, 2006) and are more closely related to one another than any other rice *ERF* genes (Gutterson and Reuber, 2004). The two *ERF* genes, *Sub1B*

and *Sub1C* are present in all *indica* and *japonica* accessions examined to date, whereas *Sub1A* appears to be limited to a subset of *indica* accessions (Xu *et al.*, 2006; S. Heuer, IRRI, Philippines, pers. comm.). Two alleles of *Sub1A* (*Sub1A-1* and *Sub1A-2*) have been recognized, which are distinguished by a single amino acid substitution within the coding region. The transcripts of *Sub1A-1* and *Sub1A-2* are highly and poorly induced under submergence, respectively (T. Fukao, S. Heuer, D. Mackill and J. Bailey-Serres, unpubl. res.). Interestingly, only submergence-tolerant accessions possess the *Sub1A-1* allele, whereas accessions that contain the less highly expressed *Sub1A-2* are submergence-intolerant. Moreover, *Sub1A* is absent from all *japonica* and some *indica* accessions, all of which are intolerant to submergence. In support of *Sub1A-1* as the key determinant of submergence tolerance, the over-expression of the allele in the intolerant *japonica* Liaogeng was confirmed to be sufficient to markedly enhance submergence tolerance (Xu *et al.*, 2006).

*Oryza rufipogon* and *O. nivara* as well as *O. sativa* belong to the A-genome group of the genus *Oryza* (Khush, 1997; Wing *et al.*, 2005; Vaughan *et al.*, 2008). *Oryza rufipogon* is a wild perennial species which adapts to persistently wet habitats, whereas *O. nivara* is an annual species which inhabits a seasonally dry environment (Li *et al.*, 2006a; Vaughan *et al.*, 2008). *Oryza nivara* is sometimes considered to be an ecotype or subspecies of *O. rufipogon* and these names are not differentiated when referring to wild rice (Londo *et al.*, 2006; Kovach *et al.*,

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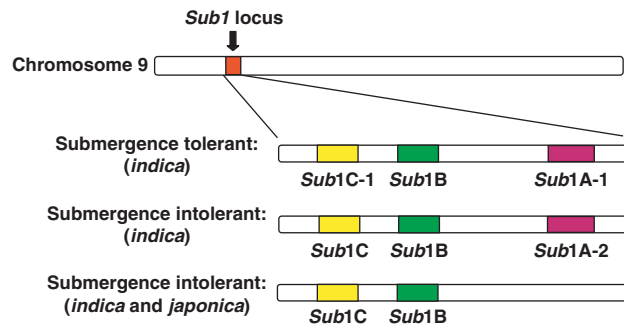


FIG. 1. *Sub1* haplotypes of *O. sativa*. The *Sub1* locus encodes two or three ethylene-responsive factors, *Sub1A*, *Sub1B* and *Sub1C*. Only submergence-tolerant accessions contain the *Sub1A-1* allele at the locus, which confers submergence tolerance to rice (Fukao *et al.*, 2006; Xu *et al.*, 2006).

2007; Sweeney and McCouch, 2007). The history of rice domestication has long been controversial. Recent genome-scale analyses demonstrate that *japonica* and *indica* cultivars of domesticated rice (*O. sativa*) are closely related to different accessions of wild rice, suggesting that the two subspecies were domesticated from divergent wild populations (Lu *et al.*, 2002; Cheng *et al.*, 2003; Garris *et al.*, 2005; Londo *et al.*, 2006; Kovach *et al.*, 2007; Sweeney and McCouch, 2007; Sang and Ge, 2007). However, allelic surveys of genes associated with shattering, pericarp colour and amylose content suggest that the key domestication genes originated only once (Yamanaka *et al.*, 2004; Li *et al.*, 2006b; Sweeney *et al.*, 2006; Kovach *et al.*, 2007; Lin *et al.*, 2007; Sang and Ge, 2007; Vaughan *et al.*, 2008). Based on these conflicting observations, single and multiple domestication hypotheses have been proposed. However, both hypotheses agree that *O. sativa* was domesticated from the gene pool of the wild-rice species, *O. rufipogon* and/or *O. nivara*.

Molecular genetic analyses have demonstrated that the polygenic *Sub1* locus of *O. sativa* consists of a variable cluster of closely related *ERF* genes (*Sub1A*, -*B*, -*C*) and confirmed that ectopic expression of at least one allele of *Sub1A* is sufficient to confer submergence tolerance (Fukao *et al.*, 2006; Xu *et al.*, 2006). The polygenic nature of the *Sub1* locus raises questions about the evolutionary timing of the gene duplication and source of the allelic variation at *Sub1* that is associated with submergence tolerance. In this study, two orthologues of *Sub1B* and *Sub1C* of *O. sativa* were identified in both *O. rufipogon* and *O. nivara*. Phylogenetic analysis of *Sub1* gene sequences in diverse accessions of domesticated rice revealed that alleles of *Sub1B* and *Sub1C* can be distinguished based on the absence of *Sub1A* and its allelic variation.

## MATERIALS AND METHODS

### Plant material and submergence treatment

Dehulled seeds of *O. nivara* (IRGC-101524 from the National Plant Germplasm System, USDA; originally collected in India) were sterilized in 70% (v/v) ethanol for 10 min and in 2% (v/v) sodium hypochlorite for 20 min.

After rinsing thoroughly with sterilized deionized water, each seed was incubated in 3 mL of 1× Murashige and Skoog medium (pH 5.8) in a 16 mm × 15 cm test tube at 20°C for 10 d (16 h light/8 h dark; light intensity, 50 μmol m<sup>-2</sup> s<sup>-1</sup>). For the submergence treatment, the test tube was filled with 18 mL of sterilized distilled water, closed with a loose plastic cap, and incubated for 3 d at 25°C in the light (50 μmol m<sup>-2</sup> s<sup>-1</sup>). After treatment, aerial organs of submerged plants were immediately frozen in liquid nitrogen and stored at -80°C until use.

### Amplification of genomic DNA by polymerase chain reaction (PCR) and cloning

Genomic DNA of *O. nivara* (IRGC-80470; originally collected in India) and *O. rufipogon* (IRGC-80506) was kindly provided by Dr Tao Sang (Michigan State University). Genomic PCR was performed in a 50-μL reaction containing 100 ng genomic DNA, 5 μL 10× PCR buffer (Qiagen, CA, USA), 10 μL Q-solution (Qiagen), 1 μM primers, 0.3 mM deoxynucleotide triphosphates and 2.5 U Proofstart DNA polymerase (Qiagen). Primers used for amplification of *Sub1* genes were: *Sub1*-forward (5'-GAVGAMTGGGAGGCCCGCTTCCRSAGATTC-3'), and *Sub1*-reverse (5'-GTCGWAGSCGGCGAGGAGGCTGTCCATC-3'), where M = A or C, R = A or G, S = C or G, V = A or C or G, and W = A or T.

After denaturing the genomic DNA template at 95°C for 5 min, PCR was performed with 30 cycles of denaturing at 95°C for 45 s, annealing at 65°C for 45 s, extension at 72°C for 60 s, and final extension incubation at 72°C for 15 min. Amplified DNA products were cloned into the pGEM-T vector (Invitrogen, CA, USA) and sequenced on both strands using standard procedures.

### 5' and 3' RACE

To isolate the full length of mRNA sequence, 5'- and 3'-RACE amplification was performed using the Gene Racer kit (Invitrogen) according to the manufacturer's protocol. Total RNA was isolated from shoots of submerged *O. nivara* (IRGC-101524) plants using the RNeasy Plant Mini Kit (Qiagen). The GeneRacer RNA oligo containing 5' adaptor was ligated to 5' decapped mRNAs and the modified mRNAs were reverse transcribed using SuperScript III reverse transcriptase and the Gene Racer oligo dT containing 3' adaptor. 5'- and 3'-RACE amplification was performed by PCR with gene-specific primers (*Sub1*-forward or *Sub1*-reverse) and Gene Racer primers supplied with the kit, as described for the amplification of genomic DNA by PCR. PCR products were gel purified, cloned into pCR4Blunt-TOPO vector (Invitrogen), and sequenced on both strands using standard procedures.

### Semi-quantitative assessment of mRNA abundance by reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA extraction from aerial tissue, cDNA synthesis and RT-PCR were performed exactly as described in Fukao *et al.* (2006). Sequences of primer pairs, annealing

temperature and cycle number used for RT-PCR are given in Table S1 in Supplementary Information available online. The level of *Actin1* mRNA was used as a loading control. The number of cycles for logarithmic amplification of RT-PCR was optimized by technical replications with a range of cycle numbers (i.e. 27, 30, 33 and 36 cycles) for each primer pair.

#### Sequence divergence and phylogenetic analyses

For direct comparison of *Sub1* genes/alleles, the nucleotide and amino acid sequences were subjected to pairwise and multiple alignment analyses using EMBOSS (Labarga *et al.*, 2007; <http://www.ebi.ac.uk/emboss/align/>) and ClustalW2 (Larkin *et al.*, 2007; <http://www.ebi.ac.uk/Tools/clustalw2/>), respectively. The *Sub1* orthologues of *O. nivara* and *O. rufipogon* used for this analysis are *OnSub1B-1* (EU429442), *OnSub1B-2* (EU429443), *OnSub1C-1* (EU429445), *OrSub1B-1* (EU429444) and *OrSub1C-1* (EU429446). Alignments were adjusted manually upon visual inspection. For phylogenetic analyses, truncated nucleotide sequences were aligned with *ERF2* (TIGR accession: LOC\_Os01g21120), which belongs to the subgroup VII of the *ERF* gene family, as the outgroup sequence (Fig. S1 in Supplementary Information available online). Neighbor-joining analysis using uncorrected distances were calculated using PHYLIP, version 3.67 (Felsenstein, 1989). The reliability of the neighbor-joining output phylogeny was estimated using bootstrapping analysis with 100 replicates and one input order per replicate.

## RESULTS

#### Sub1 orthologues of *O. nivara* and *O. rufipogon*

To isolate any orthologues of *Sub1* genes from *O. nivara* and *O. rufipogon* by PCR, degenerate primers expected to bind to the two most highly conserved regions present in all rice *Sub1* genes were designed based on the nucleotide sequence alignment of 16 alleles (two alleles of *Sub1A*, nine alleles of *Sub1B* and five alleles of *Sub1C*) (Fig. S2 in Supplementary Information available online). All *Sub1* genes are classified into subgroup VII of the *ERF* transcription factor family (Gutterson and Reuber, 2004; Nakano *et al.*, 2006). It was anticipated that the two degenerate primers would bind only to the *Sub1* genes and not to subgroup VII or other *ERF* genes due to sequence dissimilarity in the primer binding region. The degenerate primers (*Sub1*-forward and *Sub1*-reverse) amplified a single band of the expected size (approx. 550 bp) from *O. nivara* and *O. rufipogon* genomic DNA (Fig. S3 in Supplementary Information available online). Following gel purification and cloning of the PCR products, plasmid DNA was extracted from 25 independent clones and the nucleotide sequences of the inserts were determined. Sequence analysis revealed that both *O. nivara* and *O. rufipogon* encode two *Sub1* orthologues which are more similar to *Sub1B* and *Sub1C* than the *Sub1A* gene of *O. sativa* (Fig. 2). The *Sub1*-like genes isolated from *O. nivara* (accession: IRGC-80470) and *O. rufipogon* (accession: IRGC-80506)

using these degenerate primers were designated *OnSub1B-1*, *OnSub1C-1*, *OrSub1B-1* and *OrSub1C-1*. To obtain the complete coding sequences of *Sub1B* and *Sub1C* genes of *O. nivara*, 5'- and 3'-RACE amplification was performed. Due to unavailability of seed from accession IRGC-80470, the *O. nivara* accession IRGC-101524 was used to obtain the full-length sequences of *Sub1* cDNAs of this species. The *Sub1B* allele cloned from IRGC-101524 was designated *OnSub1B-2* since a single non-conservative amino acid substitution distinguishes the allele from *OnSub1B-1* of *O. nivara* accession (IRGC-80470; Fig. 2). The *Sub1C* sequence was identical in the region sequenced in both *O. nivara* accessions.

Nucleotide and amino acid sequences of the *Sub1* orthologues isolated from *O. nivara* and *O. rufipogon* were compared with the reported sequences of *Sub1B* and *Sub1C* alleles of *O. sativa* (Fig. 2 and Table 1, and Fig. S4 in Supplementary Information available online; Xu *et al.*, 2006). Direct sequence comparison revealed that the *Sub1*-like genes of wild rice are highly similar to alleles of *Sub1B* and *Sub1C* genes in *O. sativa* (approx. 99% identity in nucleotide and amino acid sequences) (Table 1). The *O. nivara* *Sub1* genes, *OnSub1B-2* and *OnSub1C-1* (complete coding sequences), are most closely related to *Sub1B-2* and *Sub1C-6* of domesticated rice, respectively. The two *Sub1* genes of *O. rufipogon*, *OrSub1B-1* and *OrSub1C-1* (partial coding sequences) are most similar to *Sub1B-2* and *Sub1C-2*, respectively. As previously shown, *Sub1B-2* and *Sub1C-2* are limited to *japonica* rice. *Sub1C-6* was found in the *indica* accessions, Habiganj aman, IR24 and IRBB21, in combination with *Sub1B-4*, *Sub1B-5* or *Sub1B-8* (Table 2; Xu *et al.*, 2006).

#### Phylogenetic analysis of *Sub1* genes in *O. sativa*, *O. nivara* and *O. rufipogon*

To estimate the evolutionary relatedness of *Sub1* genes in *O. sativa*, *O. nivara* and *O. rufipogon*, a neighbor-joining method of phylogenetic analyses was used (Fig. 3). The three *Sub1* genes, *Sub1A*, *Sub1B* and *Sub1C*, were separated into three distinct clades with significant bootstrap values supporting the phylogeny. The *Sub1A* gene, which is present only in some *indica* varieties, was more related to *Sub1B* than *Sub1C*. Interestingly, the *O. sativa* *Sub1B* alleles were resolved into two subgroups, which corresponded to the *Sub1* locus haplotype. The subgroup with the *Sub1B-1*, *-B-3*, *-B-6* and *-B-7* alleles includes only rice accessions that encode *Sub1A* in addition to *Sub1B* and *Sub1C* (Table 2; Xu *et al.*, 2006). By contrast, the *O. sativa* accessions with the *Sub1B-2*, *-B-4*, *-B-5*, *-B-8* and *-B-9* alleles do not possess the *Sub1A* gene. The *Sub1B* alleles of *O. nivara* and *O. rufipogon* co-clustered with the alleles of rice accessions that lack *Sub1A*, which is in accordance with the absence of the *Sub1A* gene in the wild-rice germplasms examined. The *Sub1C* alleles were resolved only in two subgroups. Unlike *Sub1B*, the *Sub1C* alleles were not distinguished by the absence and presence of *Sub1A*, due to high sequence similarity among alleles. Notably, the *Sub1C-1* allele, which is limited to submergence-tolerant accessions (*Sub1A-1*



TABLE 1. Percentage identity of Sub1 gene coding sequences in *O. nivara*, *O. rufipogon* and *O. sativa*

		GenBank accession	Most similar <i>O. sativa</i> allele	Nucleotide (% identity)	Amino acid (% identity)
<i>O. nivara</i>	<i>OnSub1B-1*</i>	EU429442	<i>Sub1B-2</i>	99.4 (524/527)	99.4 (175/176)
	<i>OnSub1B-2</i>	EU429443	<i>Sub1B-2</i>	99.1 (746/753)	99.2 (249/251)
	<i>OnSub1C-1</i>	EU429445	<i>Sub1C-6</i>	98.9 (700/708)	98.7 (233/236)
<i>O. rufipogon</i>	<i>OrSub1B-1*</i>	EU429444	<i>Sub1B-2</i>	99.8 (526/527)	100 (176/176)
	<i>OrSub1C-1*</i>	EU429446	<i>Sub1C-2</i>	100 (509/509)	100 (170/170)

\* Partial coding sequences lacking 5' (N-terminal) and 3' (C-terminal) sequences.

TABLE 2. Sub1 haplotypes of *O. sativa*, *O. nivara* and *O. rufipogon*

Species	Accession	Subspecies	Origin	Sub1 haplotype		
				<i>Sub1A</i>	<i>Sub1B</i>	<i>Sub1C</i>
<i>O. sativa</i>	FR13A	<i>indica/ausa</i>	India	A-1	B-1	C-1
	Kurkaruppan	<i>indica</i>	Sri Lanka	A-1	B-3	C-1
	Goda Heenati	<i>indica</i>	Sri Lanka	A-1	B-6	C-1
	Teqing	<i>indica</i>	China	A-2	B-1	C-3
	IR64	<i>indica</i>	Philippines	A-2	B-1	C-3
	93-11	<i>indica</i>	China	A-2	B-1	C-5
	CO39	<i>indica</i>	India	A-2	B-7	C-3
	Habiganj aman	<i>indica</i>	Bangladesh	Absent	B-4	C-6
	IRBB21	<i>indica</i>	Philippines	Absent	B-5	C-6
	IR24	<i>indica</i>	Philippines	Absent	B-8	C-6
	Nipponbare	<i>japonica</i>	Japan	Absent	B-2	C-2
	Taipei309	<i>japonica</i>	Taiwan	Absent	B-2	C-2
	Liaogeng	<i>japonica</i>	China	Absent	B-2	C-2
	M202	<i>japonica</i>	USA	Absent	B-2	C-2
	<i>O. nivara</i>	IRGC-80470		India	Absent	B-2
IRGC-101524			India	Absent	B-2	C-6
<i>O. rufipogon</i>	IRGC-80506		India	Absent	B-2	C-2

Most similar *O. sativa* allele

present), is distinct from the other known *Sub1C* alleles. The *Sub1C-1* allele contains 16 non-synonymous and seven synonymous substitutions as compared with *Sub1C-2*, which is the most closely related allele. This amino acid sequence variation between these two alleles is concentrated in the region just carboxyl to the ERF binding domain and includes two potential phosphorylation sites (Fig. 2). Amino acid substitutions in this region are also observed amongst the other *Sub1C* alleles, particularly in the variable proline-rich region (Fig. S4 in Supplementary Information available online).

#### Accumulation of transcripts encoding the Sub1 orthologues of *O. nivara* is promoted by submergence

Transcript levels of all three *ERF* genes of the *Sub1* locus are elevated in response to submergence in aerial tissue of *O. sativa* (Fukao et al., 2006; Xu et al., 2006). To discern whether mRNA accumulation of the *Sub1* orthologues is induced by submergence in *O. nivara*, RT-PCR analysis was carried out using gene-specific primers for *OnSub1B* and *OnSub1C* (Fig. 4). As reported previously for *O. sativa* (Fukao et al., 2006), 3 d of complete submergence increased the levels of *OnSub1B* and *OnSub1C* transcripts in aerial tissue of *O. nivara*. Transcript accumulation of

orthologues of other submergence-inducible genes, such as  $\alpha$ -amylase-3C (*Amy3C*) and alcohol dehydrogenase-2 (*Adh2*) were also examined in *O. nivara* using primers that are designed based on the genomic sequences of *O. sativa* (Fig. 4). The transcript levels of these two representative submergence inducible genes were also elevated in response to the stress.

## DISCUSSION

The *Sub1* locus of *O. sativa* encodes a cluster of ERF proteins and is known to regulate responses to submergence. It is shown here that the two *ERF* genes that are orthologues of *Sub1B* and *Sub1C* are present in accessions of *O. nivara* and *O. rufipogon*, consistent with the detection of these genes in all *O. sativa* accessions examined to date (Xu et al., 2006; S. Heuer and D. Mackill, IRRI, Philippines, pers. comm.). The presence and submergence-induced expression of the *Sub1B* and *Sub1C* orthologues of *O. nivara* and *O. rufipogon* suggest that an orthologous *Sub1* locus regulates submergence responses in these wild *Oryza* species. Direct sequence comparison revealed that *Sub1B-2* and *Sub1C-2* which are limited to *japonica* rice were most closely related to the *Sub1B* and *Sub1C* alleles in the *O. rufipogon* accession (Tables 1 and 2). By contrast,

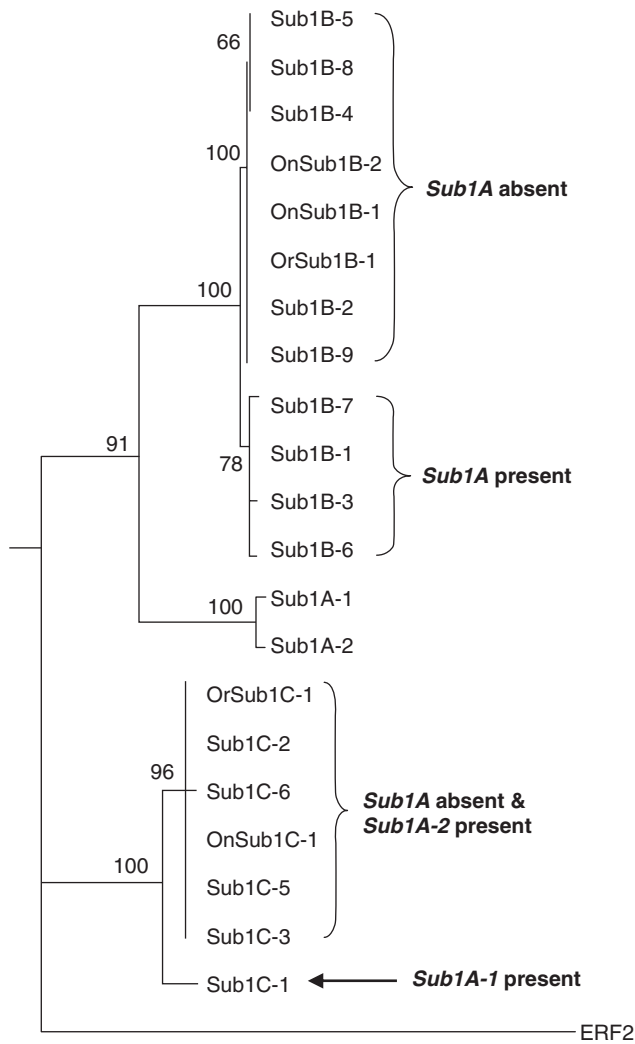


FIG. 3. Phylogenetic tree based on nucleotide sequences of *Sub1* gene alleles of *O. sativa*, *O. nivara* and *O. rufipogon*. A phylogenetic tree was generated by the neighbor-joining method using uncorrected distances in PHYLIP, version 3.67. *ERF2* (TIGR accession: LOC\_Os01g21120), another member of subgroup VII of the *ERF* gene family, was used as an outgroup. The central portion of the coding sequence was analysed (Fig. S1 in Supplementary Information available online). The length of each branch is proportional to sequence divergence. The numbers above branches indicate bootstrap values from 100 replicates.

an *O. nivara* accession had a *Sub1C* allele that was most closely related to that of *indica* accessions. These observations are consistent with genome-scale analyses that show *indica* and *japonica* rice are more closely related to certain accessions of *O. rufipogon* and *O. nivara* than to each other (Lu *et al.*, 2002; Cheng *et al.*, 2003; Londo *et al.*, 2006; Kovach *et al.*, 2007; Sang and Ge, 2007; Sweeney and McCouch, 2007). Exceptionally, the *O. nivara* *Sub1B* allele isolated here was most related to *Sub1B-2* which is restricted in *japonica* rice (Tables 1 and 2). Haplotype analysis of diverse domesticated and wild-rice accessions would provide further information to elucidate phylogenetic relatedness of polygenic *Sub1* locus amongst *O. sativa*, *O. nivara* and *O. rufipogon*.

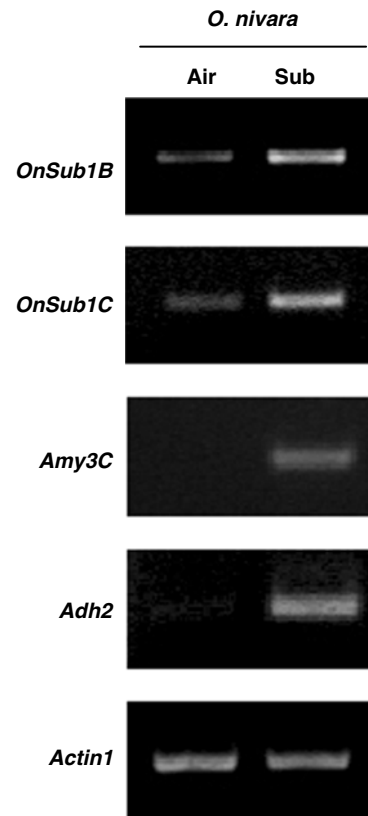


FIG. 4. Accumulation of the *Sub1* orthologue and representative gene mRNAs induced by submergence stress in *O. nivara*. Air, air-grown plants; Sub, submerged plants. Ten-day-old plants were completely submerged or aerobically grown in test tubes for 3 d. After treatment, total RNA was extracted from aerial tissue and analysed by RT-PCR. Gene-specific primers for *OnSub1B* and *OnSub1C* were designed based on the coding sequences of these genes in *O. nivara*. Primers for *Amy3C*, *Adh2* and *Actin1* were identical to those used for the transcript analysis in *O. sativa* (Fukao *et al.*, 2006). The level of *Actin1* mRNA was used as a loading control.

The three *ERF* genes of the *Sub1* locus are most closely related to one another, as compared with the 139 other *ERF*-domain containing genes of rice (Gutterson and Reuber, 2004; Nakano *et al.*, 2006; T. Fukao and J. Bailey-Serres, unpubl. res.), indicating that the polygenic locus arose via tandem duplication. The three *SUB1* proteins are highly conserved within the *ERF* DNA binding domain, but are considerably divergent in the amino- and carboxyl-terminal regions (Fig. 2, and Fig. S4 in Supplementary Information available online). Although the data do not identify a founding member of this cluster of paralogues, the nucleotide and amino acid sequence analyses provide some intriguing insight into these genes. First, the phylogenetic analysis exposes diversification within the *Sub1B* and *Sub1C* clades that greatly exceeds that observed in the *Sub1A* clade, suggesting that the duplication that gave rise to the *Sub1A* gene is the most recent event (Fig. 3). This is also supported by the finding that the *Sub1A* gene is not present in all domestic and wild-rice accessions. In accordance with this, the subgroups of *Sub1B* alleles corresponded to accessions that either possess or lack the

*Sub1A* gene (Fig. 3). Secondly, the phylogenetic analysis indicates that *Sub1A* is more closely related to *Sub1B* than *Sub1C*. This is consistent with the high amino acid sequence similarity at the N-terminus shared by *Sub1A* and *Sub1B*, but not *Sub1C* (Fig. 2 and Fig. S4 in Supplementary Information available online). This conserved N-terminal region resembles the MCGGAI(IL) motif present in most known Group VII ERFs of diverse plant species (Nakano *et al.*, 2006). Further support that *Sub1A* is more closely related to *Sub1B* than *Sub1C* is based on the site of the single intron in each of these genes. In *Sub1A* and *Sub1B*, the insertion is within the final portion of the coding sequence, whereas it is located at the 3'-untranslated region in *Sub1C* (Fig. S2 in Supplementary Information available online). Together, these results indicate that *Sub1A* arose by duplication of *Sub1B*.

Two *Sub1A* alleles have been recognized based on two single nucleotide polymorphisms within the coding region, one of which confers a non-conservative variation of a Ser (*Sub1A-1*) or Pro (*Sub1A-2*) (Xu *et al.*, 2006). The *Sub1A-2* allele is present in a subset of the *indica* accessions, all of which initiate an elongation growth response to submergence. The *Sub1A-1* allele was identified in the submergence-tolerant accession FR13A collected in Orissa State of eastern India (Xu *et al.*, 2006). FR13A is a member of the *aus* varietal group of domesticated rice. *Aus* varieties are distinguishable from *indica* by simple sequence repeat and single nucleotide polymorphism markers, but are considered a subgroup of *indica* (Garris *et al.*, 2005; McNally *et al.*, 2006). The presence of *Sub1A-1* in FR13A and two *indica* cultivars from Sri Lanka (Goda Heenati and Kurkaruppan) is correlated with a submergence response that includes carbohydrate conservation and limited elongation growth (Fukao *et al.*, 2006; Xu *et al.*, 2006). The confirmation of *Sub1A-1* or *Sub1A-2* alleles in the genomes of a subset of *indica* and *aus* accessions indicates that *Sub1A* most likely arose from gene duplication at this locus prior to the divergence of the *indica/aus* lineages.

Submergence dramatically increases the transcript abundance of *Sub1C* as well as *Sub1A* in aerial tissue of submergence-tolerant and intolerant rice accessions (Fukao *et al.*, 2006; Xu *et al.*, 2006). Interestingly, the *Sub1C-1* allele, which is limited to the submergence-tolerant accessions with *Sub1A-1*, is the most divergent of all of the *Sub1C* alleles (Fig. 3). The unusual *Sub1C* allele in submergence-tolerant rice could have arisen from positive nucleotide selection on this allele as a consequence of the function of *Sub1A-1*. In contrast to the observation for the *Sub1C* gene, more than one *Sub1B* allele was found in association with *Sub1A-1* (Table 2) (Xu *et al.*, 2006). Experimental analysis of the molecular function of the SUB1 proteins is required to understand better the observed diversity of the *Sub1* genes. Of particular interest will be consideration of the variation in potential mitogen-activated protein kinase phosphorylation sites located immediately carboxyl-terminal to the ERF DNA binding domain (Fig. 2, and Fig. S4 in Supplementary Information available online; Xu *et al.*, 2006).

Recent allelic survey of cloned domestication genes revealed that alleles which encode genes associated with shattering, pericarp colour and amylose content are common to all *O. sativa* accessions including *indica* and *japonica* (Yamanaka *et al.*, 2004; Li *et al.*, 2006b; Kovach *et al.*, 2007; Sang and Ge, 2007; Sweeney *et al.*, 2006). Unlike key domestication alleles, the submergence tolerance conferring *Sub1A-1* allele is restricted to limited *aus/indica* accessions and is uncommon in modern cultivars. Interestingly, the occurrence of the submergence tolerance-conferring haplotypes of the *Sub1* locus (*Sub1A-1*, *Sub1B-1*, -3, -6, *Sub1C-1*) in both *aus* (FR13A; Orissa, India) and *indica* (Goda Heenati and Kurkaruppan; Sri Lanka) most likely reflects human selection of the submergence tolerance trait in two geographic regions. An orally chronicled migration of followers of an outcast prince from the Orissa region to Sri Lanka circa 500 BC is supported by recent molecular studies of the frequency of subtype alleles encoding human leucocyte antigens (HLA-A\*02) in Sri Lankan and eastern Indian populations (Malavige *et al.*, 2007). Hence, it is feasible that rice with the submergence tolerance-conferring *Sub1* haplotype was transported by immigrants and subsequently introgressed into local *indica* varieties grown in Sri Lanka. The *Sub1* haplotypes that confer submergence tolerance has limited distribution amongst rice cultivars but may have been prized by rice farmers with submergence-prone paddies in Orissa and Sri Lanka.

#### SUPPLEMENTARY INFORMATION

Supplementary information is available online at [www.oxfordjournals.org](http://www.oxfordjournals.org) and consists of the following. Figure S1: nucleotide sequence alignment of truncated *Sub1* genes in *O. sativa*, *O. nivara*, and *O. rufipogon*. Figure S2: nucleotide sequence alignment of *Sub1* genes in *O. sativa*. Figure S3: *Sub1* orthologues amplified by genomic PCR in *O. nivara* and *O. rufipogon*. Figure S4: Amino acid alignment of *Sub1* genes of *O. sativa*, *O. nivara*, and *O. rufipogon*.

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