LETTERS

Sub1A is an ethylene-response-factor-like gene that confers submergence tolerance to rice

Kenong Xu[1], Xia Xu[1], Takeshi Fukao[2], Patrick Canlas[1], Reycel Maghirang-Rodriguez[3], Sigrid Heuer[3], Abdelbagi M. Ismail[3], Julia Bailey-Serres[2], Pamela C. Ronald[1] & David J. Mackill[3]

Most *Oryza sativa* cultivars die within a week of complete submergence—a major constraint to rice production in south and southeast Asia that causes annual losses of over US$1 billion and affects disproportionately the poorest farmers in the world[1,2]. A few cultivars, such as the *O. sativa* ssp. *indica* cultivar FR13A, are highly tolerant and survive up to two weeks of complete submergence owing to a major quantitative trait locus designated *Submergence 1* (*Sub1*) near the centromere of chromosome 9 (refs 3–6). Here we describe the identification of a cluster of three genes at the *Sub1* locus, encoding putative ethylene response factors. Two of these genes, *Sub1B* and *Sub1C*, are invariably present in the *Sub1* region of all rice accessions analysed. In contrast, the presence of *Sub1A* is variable. A survey identified two alleles within those *indica* varieties that possess this gene: a tolerance-specific allele named *Sub1A-1* and an intolerance-specific allele named *Sub1A-2*. Overexpression of *Sub1A-1* in a submergence-intolerant *O. sativa* ssp. *japonica* conferred enhanced tolerance to the plants, downregulation of *Sub1C* and upregulation of *Alcohol dehydrogenase 1* (*Adh1*), indicating that *Sub1A-1* is a primary determinant of submergence tolerance. The FR13A *Sub1* locus was introgressed into a widely grown Asian rice cultivar using marker-assisted selection. The new variety maintains the high yield and other agronomic properties of the recurrent parent and is tolerant to submergence. Cultivation of this variety is expected to provide protection against damaging floods and increase crop security for farmers.

Submergence of plants inhibits aerobic respiration and photosynthesis, and stimulates a variety of responses that can enhance survival, such as a switch from aerobic to anaerobic respiration[7]. In contrast to deep-water rice cultivars that avoid submergence stress by growing above the water surface and thereby restoring gas exchange[8], submergence-tolerant rice can survive 10–14 days of complete submergence and renew growth when the water subsides[9]—although the duration of survival is also influenced by environmental factors such as water turbidity, temperature and light levels[10]. The *Sub1* locus was mapped to an interval of 0.06 centimorgans on chromosome 9 using a mapping population (DX202) of 4,022 plants developed from the hybridization of a tolerant *indica* derivative of the FR13A cultivar (IR40931-26) and the intolerant *japonica* cultivar M-202 (Supplementary Fig. 1a, b; Supplementary Tables 1–3). Physical coverage of this region was obtained with five overlapping bacterial artificial chromosome (BAC) clones derived from submergence-intolerant *indica* rice varieties and a nearly complete contig of 13 binary clones from IR40931-26 (Supplementary Fig. 1b). The *Sub1* region, bordered by the markers CR25K and SSR1A, physically spans over 182 kilobases (kb). This interval encodes three genes containing ethylene-response-factor (ERF) domains and designated *Sub1A*, *Sub1B* and *Sub1C*, ten non-ERF genes including four transcribed and six hypothetical protein-coding genes, and >50% retrotransposon-related sequences (Fig. 1a; Supplementary Fig. 1; Supplementary Table 4). The corresponding region of the *japonica* genome represented by the sequenced variety Nipponbare spans 142 kb and is considerably rearranged. Notably, *Sub1A* is absent from the Nipponbare genome[11]. Recombination was suppressed in this region in the mapping population, as revealed by the 10.7-fold higher-than-average recombination ratio (3,030 kb cM^{-1} in the *Sub1* region versus 282 kb cM^{-1} for the entire genome)[5,12]. This could reflect the proximity of the *Sub1* locus to the centromere and/or the presence of genomic rearrangements that have altered continuity in this region in the two rice subspecies[13].

Plant proteins that contain ERF domains are known regulators of abiotic and biotic stress responses[14,15]. The accumulation of *Sub1A* and *Sub1C* messenger RNAs was strongly but transiently promoted by submergence and further reduced on de-submergence in seedling leaves of tolerant FR13A (Fig. 1b). *Sub1C* mRNA induction was earlier and more pronounced in intolerant Nipponbare compared with FR13A (Fig. 1b), suggesting that the rapid induction of *Sub1A* limits expression of *Sub1C*. *Adh1* gene transcript levels were more strongly induced in the tolerant line, indicating that *Sub1A* may

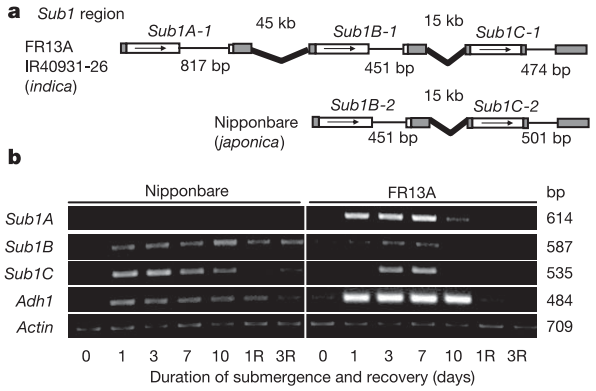

Figure 1 | *Sub1* region gene composition and submergence-induced mRNA accumulation in rice. a, ERF gene structure and organization in tolerant *indica* (IR40931-26) and intolerant *japonica* (Nipponbare). Arrows: direction of transcription; shaded boxes: untranslated regions; open boxes: coding sequence; thin lines: introns; thick lines: intergenic regions. Gene structure was determined by comparison of genomic and cDNA sequences of *Sub1B* (AK106057, AK068688) and *Sub1C* (AK060090, AK072749). **b**, Semi-quantitative RT–PCR assessment of gene transcript levels in shoot tissue from tolerant (FR13A) and intolerant (Nipponbare) genotypes following 1–10-d submergence and a subsequent 1–3-d recovery (1R and 3R, respectively).

[1]Department of Plant Pathology, University of California, Davis, California 95616, USA. [2]Center for Plant Cell Biology, Department of Botany and Plant Sciences, University of California, Riverside, California 92521-0124, USA. [3]International Rice Research Institute, DAPO Box 7777, Metro Manila, The Philippines.

positively regulate certain acclimation responses (Fig. 1b). In contrast, *Sub1B* transcripts increased only slightly during submergence (Fig. 1b; Supplementary Fig. 2a). The ten non-ERF genes in the *indica Sub1* region showed no evidence of expression in seedling leaves before or during submergence in IR40931-26 or the intolerant variety M-202 (data not shown). These results indicated that the three ERF-domain-containing genes, particularly *Sub1A*, were strong candidates for the genetic determinant controlling submergence tolerance.

The three SUB1 proteins each possess a single copy of the 56-amino-acid DNA-binding domain characteristic of the ERF subfamily of the plant *Apetela2*-like transcription factors (Supplementary Fig. 3). SUB1B and SUB1C have the two signature amino acids—alanine and aspartic acid—at positions 13 and 18, respectively, of the ERF domain that are characteristic of the B2 subgroup of ERF proteins[16]. In contrast, SUB1A has a serine at position 13, as found in two members of the B1b subgroup[15], but falls in the same clade as the other two SUB1 proteins[14]. Within the ERF domain, SUB1A shares 87.7% and 77.2% sequence identity with SUB1B and SUB1C, respectively (Supplementary Fig. 3). A full-length *Sub1A* transcript of 1312 nucleotides was obtained from mRNA of submerged IR40931-26 plants by reverse transcription–polymerase chain reaction (RT–PCR). The transcript consists of a 5′ untranslated region (UTR) of 149 nt, an open reading frame (ORF) of 846 nucleotides encoding a deduced protein of 281 amino acids, and a 3′ UTR of 317 nucleotides (Fig. 1a; Supplementary Fig. 4). The 5′ end of the *Sub1A* complementary DNA includes 30 nucleotides of ORF not predicted by gene-prediction algorithms. The *Sub1A* gene has one intron of 817 base pairs (bp) and two exons, the second exon comprising only 11 bp including the stop codon. A similar genomic structure was found for *Sub1B* (second exon 15 bp), whereas *Sub1C* has only one intron, located in the 3′ UTR (Fig. 1a).

A survey of *Sub1* locus haplotypes in 17 *indica* and four *japonica* varieties identified two *Sub1A*, nine *Sub1B* and seven *Sub1C* alleles on the basis of variation in amino-acid sequence (Table 1; Supplementary Figs 3, 5 and 6). The *Sub1A-1* and *Sub1C-1* alleles are limited to all six submergence-tolerant accessions, three of which were independently isolated submergence-tolerant varieties, including FR13A. There was no *Sub1B* allele identified as being specific to submergence tolerance. Variations in putative mitogen-activated protein kinase (MAPK) sites distinguish the tolerant and intolerant alleles of *Sub1A* and *Sub1C*. In the tolerant *Sub1A-1* allele a single nucleotide polymorphism at position 556 is responsible for a Pro 186 (intolerant) to Ser 186 (tolerant) substitution in a MAPK site (PXS/TP, where "X" is any amino acid[17]). Conversely, the *Sub1C-1* allele of tolerant lines lacks a MAPK phosphorylation site (underlined) present in the alleles of the intolerant accessions (tolerant: $\underline{S}PPP_{175}PEQPAAPV$; intolerant: $\underline{S}L/PP\underline{T}_{175}PPPP/E(P)_{0–3}$; (where P can be 0 or 3 in intolerant rice varieties) Table 1; Supplementary Figs 3 and 6). These potential phosphorylation sites are located in variable regions immediately carboxy-terminal to the ERF domain and may be of significance as phosphorylation can modulate DNA binding by ERF proteins[14,18].

The allelic survey further revealed that *Sub1A* is absent from five out of seventeen *indica* varieties and all four *japonica* varieties analysed, including the fully sequenced genome of Nipponbare[11] (Table 1). Assay of gene expression in a selection of *indica* varieties with *Sub1A* revealed that submergence tolerance is correlated with possession of the strongly submergence-induced *Sub1A-1* and intolerance is associated with the poorly submergence-induced *Sub1A-2* or complete absence of this gene (Fig. 1b; Supplementary Fig. 2a). Nucleotide polymorphisms in the *Sub1A* alleles, including the 5′-flanking regions (Supplementary Fig. 4), could be responsible for their differential expression. In contrast, submergence tolerance is correlated with limited induction of *Sub1C* under submergence, whereas intolerance is associated with high levels of *Sub1C* mRNA (Fig. 1b; Supplementary Fig. 2a). Together, these data suggested that stable transformation of *japonica* rice with an ectopically expressed *Sub1A-1* would downregulate *Sub1C* and confer submergence tolerance.

To test this hypothesis, we transformed the intolerant *japonica* variety Liaogeng with a *Sub1A-1* full-length cDNA under the control of the maize *Ubiquitin1* promoter[19,20] (*Ubi:Sub1A*). A screen of seedlings after 11 days of submergence identified four T_1 families, derived from independent T_0 *Ubi:Sub1A*$^+$ lines, with submergence-tolerant transgenic individuals, and progeny from two families were examined in detail. T_1 families 1 and 3 showed a correlation between high expression of the *Sub1A-1* transgene and submergence tolerance (Fig. 2c; Supplementary Fig. 2b). As observed in the FR13A descendant IR40931-26, tolerant *Sub1A-1*$^+$ plants showed a significant impairment of shoot elongation under submergence compared with the intolerant parent Liaogeng and non-transgenic siblings (Fig. 2a, b; Supplementary Fig. 2c). *Ubi:Sub1A-1* conferred a pleiotropic phenotype including reduced plant height under normal and submergence conditions (Fig. 2a, b) and enhanced expression of *Adh1* under normal growth conditions (Fig. 2c). Under submergence, the transgenic progeny showed reduced *Sub1C* (Fig. 2d) and enhanced *Adh1* (Supplementary Fig. 2d) mRNA accumulation concomitant with increased survival, as characteristic of tolerant *indica* varieties (Fig. 1b; Supplementary Fig. 2a). Although ectopic expression of *Ubi:Sub1A-1* reduced plant height, submergence tolerance was independent of plant height at the time of inundation (Fig. 2a; Supplementary Fig. 2c). It is known that entrapment of ethylene during submergence leads to decreased abscisic acid levels and increased gibberellin sensitivity, and has ramifications on cellular metabolism as well as stem and leaf elongation[10,21,22]. *Sub1* genotype controls ethylene and gibberillin mediated changes in gene expression including regulation of genes that control carbohydrate consumption and

Table 1 | Haplotypes of the *Sub1* locus based on alleles of the ERF-like genes in rice varieties

Line or cultivar	Submergence phenotype	Subspecies	*Sub1A* allele	*Sub1B* allele	*Sub1C* allele
FR13A, IR40931-26, DX18-121, IR48930	Tolerant	*indica*	A-1	B-1	C-1
Goda Heenati	Tolerant	*indica*	A-1	B-6	C-1
Kurkaruppan	Tolerant	*indica*	A-1	B-3	C-1
LMNIII	ND	*indica*	A-2	B-1	C-4
Teqing, CO39, IR64, IR64-M6D6-933-1-2, 93-11	Intolerant	*indica*	A-2	B-1, B-7	C-3, C-5
IR24, IRBB21, Swarna*	Intolerant	*indica*	Absent	B-8, B-5	C-6
IR50	Intolerant	*indica*	Absent	B-9	C-7
Habiganj aman	Intolerant	*indica*	Absent	B-4	C-6
Nipponbare, Liaogeng, M-202, Taipei309	Intolerant	*japonica*	Absent	B-2	C-2

Allele designations were based on the amino-acid sequence of the putative proteins (Supplementary Figs 3, 5 and 6). The submergence-tolerant *indica*-like variety FR13A is from Orissa, in eastern India. DX18-121 is an *indica/japonica* hybrid derivative. The submergence-tolerant varieties Kurkaruppan and Goda Heenati are from Sri Lanka. IR48930, IR40931-26 and DX18-121 are derivatives of FR13A. The primary locus conferring tolerance in FR13A and Kurkaruppan was reported to be similar but different from Goda Heenati[30]. However, submergence tolerance in Goda Heenati is also largely controlled by the *Sub1* locus (K.X. and D.J.M. unpublished data). Molecular marker studies indicate considerable divergence between Goda Heenati and FR13A (D.J.M. unpublished data). GenBank accessions of 93-11 containing *Sub1A*, *Sub1B* and *Sub1C* are AAAA01009971, AAAA01020021 and AAAA01005744, respectively. ND, not determined. The varieties are grouped based primarily on common alleles of Sub1A and Sub1C.

*Swarna lacks *Sub1A* and its alleles of *Sub1B* and *Sub1C* were not determined.

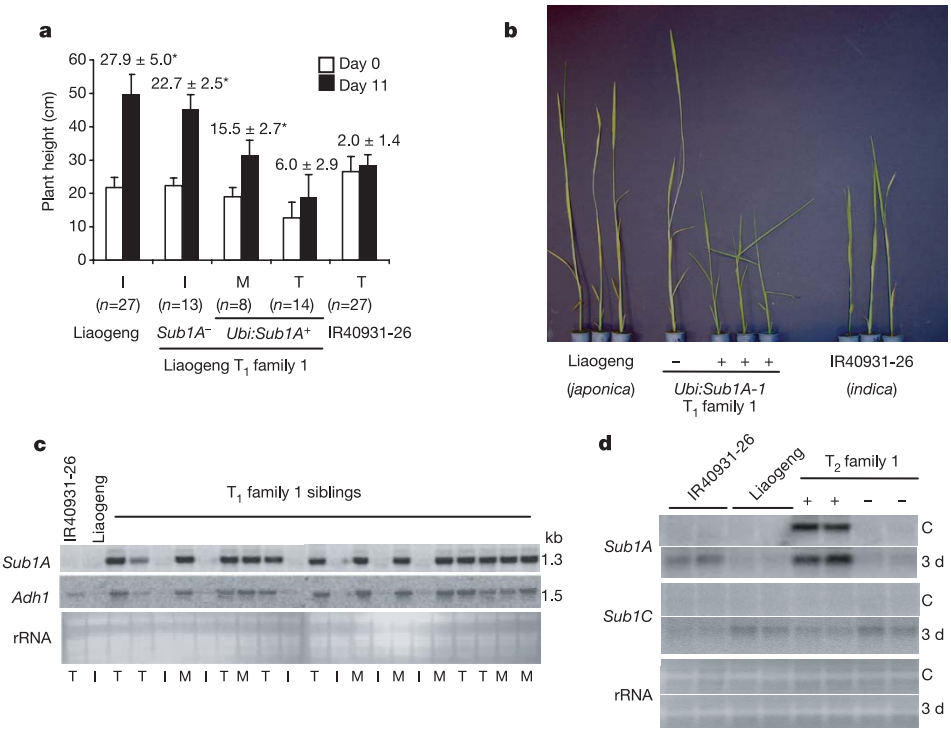

Figure 2 | Characterization of submergence response in transgenic rice ectopically expressing *Sub1A-1*. **a**, Comparison of plant height before submergence and after 11 days of submergence of the controls (non-transformed Liaogeng and IR40931-26) and siblings from T_1 family 1, including non-transgenic (*Sub1A*$^-$) intolerant (I) plants, and transgenic (*Sub1A*$^+$) intermediate tolerant (M) and tolerant (T) plants. At day 0 *Sub1A*$^+$ plants were significantly shorter than *Sub1A*$^-$ plants ($P < 0.0001$, unpaired *t*-test). Values above the bars are the mean and s.d. of the change in plant height following submergence. Error bars are also s.d. An asterisk indicates a significant increase in plant height in response to submergence at day 11 ($P < 0.0001$, unpaired *t*-test). **b**, Comparison of plant height of T_1 family 1 siblings and control plants following 11 days of submergence. **c**, Northern blot analysis of *Sub1A-1* and *Adh1* mRNA levels in shoot tissue harvested 10 days following de-submergence in T_1 family 1 siblings and control plants. Hybridization was performed with rice *Sub1A-1* and *Adh1* cDNA probes. **d**, Northern blot analysis of *Sub1A* and *Sub1C* mRNA levels in shoot tissue harvested immediately following submergence for 3 days or in control non-submerged tissue (C) from four T_2 siblings generated by self-pollination of a T_1 family 1 hemizygote. Siblings with the *Ubi:Sub1A* transgene (+); siblings lacking the transgene (−). Hybridizations were to *Sub1A-1* cDNA and *Sub1C-1* 3′-UTR probes, respectively.

cell elongation[23]. A genetic interaction between *Sub1A* and *Sub1C* may be of relevance because antagonistic relationships between ERFs have been recognized[15,24,25]. Detailed analyses of the function of *Sub1A* and *Sub1C* in the submergence response by targeted RNA interference (RNAi) and overexpression constructs are ongoing.

We used polymorphic molecular markers for *Sub1* with markers that flanked the locus to introgress the *Sub1* genes into the widely grown Indian variety Swarna, which lacks *Sub1A*. *Sub1* markers were used for selection of tolerant progenies, in combination with 5–12 background markers for each of the 12 rice chromosomes[26–28]. Marker assisted selection (MAS) application in the first backcross generation (BC_1F_1) was used to identify individual plants with the fewest IR49830-7-1-2-3 (FR13A descendant) chromosomal segments. Selected BC_1F_1 plants were used to generate BC_2F_3 and BC_3F_2 Swarna-*Sub1* lines that were genotypically identical to Swarna, except for the *Sub1* haplotype and adjacent markers. Both Swarna-*Sub1* lines showed strong submergence tolerance (Fig. 3). Field trials with the BC_2F_3 plants grown under control (non-submerged) conditions in the Philippines indicated no differences between the two varieties in terms of yield (Swarna: $6.3 \pm 0.1\,t\,ha^{-1}$; Swarna-*Sub1*: $6.4 \pm 0.1\,t\,ha^{-1}$), plant height (Swarna: 105 ± 1.4 cm; Swarna-*Sub1*: 106 ± 1.2 cm), harvest index (both 0.35) and grain quality as indicated by amylose content (Swarna: 26.4%; Swarna-*Sub1*: 25.9%). Development of submergence-tolerant varieties using these procedures is at an advanced stage for Laos, Bangladesh and India, and has already been reported in Thailand[29].

The results presented here confirm that submergence tolerance is conferred by a haplotype of the complex *Sub1* locus, with ectopic

Figure 3 | Introgression of the FR13A *Sub1* haplotype into an intolerant variety by MAS confers submergence tolerance. The *Sub1* region donor line IR49830 (an FR13A derivative) was introduced into the submergence-intolerant *indica* variety Swarna by backcrossing (BC) with MAS using markers for the *Sub1* region (SSR1, RM316, RM464, RM464A, RM219 and RM524) and the 12 chromosomes[25–27]. Individual F_1 plants were selected from BC_1, BC_2 and BC_3 that carried the FR13A *Sub1* haplotype with the least IR49830 background. Fourteen-day-old seedlings were submerged for 14 days and photographed 14 d after de-submergence.

Sub1A-1 expression being sufficient to enhance tolerance. The finding of the identical *Sub1* haplotype in accessions from submergence-prone areas in Sri Lanka and eastern India (Table 1) suggests that rice grains from submergence-tolerant plants may have been transported over 1,000 km and subsequently introgressed into local varieties, further indicating the agronomic importance of the *Sub1* locus.

METHODS

***Sub1* characterization.** Details of rice genotypes, growth and treatment conditions, and mapping are provided in Supplementary Methods. An F_2 mapping population of 4,022 plants expanded from DX202 (ref. 4), derived from a cross between DX18-121 (a tolerant line derived from *indica* IR40931-26, a descendant of FR13A) and the intolerant *japonica* cultivar M-202, was used. Submergence treatment was conducted as described previously[4]. The fine-mapping of the *Sub1* locus was accomplished with 24 markers specific to the *Sub1* region. For gene expression analyses, total RNA was isolated from seedling leaves and analysed as detailed in Supplementary Methods.

Generation of submergence-tolerant rice. To overexpress *Sub1A-1*, a binary construct *Ubi:Sub1A-1*-C1300 carrying the full-length *Sub1A-1* cDNA was transformed into an intolerant *japonica* cultivar, Liaogeng, using *Agrobacterium tumefaciens* (EHA105) as described in Chern *et al.*[20]. Integration of the *Ubi:Sub1A-1* transgene was verified by PCR using a maize *Ubiquitin1* promoter-specific primer and a *Sub1A* specific primer. Submergence tolerance and gene expression in the transgenic rice was evaluated as described in Supplementary Methods. Swarna-*Sub1* was produced by crossing the Indian variety Swarna to the FR13A descendant IR49830-7-1-2-3, followed by subsequent backcrossing to Swarna. MAS of progeny with polymorphic markers was performed by PCR analysis of genomic DNA.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Information Sequences were submitted to GenBank/EMBL/DDJB under accession numbers DQ011597–DQ011607 and DQ453964–DQ453966. Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to P.C.R. (pcronald@ucdavis.edu) or D.J.M. (d.mackill@cgiar.org).