Growth responses of seedlings under complete submergence in rice cultivars carrying both the submergence-tolerance gene \textit{SUB1A-1} and the floating genes \textit{SNORKELs}

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\textbf{ABSTRACT}
We screened 80 Asian rice (\textit{Oryza sativa} L.) cultivars for the presence of the submergence-tolerance gene \textit{SUB1A-1} and the floating genes \textit{SNORKEL1} (SK1) and \textit{SNORKEL2} (SK2), and found that the deepwater rice cultivar Baisbish (BSB) and the submergence-tolerant cultivar Flood Resistant 13A (FR13A) both possess the \textit{SUB1A-1} and the \textit{SK1}/\textit{SK2}. When BSB and FR13A seedlings were completely submerged, spindly growth of shoots was induced in BSB but not in FR13A. Submergence significantly increased the \textit{SUB1A-1} transcript abundance in BSB and FR13A shoots, but the expression level in BSB was much lower than that of FR13A. Submergence also induced the expression of both \textit{ERF66} and \textit{ERF67}, the transcriptional targets of \textit{SUB1A-1}, in FR13A shoots, whereas it upregulated the expression of \textit{ERF67} but not that of \textit{ERF66} in BSB shoots. These results suggest that BSB could not display submergence tolerance due to the low expression of \textit{SUB1A-1} and/or \textit{ERF66} under submergence.
Introduction

Flooding is one of the environmental stresses that constrains plant growth. Flooding imposes hypoxia on plants and consequently severely restricts aerobic respiration. Some plant species respond to this unfavourable environmental condition by promoted elongation growth of organ to keep or resume contact with the atmosphere. This elongation response to flooding is referred to as an ‘escape strategy’, which is typified by deepwater rice (also known as floating rice; *Oryza sativa*), the only crop that can be cultivated in flood-prone areas in South and Southeast Asia. Deepwater rice cultivars distinguish themselves from most modern rice cultivars by their ability to survive in water depths of more than 50 cm for at least 1 month (Catling, 1992). The elongation response of deepwater rice to submergence is mainly achieved by internodal elongation during the vegetative growth stage (Kende et al., 1998). This submergence-induced internodal elongation is also referred to as ‘floating ability’, and is evoked by ethylene (Kende et al., 1998). Two genes that confer floating ability to rice, *SNORKEL1* (SK1) and *SNORKEL2* (SK2), have been previously identified, encoding ethylene response factor (ERF) transcription factor (Hattori et al., 2009). SK genes are also found in *Oryza rufipogon*, *Oryza nivara*, and *Oryza glumaepatula* (Hattori et al., 2009; Sasayama et al., 2018). In addition, the existence of a mechanism of floating ability independent of SK genes has been suggested in a wild rice species *Oryza grandiglumis* (Okishio et al., 2014).

In contrast to the escape strategy of deepwater rice, some *indica* varieties of *O. sativa* have the ability to tolerate flooding via a different mechanism, referred to as ‘quiescence strategy’. These varieties, including the representative Flood Resistant 13A (FR13A), display reduced underwater shoot elongation at the seedling stage, which allows them to survive for 14 days during complete submergence caused by flash flooding (Bailey-Serres et al., 2010). This adaptation is composed of restriction of metabolism and growth under submergence and resumption of growth after flood subsidence using conserved energy (Fukao & Bailey-Serres, 2008; Setter & Laureles, 1996). Such submergence tolerance based on the quiescence strategy is known to be conferred by the major quantitative trait locus (QTL) *SUBMERGENCE1* (*SUB1*), which contains two or three ERF genes: *SUB1A, SUB1B*, and *SUB1C* (Xu et al., 2006). *SUB1B* and *SUB1C* are present in all *O. sativa* accessions investigated so far, and the level of *SUB1C* mRNA is considered to be positively correlated with underwater elongation via carbohydrate metabolism (Fukao & Bailey-Serres, 2008; Fukao et al., 2006). On the other hand, the presence of *SUB1A* is limited to some accessions. Two alleles, *SUB1A-1* and *SUB1A-2*, have been identified. There are two SNPs in their coding regions, one of which at position 556 bp is responsible for the difference in the residue at position 186 in the amino acid sequence encoded by the alleles, Ser in *SUB1A-1* and Pro in *SUB1A-2*. This substitution is predicted to result in a specific phosphorylation site for *SUB1A-1* (Xu et al., 2006). Due to its presence in submergence-tolerant accessions, *SUB1A-1* has been identified as a submergence-tolerant allele to limit underwater elongation for quiescence strategy (Xu et al., 2006). The reduced elongation by *SUB1A-1* has been, at least in part, associated with the downregulation of *SUB1C* (Fukao & Bailey-Serres, 2008; Fukao et al., 2006). Recently, Lin et al. (2019) reported that two rice ERF transcription factor genes, *ERF66* and *ERF67*, were highly upregulated in the presence of *SUB1A-1* under submergence. The protoplast transient assay and ChiP assay showed that *SUB1A-1* transcriptionally activated *ERF66* and *ERF67*. Overexpression of *ERF66* or *ERF67* in the submergence-tolerant cultivar Tainung 67 led to enhanced submergence tolerance. Thus, Lin et al. concluded that *ERF66* and *ERF67* function as direct downstream targets of *SUB1A-1* to form a regulatory cascade for submergence tolerance. In addition to *O. sativa*, *SUB1A* genes were also found in *O. rufipogon* and *O. nivara* (Niroula et al., 2012). In addition, the existence of a mechanism of submergence tolerance independent of *SUB1A* has been suggested in several wild rice species such as *Oryza rhizomatis*, *Oryza eichingeri*, and *Oryza grandiglumis* (Niroula et al., 2012; Okishio et al., 2014).

Although escape and quiescence strategies are opposite adaptation to flood, this does not exclude the possibility that these adaptations coexist in one plant species or accessions. In fact, we previously found that accessions from the Amazon river basin of South American wild rice species *O. grandiglumis* have both floating ability at mature stage, defined as the stage in which internodes have already formed during vegetative growth, and submergence tolerance at seedling stage (Okishio et al., 2014). However, currently, no cultivated rice varieties that have both floating ability at the mature stage and submergence tolerance at the seedling stage have been reported. Such varieties would contribute to improve the stability of rice cultivation in flood-prone areas. To find rice cultivars with the floating ability and the submergence tolerance, we screened 80 rice cultivars for the presence of the *SUB1A, SK1*, and SK2 genes. Consequently, we found two cultivars carrying both the *SUB1A-1* gene (which confers submergence tolerance in rice) and the SK genes (which confer floating ability in rice). One of the cultivars was FR13A, which has
been most widely used as a source of submergence tolerance (Xu et al., 2004). The other was Baisbish (BSB), which is one of the strains identified in Bangladesh as the highest yielders among deepwater rice cultivars suited to a flood depth of 300 cm at the mature stage (Zaman, 1977); however, its submergence tolerance at seedling stage has not been characterized. The aim of the present study was to evaluate submergence tolerance of BSB at seedling stage to provide insight into the relationship between floating ability and submergence tolerance in rice. Furthermore, we examined the relationship between the growth response of seedlings and the expression level of the SUB1A, ERF66, and ERF67 genes under submergence.

Materials and methods

Plant materials

Eighty rice cultivars, representing 24 japonica types, 3 japonica-indica hybrid types, 35 indica non-deepwater types, and 18 deepwater types (Supplementary Table 1), were screened for the presence of SUB1A, SK1, and SK2 genes. For complete submergence treatment of seedlings and expression analysis, three indica rice cultivars (Oryza sativa L.), namely, the deepwater rice cultivars Habiganj Aman II (HA II) and Baisbish (BSB) from Bangladesh and the submergence-tolerant cultivar Flood Resistant 13A (FR13A) from eastern India, were used in this study.

Rice caryopses were surface sterilized in 0.5% sodium hypochlorite solution for 30 min and then rinsed several times with tap water. The caryopses were then sown in pairs in 0.1 L plastic pots (diameter 5 cm, height 7 cm) filled with paddy soil containing 0.1 g N, 0.1 g P2O5, and 0.1 g K2O per liter of soil. The plants were grown outdoors under natural conditions from May to September in the experimental field of Kobe University, Hyogo, Japan.

DNA analysis

Genomic DNA of 80 rice cultivars (Supplementary Table 1) was extracted from leaves of the seedlings with 200 mM Tris–HCl (pH 7.5) containing 250 mM NaCl, 25 mM EDTA, and 0.5% SDS. For genotype analysis of SUB1A, SK1, and SK2, PCR analysis was performed with TaKaRa Ex Taq (Takara Bio, Kusatsu, Shiga, Japan) using gene-specific three primer sets listed in Supplementary Table 2. The genotypes were determined by the presence of the bands with the expected size. In addition, to distinguish between SUB1A-1 and SUB1A-2, the SUB1A-1-specific primers (Singh et al., 2010; Supplementary Table 2) were also used. The PCR products were separated on 1.5% agarose gels and visualized under UV light with ethidium bromide.

For sequence analysis of SUB1A, SK1, SK2, and SUB1C in rice cultivars BSB and FR13A, the full-length sequence of the genes was amplified by Tks Gflex DNA Polymerase (Takara Bio) using gene-specific primers listed in Supplementary Table 2. The PCR products were gel-purified and sequenced.

Complete submergence treatment of seedlings

At fourth-leaf stage, the plants grown in 0.1 L pots were completely submerged in 200 L semi-transparent plastic tanks (55 cm diameter and 90 cm height) filled with tap water. The control plants were allowed to continue growing aerobically. The initial plant lengths of HA II, BSB, and FR13A were 31.2, 32.0, and 22.4 cm, respectively. After 7 days of complete submergence, the test plants were removed from the tanks and grown aerobically for a further 7 days. During the submergence treatment, a small amount of water was kept pouring into and overflowing from the tank to keep the water in the tanks transparent. The ambient temperature during the treatment was 25–31°C. Plant length was measured before and after submergence, and again 7 days after desubmergence. Plant length was measured from the soil surface to the top of the straightened living shoot/leaf. For expression analysis of the SUB1 and SK genes, the basal 1-cm regions of the shoots of three seedlings grown in air or submerged for 12, 24, and 72 h were excised, frozen in liquid N2, and stored at −80°C until use.

RNA isolation and expression analysis

Total RNA was extracted from the basal sections of the shoots described above using an ISOSPIN Plant RNA (NIPPON GENE, Chiyoda, Tokyo, Japan), according to the manufacturer’s protocol. First-strand cDNA was synthesized from 1 µg of total RNA using a PrimeScript 1st strand cDNA Synthesis Kit (TaKaRa Bio, Japan), following the manufacturer’s protocol. Expression analysis was performed as follows with three biological replicates.

Expression analysis using quantitative RT-PCR for the SUB1A-1, ERF66, ERF67, SK1, SK2, and 17s rRNA genes was performed using TB Green Premix Ex Taq GC (Takara Bio, Japan) with MyGo Pro Real Time PCR (Funakoshi, Bunkyo, Tokyo, Japan) according to manufacturer’s instruction. The primers used are listed in Supplementary Table 2. 17s rRNA was used as an internal control for normalization.
Expression analysis using semi-quantitative RT-PCR for the SUB1C and Actin1 genes was performed using Takara Ex Taq (Takara Bio, Japan). The primers used are listed in Supplementary Table 2. The PCR products were separated on 1.5% agarose gels and visualized under UV light with ethidium bromide. OsActin1 was used as an internal control.

Results

Germlasm survey for the presence of the SUB1A-1, SK1, and SK2 genes

We checked 80 rice cultivars for the presence of the SUB1A, SK1, and SK2 genes by PCR analysis using gene-specific three primer sets (Supplementary Table 2). Each primer sets provided consistent results summarized in Supplementary Table 1. For japonica types, neither SUB1A nor SKs were amplified in any cultivars. For japonica–indica hybrid types, the SUB1A was amplified in two out of three cultivars examined, whereas the SKs were not amplified in any cultivars. For indica non-deepwater types, the SUB1A, SK1, and SK2 were amplified in 18, 2, and 1 cultivars, respectively. For indica deepwater types, the SK1 and SK2 were amplified in all 18 cultivars examined, whereas the SUB1A was amplified in seven cultivars. In total, the SUB1A gene was amplified in 27 cultivars, the SK1 gene in 20 cultivars, and the SK2 gene in 19 cultivars. Two SUB1A alleles have been identified previously: a tolerance-specific allele named SUB1A-1 and an intolerance-specific allele named SUB1A-2 (Xu et al., 2006). PCR analysis using the SUB1A-1 specific primers (Singh et al., 2010; Supplementary Table 2) indicated that this allele was present in the genomes of 6 out of the 27 cultivars. Among the six cultivars, the deepwater rice cultivar BSB and the submergence-tolerant cultivar FR13A also showed amplification of both SK1 and SK2 by PCR from genomic DNA (Figure 1). Nucleotide comparison of the SK1 and SK2 genes between BSB and FR13A revealed 100% identity. In addition, sequence analysis of SUB1A revealed that deepwater rice cultivar BSB possesses the SUB1A-1 gene, with a nucleotide sequence completely identical to that of the SUB1A-1 gene that was responsible for submergence tolerance in FR13A (Xu et al., 2006).

Submergence responses of seedlings

Plants at the fourth-leaf stage were completely submerged for 7 days and then allowed to recover in the air for 7 days. The deepwater rice cultivar HA II, which possessed SK1 and SK2 but not SUB1A (Figure 1), was also used for comparison. Complete submergence significantly increased the height of HA II and BSB plants by 1.3-fold compared with the air-grown control, whereas the height of submerged FR13A plants was similar to that of the air-grown control (Figure 2). After the submerged plants were transferred to aerobic conditions, HA II and BSB, which had displayed enhanced shoot elongation during submergence, showed symptoms of lodging. Because the leaves that had elongated during submergence withered within the 7-day recovery period, the lengths of the new leaves that emerged during the recovery period contributed to the lengths of the plants, resulting in a reduction in the lengths of the HA II and BSB plants during the recovery period. In contrast, when the submerged seedlings of FR13A were transferred to aerobic conditions, they showed no lodging and continued to grow.

Next, we examined the expression of SUBA-1, SK1, and SK2 in the 1-cm-long basal region of the shoots of seedlings submerged for 12, 24, and 72 h. Submergence significantly increased the SUB1A-1 transcript abundance in FR13A and BSB after 12 h, but the expression was more strongly induced in submerged FR13A seedlings; the induced level of SUB1A expression in FR13A seedlings submerged for 12, 24, and 72 h was approximately 11-, 5-, and 6-fold higher than that of BSB, respectively (Figure 3). The expression of SK1 was not significantly upregulated by submergence and that of SK2 was significantly upregulated in HA II and FR13A seedlings submerged for 12 h (Supplementary Figure 1), but submerged FR13A seedlings failed to display significant shoot elongation unlike the case of HA II seedlings (Figure 2). We also examined the expression of ERF66 and ERF67, recently characterized as direct transcriptional targets of SUB1A-1 (Lin et al., 2019). Submergence also significantly induced the expression of both ERF66 and ERF67 in FR13A as reported by Lin

![Figure 1. PCR analysis of the SUB1A-1, SK1, and SK2 genes in the deepwater rice cultivars HA II and BSB, and the submergence-tolerant cultivar FR13A.](image-url)
et al. (2019), but induced only the expression of ERF67 in BSB (Figure 3).

We then examined the nucleotide sequence and expression of SUB1C, which is also located at the SUBMERGENCE 1 locus. There are seven known SUB1C alleles in rice cultivars and FR13A possesses the SUB1C-1 allele (Xu et al., 2006). Our germplasm survey revealed that the nucleotide sequence of the SUB1C gene of BSB was identical to that of FR13A, indicating that it possessed the SUB1C-1 allele; by contrast, HA II possessed the SUB1C-6 allele (data not shown). Submergence increased the transcription of SUB1C in HA II and BSB, but did not noticeably affect it in FR13A (Figure 4).

Discussion
Submerged BSB seedlings, in which the expression of SUB1A-1 was significantly increased but the level was approximately 5- to 11-fold lower than that of submerged FR13A seedlings (Figure 3), displayed shoot elongation similar to that of submerged HA II seedlings, which lack the SUB1A gene (Figures 1 and 2). The induction of SUB1A-1 gene expression has been proposed to lead to accumulation of the GA signaling repressors SLR1 and SLRL1, which have been implicated in the quiescence response of submergence-tolerant rice seedlings to complete submergence (Fukao & Bailey-Serres, 2008). Therefore, the transcript of SUB1A-1 in BSB might be not high enough to elicit the negative regulation of GA signaling, and thus not suppress the shoot elongation of submerged seedlings in this cultivar. A possible reason for the lower expression level of SUB1A-1 in BSB may be differences of its promoter sequence compared to that of FR13A. In this study, we analyzed 100 bp upstream of the translation start site and found no difference between BSB and FR13A. Thus, more upstream regions could affect the expression of SUB1A-1. The mechanism that regulates the expression of SUB1A-1 remains to be discovered and analysis of the promoter regions in these two cultivars may help elucidate it.

Our results show that both ERF66 and ERF67, downstream transcriptional targets of SUB1A-1 (Lin et al., 2019), were upregulated by submergence in FR13A, whereas in BSB, ERF66 was not significantly upregulated and only the transcript of ERF67 was increased comparable to that in FR13A under submergence (Figure 3). This might suggest that the lower expression level of SUB1A-1 in BSB was not sufficient to induce ERF66 expression and that ERF67 has a lower threshold for expression induction than ERF66. Although the overexpression of ERF67 led to enhanced submergence tolerance (Lin et al., 2019), the upregulated expression of ERF67 in submerged BSB seedlings seems to be insufficient to induce submergence tolerance (Figures 2 and 3). The comparable levels of ERF67 expression in BSB and FR13A and the upregulated expression of ERF66 only in FR13A under submergence (Figure 3) might suggest that ERF66 plays
Figure 3. Expression of the SUB1A-1, ERF66, and ERF67 genes in submerged seedlings of the deepwater rice cultivars HA II and BSB, and the submergence-tolerant cultivar FR13A detected by real-time quantitative RT-PCR. Values represent the mean ± SE of three biological replicates. Asterisks indicate a significant difference (p < 0.05; Student’s t-test) between air-grown control and submerged seedlings.
more substantial role for submergence tolerance. Further study is required to confirm these possibilities.

All rice cultivars have SUB1B and SUB1C in the SUB1 locus and some indica cultivars additionally contain SUB1A (Fukao et al., 2009). Almost all submergence-tolerant genotypes, such as FR13A, possess the tolerant SUB1 haplotype SUB1A-1/SUB1C-1 (Singh et al., 2010). In the present study, the BSB cultivar was also found to possess the tolerant SUB1 haplotype SUB1A-1/SUB1C-1 (data not shown). In rice plants lacking the SUB1A-1 gene, submergence increases the expression of SUB1C, which might lead to increased carbohydrate catabolism and provide the energy needed for shoot elongation (Fukao et al., 2006). In rice plants carrying the SUB1A-1 gene, however, submergence-induced expression of SUB1A-1 may negatively regulate SUB1C expression, resulting in a limitation of carbohydrate consumption and restriction of elongation (Fukao et al., 2006). In the present study, although the expression of SUB1A-1 increased in both FR13A and BSB seedlings during submergence (Figure 3), SUB1C gene showed enhanced expression in BSB seedlings but not in FR13A seedlings (Figure 4). Therefore, the inability of BSB seedlings to restrict their growth under complete submergence might also be related to the increased expression of SUB1C, despite the increased expression of SUB1A-1.

In conclusion, despite the presence of SUB1A-1, the deepwater rice cultivar BSB is unlikely to have submergence tolerance at the seedling stage, different from FR13A. Our expression analyses suggest that the difference in growth responses in BSB and FR13A seedlings might be related to the expression levels of SUB1A-1 or ERF66 in these cultivars under complete submergence. Our findings would provide valuable information contributing to the development of rice cultivars that possess both submergence tolerance and floating ability.

Disclosure statement

No potential conflict of interest was reported by the authors.

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