Adaptive responses to flooding in wild rice species with various genomes other than AA

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ABSTRACT

In the present study, 15 accessions of wild rice species belonging to genome groups different from that of Oryza sativa were tested for their adaptive response to flooding at the seedling and mature stages. Under complete submergence at the seedling stage, reduced underwater shoot elongation and high survival rate after the recovery period were observed in the accessions of O. minuta as well as tetraploid O. punctata, O. euchingeri, O. officinalis, O. alta, O. grandiglumis, O. latifolia, and O. australiensis. This suggests that these species exhibit submergence tolerance at the seedling stage. During gradual submergence at mature stage, promoted internodal elongation was observed in the accessions of diploid and tetraploid O. punctata, O. alta, O. grandiglumis, O. latifolia, and O. brachyantha. This suggests that these species possess floating ability. Remarkably, two CCDD genome species, namely O. grandiglumis and O. latifolia, strongly displayed both submergence tolerance at the seedling stage and floating ability at mature stage, suggesting that these species are valuable genetic resources for improving rice adaptability to flooding. SUB1A and SNORKEL genes are known to confer submergence tolerance and floating ability to O. sativa, respectively. However, SUB1A, SNORKEL1, and SNORKEL2 genes were not detected in any of the wild rice accessions investigated in the present study. Our results suggest that adaptive responses to flooding in the investigated wild rice species can be achieved independently of the presence or absence of SUB1A and SNORKEL genes.

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**Introduction**

Flooding is an environmental stress that constrains plant growth by imposing hypoxia on plants and consequently severely restricting aerobic respiration. Some *indica* cultivars of Asian cultivated rice species *Oryza sativa* have the ability to tolerate flooding, which is referred to as ‘quiescence strategy’. These cultivars, 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 submerged conditions and resumption of growth after flood subsidence using conserved energy (Fukao & Bailey-Serres, 2008; Setter & Laureles, 1996). Such submergence tolerance is known to be conferred by the major quantitative trait locus SUBMERGENCE1 (SUB1), which contains two or three ethylene response factor (ERF) transcription factor genes: SUB1A, SUB1B, and SUB1C (Xu et al., 2006). SUB1B and SUB1C are present in all *O. sativa* cultivars studied to date, 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). However, the presence of SUB1A is limited to some *indica* cultivars. Besides it being present in *O. sativa*, SUB1A gene was also found in *Oryza rufipogon* and *Oryza nivara* accessions (Niroula et al., 2012).

In contrast to the quiescence strategy, some plant species respond to flooding by promoted elongation growth to maintain or resume contact with the atmosphere. This elongation response to flooding is referred to as ‘escape strategy’, which is typified by deepwater rice (also known as floating rice), the only crop that can be cultivated in flood-prone areas of South and Southeast Asia. The elongation response of deepwater rice to submergence is mainly achieved through internodal elongation at mature stage during vegetative growth (Kende et al., 1998). This submergence-induced internodal elongation is also referred to as ‘floating ability’. Two genes that confer floating ability to rice, namely SNORKEL1 (SK1) and SNORKEL2 (SK2), have been previously identified, encoding ERF transcription factor (Hattori et al., 2009). SK genes were also found in *O. rufipogon* and *O. nivara* (Hattori et al., 2009), and in *O. glumaepatula* (Sasayama et al., 2018).

The genus *Oryza* comprises more than 20 species, and it includes different genome types. Submergence tolerance and floating ability in this genus have been studied mainly in the cultivated rice species *O. sativa* (the AA genome) and several wild rice species that share the AA genome with *O. sativa*. However, little is known about the adaptive responses of other wild rice species to flooding. We previously found that two accesions of South American wild rice species *Oryza grandiglumis* (the CCDD genome) do not possess SUB1A and SK genes, but display both submergence tolerance at the seedling stage and floating ability at the adult plant stage (Okishio et al., 2014). This suggested the possibility that submergence tolerance and floating ability in *O. sativa* and wild rice species characterized by genome types different from the AA genome could be exerted by distinct mechanisms. In the present study, we investigated the presence of SUB1A and SK genes and the growth response to flooding in accessions of wild rice species belonging to genome groups different from that of *O. sativa*, including BB (diploid *Oryza punctata*), BBCC (*Oryza minuta* and tetraploid *O. punctata*), CC (*Oryza eichingeri* and *Oryza officinalis*), CCDD (*Oryza alta*, *Oryza grandiglumis*, and *Oryza latifolia*), EE (*Oryza australiensis*), and FF genome species (*Oryza brachyantha*).

**Materials and methods**

**Plant materials**

Fifteen accessions of nine wild rice species, namely diploid *O. punctata* (W1577; BB genome), tetraploid *O. punctata* (W1409; BBCC), *O. minuta* (W1318; BBCC), *O. eichingeri* (W1525; CC), *O. officinalis* (W0002 and W1930; CC), *O. alta* (W1147; CCDD), *O. grandiglumis* (RR8-H, W0613 and W1194; CCDD), *O. latifolia* (W2200; CCDD), *O. australiensis* (W0008 and W1538; EE), and *O. brachyantha* (W0656 and W1401; FF) were used in the present study (Supplementary Table S1). In the experiments for submergence tolerance at the seedling stage, the *Oryza sativa* submergence-tolerant cultivar FR13A was used as a control. In the experiments for floating ability at mature stage, the deepwater rice cultivar Habiganj Aman II (HA II) was used as a control. The wild rice accessions were obtained from the National Institute of Genetics supported by the National Bioresource Project, MEXT, Japan.

For wild rice accessions, caryopses were pre-treated at 42°C to break dormancy. Caryopses were surface sterilized in 0.5% sodium hypochlorite solution for 30 min and then rinsed several times with tap water, after which they were germinated by soaking in water at 30°C for 2–3 days. The germinated caryopses were then sown in 0.1 L or 1 L plastic pots filled with paddy soil containing 0.2 g N, 0.2 g P2O5, and 0.2 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. The plants remained in the vegetative growth stage throughout the experimental period.

**DNA analysis**

Genomic DNA of rice cultivars and wild rice accessions (Supplementary Table S1) was extracted from the 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, SUB1B, SUB1C, SK1, and SK2, PCR analysis was performed with TaKaRa Ex Taq (Takara Bio, Shiga, Japan) using gene-specific primers listed in Supplementary Table S2. The PCR products were separated on 1% agarose gels and visualized under blue LED light using SAFELOOK Green Nucleic Acid Strain (FUJIFILM Wako, Osaka, Japan).

**Complete submergence treatment at the seedling stage**

At 14 days after germination, the seedlings grown in 0.1 L pots were completely submerged in 200 L semi-transparent plastic tanks (diameter 55 cm, height 90 cm) filled with tap water. The control plants continued growing aerobically. After 14 days of complete submergence, the test plants were removed from the tanks and grown aerobically for another 14 days. During the submergence treatment, a small amount of water was constantly poured into and overflowing from the tanks to keep the water in the tanks transparent. Plant length was measured before and after submergence, and again 14 days after desubmergence. It was measured from the soil surface to the top of a straightened living shoot/leaf.

**Gradual submergence treatment at mature stage**

At 50 days after germination, the plants grown in 1 L pots were submerged in the 200 L plastic tanks filled with tap water. The plants were submerged at the depth of 25 cm, after which the water level was raised at a rate of 5 cm per day for 10 days to a final depth of 75 cm. The control plants continued growing aerobically. Plant length, internode length, and the number of leaves and internodes on the main stem were measured before and after submergence. To measure internode length and the number of internodes, the plants were dissected longitudinally.

**Results**

**Germplasm survey for the presence of the SUB1A, SK1, and SK2 genes**

A total of 15 wild rice accessions (Supplementary Table S1) was checked for the presence of SUB1A, SK1, and SK2 genes. PCR analysis using three gene-specific primer sets (Supplementary Table S2) showed that SUB1A gene was amplified in the submergence-tolerant cultivar FR13A, but not in any wild rice accessions (Figure 1). In addition to SUB1A gene, the SUB1 locus in O. sativa also contains SUB1B and SUB1C genes (Xu et al., 2006). Therefore, the presence of SUB1B and SUB1C genes in the investigated wild rice accessions was also examined. SUB1B gene was also not amplified in any wild rice accession, whereas SUB1C gene was amplified in all wild rice accessions (Figure 1). Furthermore, the PCR analysis of SK1 and SK2 genes using three gene-specific primer sets (Supplementary Table S2) also showed that SK1 and SK2 genes were amplified in the deepwater rice cultivar HA II, but not in any wild rice accessions (Figure 2).

**Response to complete submergence at the seedling stage**

To determine whether the wild rice accessions show submergence tolerance, 14-day-old plants were completely submerged for 14 days and then allowed to recover in aerobic conditions for another 14 days. Shoot elongation during the treatment and survival rate after the recovery period of the seedlings are shown in Figure 3. In the submergence-tolerant cultivar FR13A, shoot elongation during complete submergence was restricted compared with that in the air-grown control, and all seedlings survived after the recovery period. Such restricted underwater elongation was also observed in accessions of BBCC (O. punctata W1409 and O. minuta W1318), CC (O. eichingeri W1525 and O. officinalis W0002 and W1930), CCDD (O. alta W1147, O. grandiglumis RS8-H, W0613, and W1194, and O. latifolia W2200), and EE genome species (O. australiensis W0008 and W1538). Except for W0002, the survival rate of these accessions exceeded 50%. In contrast, shoot elongation in accessions of FF genome species (O. brachyantha W0656 and W1401) was enhanced by complete submergence compared with that in the air-grown control, and none of the seedlings survived after the recovery period. In the accession of diploid O. punctata (W1577; BB genome), shoot elongation during complete submergence was similar to that in the air-grown control, and almost none of the seedlings survived after the recovery period.
Response to gradual submergence at mature stage

The effect of gradual submergence at mature stage on the growth of the wild rice accessions was investigated by submergence of 50-day-old plants. The total internode length on the main culm of 50-day-old wild rice accessions is shown in Supplementary Table S3. Internodes were not formed in the accessions of BB (O. punctata W1577), CC (O. eichingeri W1525 and O. officinalis W0002 and W1930), and EE genome species (O. australiensis W0008 and W1538).

Plant length of the wild rice accessions was measured before and after the gradual submergence treatment (Figure 4). Plant length after the treatment in accessions of O. minuta (W1318; BBCC genome) and O. officinalis (W0002 and W1930; CC) was below the final water level of

| Genome type | AA | BB | BBCC | CC | CCDD | EE | FF |
|-------------|----|----|------|----|------|----|----|
| Cultivars/accessions | FR13A | W1577 | W1409 | W1318 | W1525 | W0002 | W1930 | W1147 | RS8-H | W0613 | W1194 | W2200 | W0008 | W1538 | W0656 | W1401 |
| Primer-1 | Primer-2 | Primer-3 |
| SUB1A | Primer-1 | Primer-2 | Primer-3 |
| SUB1B | Primer-1 | Primer-2 | Primer-3 |
| SUB1C | Primer-1 | Primer-2 | Primer-3 |
| 17s rRNA | Primer-1 | Primer-2 | Primer-3 |

Figure 1. Results of genomic PCR analysis of the SUB1A, SUB1B, and SUB1C genes in 15 wild rice accessions and the submergence-tolerant cultivar FR13A.

| Genome type | AA | BB | BBCC | CC | CCDD | EE | FF |
|-------------|----|----|------|----|------|----|----|
| Cultivars/accessions | HA II | W1577 | W1409 | W1318 | W1525 | W0002 | W1930 | W1147 | RS8-H | W0613 | W1194 | W2200 | W0008 | W1538 | W0656 | W1401 |
| Primer-1 | Primer-2 | Primer-3 |
| SK1 | Primer-1 | Primer-2 | Primer-3 |
| SK2 | Primer-1 | Primer-2 | Primer-3 |
| 17s rRNA | Primer-1 | Primer-2 | Primer-3 |

Figure 2. Results of genomic PCR analysis of the SK1 and SK2 genes in 15 wild rice accessions and the deepwater rice cultivar HA II.
In O. brachyantha accessions (W0656 and W1401; FF genome), plant length after the treatment was almost the same as the final water level. In contrast, in all accessions of the other investigated species, plant length after the treatment exceeded the final water level, and their leaves remained above the water surface.

Internodal elongation in the wild rice accessions after the treatment was also investigated (Figure 5). In the deepwater rice cultivar HA II, the internodes elongated by approximately 50 cm under submergence. Internodal elongation comparable to that of HA II was observed in the accessions of diploid O. punctata (W1577; BB genome), O. grandiglumis (RS8-H, W0613, and W1194; CCDD), and O. latifolia (W2200; CCDD). In the accessions of tetraploid O. punctata (W1409; BBCC), O. alta (W1147; CCDD), and O. brachyantha (W0656 and W1401; FF), the internodal elongation was less than half of that of HA II. In contrast, internodal elongation was not significantly promoted under submergence in the accessions of O. minuta (W1318; BBCC) and O. australiensis (W0008 and W1538; EE), whereas little or no internodal elongation was observed in the accessions of the CC genome species (O. eichingeri W1525 and O. officinalis W0002 and W1930). The effect of gradual submergence on the number of internodes and leaves is shown in Supplementary Tables S4 and S5. In all accessions, the number of internodes and leaves increased slightly after the treatment compared to that in the control.

**Discussion**

The seedlings of diploid O. punctata (BB genome) and O. brachyantha accessions (FF genome) did not show reduced shoot elongation under complete submergence,
and most or all seedlings did not survive after the recovery period (Figure 3). In contrast, reduced shoot elongation in completely submerged seedlings was observed in the accessions of tetraploid O. punctata (BBCC), O. minuta (BBCC), O. eichingeri (CC), O. officinalis (CC), O. alta (CCDD), O. grandiglumis (CCDD), O. latifolia (CCDD), and O. australiensis (EE), with survival rates ranging from 38% to 100%. This result indicates that these species have the ability to tolerate complete submergence at the seedling stage. It has been reported that there is a correlation between reduced shoot elongation and survival in completely submerged seedlings of cultivated rice species O. sativa and O. glaberrima (Luo et al., 2020; Setter & Laureles, 1996). In the present study, there is a significantly negative correlation between shoot elongation and survival rate in submerged seedlings of wild rice accessions (Figure 6). Therefore, suppression of growth under complete submergence also may lead to an increase in the survival rate after flood subsidence in wild rice species other than AA genome type. On the other hand, another possibility is that plants with longer height at the start of experiment may be more advantageous for survival under complete submergence since the closer to the surface of water, the greater availability oxygen in the water. However, we failed to find a positive correlation between them (Supplementary Figure S1).

Previously, Niroula et al. (2012) reported that several accessions of the CC genome species O. eichingeri and O. rhizomatis showed submergence tolerance at the seedling stage. In the present study, all the accessions of wild rice species with the CC genome, namely BBCC (O. punctata and O. minuta), CC (O. eichingeri and O. officinalis), and CCDD genome species (O. alta, O. grandiglumis, and O. latifolia), displayed submergence tolerance (Figure 3). It is noteworthy that submergence tolerance was observed in tetraploid O. punctata (BBCC genome), but not in diploid O. punctata (BB). Our results suggest that the CC genome is involved in submergence tolerance.

Niroula et al. (2012) also reported that all the tested submergence-tolerant accessions of O. eichingeri and O. rhizomatis did not have SUB1A gene, suggesting that
they showed SUB1A-independent response to submergence. We previously reported that two accessions of *O. grandiglumis* (RS8-H and W0613) displayed submergence tolerance, but did not possess SUB1A gene (Okishio et al., 2014). In the present study, SUB1A gene was not detected in any submergence-tolerant accessions of the BBCC, CC, CCDD, and EE genome species (Figures 1 and 3), suggesting that SUB1A-independent mechanisms function to allow submergence tolerance at the seedling stage in these wild rice species.

The SUB1 locus accounts for 69% of the phenotypic variance in submergence tolerance, and SUB1A gene is the causative gene at this locus (Xu & Mackill, 1996; Xu et al., 2006). To date, SUB1A has been found in rice species that contain the AA genome, such as *O. sativa*, *O. glaberrima*, *O. nivara*, and *O. rufipogon* (Luo et al., 2020; Niroula et al., 2012; Xu et al., 2006), but not in wild rice species that contain genome types other than the AA genome (Figure 1; Niroula et al., 2012; Okishio et al., 2014). The BB and CC genomes are relatively closely related to the AA genome (Atwell et al., 2014); however, in the present study, SUB1A was not detected even in wild rice species with the BB or CC genomes (Figure 1). Thus, it may be possible that the emergence of SUB1A and establishment of submergence tolerance via SUB1A occurred after the divergence of the AA genome species in the genus *Oryza*. A previous phylogenetic study suggested that SUB1A arose from a duplication of
SUB1B gene present at the same locus (Fukao et al., 2009). SUB1B has been found in all tested cultivars/accessions of AA genome species (Fukao et al., 2009; Xu et al., 2006). In the present study, the presence of SUB1B in the wild rice accessions was also investigated, and SUB1B was not detected in wild rice species containing genomes other than the AA genome (Figure 1). Thus, it may be possible that in addition to SUB1A, the emergence of SUB1B also occurred after divergence of the AA genome species in the genus Oryza.

In the present study, floating ability of the wild rice species was investigated by gradual submergence treatment of 50-day-old plants. After the treatment, plant length of the accessions of the BBCC genome species O. minuta and the CC genome species O. officinalis was below the final water level (Figure 4). In the CC genome species O. eichingeri and the EE genome species O. australiensis, internodal elongation was not significantly promoted by submergence (Figure 5). In contrast, internodal elongation was highly promoted in BB and CCDD genome species (diploid O. punctata, O. grandiglumis, and O. latifolia) to the extent of internodal elongation in the deepwater rice cultivar HA II (Figure 5), suggesting that these species possess strong floating ability. On the other hand, the floating ability of tetraploid O. punctata (BBCC genome), O. alta (CCDD), and O. brachyantha (FF) may be weak because the internodal elongation of these accessions was less than half of that of HA II (Figure 5).

Tetraploid O. punctata and O. minuta are both BBCC genome species but showed different responses to the deepwater condition (Figures 4 and 5). Tetraploid O. punctata arose from polyploidization of diploid O. punctata (BB) and O. eichingeri (CC), whereas O. minuta arose from polyploidization of diploid O. punctata (BB) and O. officinalis (CC). According to the previous phylogenetic study (Zou et al., 2015), the BB genomes of tetraploid O. punctata and O. minuta belong to different clades. In addition, the CC genomes of O. eichingeri and O. officinalis also belong to different clades. These suggest that the BBCC genomes of tetraploid O. punctata and O. minuta are relatively distantly related in their genotypes, both in terms of BB and CC genomes. This might have led to the difference in floating ability between tetraploid O. punctata and O. minuta.

Neither of the two diploid CC genome species (O. eichingeri and O. officinalis) showed floating ability, whereas all tetraploid CCDD genome species (O. alta, O. grandiglumis, and O. latifolia) showed floating ability. Thus, the floating ability of these CCDD genome species may have originated from the DD genome. Although currently, there are no diploid DD genome species in the genus Oryza, previous phylogenetic study has suggested that the DD genome may be derived from an ancestor similar to the EE genome of O. australiensis (Bao & Ge, 2004; Kellogg, 2009). However, floating ability was not observed in O. australiensis (Figure 5). Thus, it might be possible that floating ability in the DD genome emerged after the divergence of the DD and EE genomes, and then spread into the CCDD genome species through polyploidization.

The accessions of diploid O. punctata (BB genome), tetraploid O. punctata (BBCC), O. alta (CCDD), O. grandiglumis (CCDD), O. latifolia (CCDD), and O. brachyantha (FF) showed floating ability (Figures 4 and 5), but SK genes were not detected in any of these wild rice species (Figure 2). To date, SKs have been found in rice species that contain the AA genome, such as O. sativa, O. glumaepatula, O. nivara, and O. rufipogon (Hattori et al., 2009; Sasayama et al., 2018). Thus, it may be possible that the establishment of floating ability via SKs occurred after the divergence of the AA genome species in the genus Oryza.

In O. sativa, the plant hormone ethylene is involved in the promotion of internodal elongation under submergence mediated by SKs, which encode the ERF transcription factor (Hattori et al., 2009). Previously, we found that two accessions of the CCDD genome species O. grandiglumis did not possess SKs, but showed floating ability (Okishio et al., 2014). Furthermore, we also found that enhanced internodal elongation of submerged O. grandiglumis plants was not triggered by ethylene (Okishio et al., 2015). In the present study, the wild rice species that displayed floating ability also lacked SK genes. Thus, these findings suggest that ethylene might not be involved in the floating ability of these wild rice species.

Table 1 summarizes the presence or absence and degree of submergence tolerance and floating ability in wild rice accessions determined in the present study.

| Genotype | Species | Cultivar/Accession | Submergence tolerance | Floating ability |
|----------|---------|--------------------|-----------------------|------------------|
| BB       | O. punctata | W1577 | - | ++ |
| BBCC     | O. punctata | W1409 | ++ | + |
|          | O. minuta  | W1318 | ++ | - |
| CC       | O. eichingeri | W1525 | + | - |
|          | O. officinalis | W0002 | + | - |
|          |            | W1930 | ++ | - |
| CCDD     | O. alta    | W1147 | ++ | + |
|          | O. grandiglumis | R58-H | ++ | ++ |
|          |            | W0613 | ++ | ++ |
|          |            | W1194 | ++ | ++ |
|          | O. latifolia | W2200 | ++ | ++ |
| EE       | O. australiensis | W0008 | + | - |
|          |            | W1538 | ++ | - |
| FF       | O. brachyantha | W0656 | + | - |
|          |            | W1401 | + | - |

+++, very strong; ++, strong; +, weak; -, intolerant
AA genome determined in the present study. Among these species, only *O. grandiglumis* and *O. latifolia* showed strong submergence tolerance at the seedling stage and floating ability at mature stage. Elucidation of the underlying mechanisms might provide new insights into the improvement of flooding adaptability in cultivated rice species.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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