The Submergence Tolerance Regulator SUB1A Mediates Crosstalk between Submergence and Drought Tolerance in Rice

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Submergence and drought are major constraints to rice (Oryza sativa) production in rain-fed farmlands, both of which can occur sequentially during a single crop cycle. SUB1A, an ERF transcription factor found in limited rice accessions, dampens ethylene production and gibberellic acid responsiveness during submergence, economizing carbohydrate reserves and significantly prolonging endurance. Here, we evaluated the functional role of SUB1A in acclimation to dehydration. Comparative analysis of genotypes with and without SUB1A revealed that SUB1A enhanced recovery from drought at the vegetative stage through reduction of leaf water loss and lipid peroxidation and increased expression of genes associated with acclimation to dehydration. Overexpression of SUB1A augmented ABA responsiveness, thereby activating stress-inducible gene expression. Paradoxically, vegetative tissue undergoes dehydration upon desubmergence even though the soil contains sufficient water, indicating that leaf desiccation occurs in the natural progression of a flooding event. Desubmergence caused the upregulation of gene transcripts associated with acclimation to dehydration, with higher induction in SUB1A genotypes. SUB1A also restrained accumulation of reactive oxygen species (ROS) in aerial tissue during drought and desubmergence. Consistently, SUB1A increased the abundance of transcripts encoding ROS scavenging enzymes, resulting in enhanced tolerance to oxidative stress. Therefore, in addition to providing robust submergence tolerance, SUB1A improves survival of rapid dehydration following desubmergence and water deficit during drought.

INTRODUCTION

Global climate change influences the frequency and magnitude of hydrological fluctuations, causing devastating events such as floods and drought. Both high and low extremes in precipitation increasingly limit food, fiber, and forest production worldwide (Easterling et al., 2007). Approximately 30% of the world’s rice (Oryza sativa) farmlands are at a low elevation and irrigated by rain (Bailey-Serres et al., 2010). Such rain-fed farming lessens groundwater depletion, water pollution, and soil salinization, which are often associated with controlled irrigation systems. However, rain-fed fields are prone to flooding and drought due to inadequate water management. Thus, submergence, drought, and the sequential events (submergence followed by drought and vice versa) are major constraints to rice production in rain-fed lowlands. Therefore, improvement of combined tolerance to submergence and drought would substantially increase rice productivity while sustaining water resources and soil quality.

Rice is a semiaquatic species that is typically cultivated under partially flooded conditions. However, flash flooding can cover the entire plant for prolonged periods, and most rice cultivars die within 7 d of complete submergence (Xu et al., 2006; Bailey-Serres et al., 2010). A limited number of rice cultivars overcome submergence through antithetical growth responses. Deepwater rice responds to submergence by promoting internode elongation to outgrow floodwaters. This escape response is regulated by a polygenic locus that encodes two APETALA2/Ethylene Response Factor (AP2/ERF) DNA binding proteins, SNORKEL1 (SK1) and SK2 (Hattori et al., 2009). By contrast, submergence-tolerant lowland rice, including Flood Resistant 13A (FR13A), restrains elongation growth, economizing carbohydrate reserves to enable development of new leaves upon desubmergence. This quiescence response is regulated by another AP2/ERF, located at the polygenic SUBMERGENCE1 (SUB1) locus (Xu et al., 2006). The highly submergence-inducible SUB1A gene that confers this tolerance is absent in all japonica and most indica accessions. Additionally, the closely related SUB1B and SUB1C genes are also submergence inducible and invariably present in rice accessions at the SUB1 locus but are not associated with submergence tolerance.

During submergence, SUB1A dampens ethylene production and enhances mRNA and protein accumulation of two negative regulators of gibberellic acid (GA) signaling, SLENDER RICE1 (SLR1) and SLR1-like 1 (SLRL1), resulting in the suppression of the energy-consuming escape response (Fukao et al., 2006; Fukao and Bailey-Serres, 2008). Consistently, global-scale transcriptome analysis revealed that SUB1A regulates the abundance of mRNAs associated with ethylene and GA production and signaling during submergence (Jung et al., 2010). SUB1A also coordinates diverse transcription factors, including a subset...
of AP2/ERFs at the mRNA accumulation level. A recent survey of 13 rice accessions containing SUB1A found that the degree of submergence tolerance positively correlates with the expression level of SUB1A in node and internode regions during submergence (Singh et al., 2010). Although the SKs and SUB1A regulate opposite growth responses under submerged conditions, all are group VII ERF subfamily members.

In general, rice is sensitive to drought due to its high water requirement, but upland and lowland rice varieties range in their tolerance. Molecular genetic analyses detected a number of quantitative trait loci that affect the components related to drought tolerance, including grain production, shoot and root morphology, and leaf water status (Lanceras et al., 2004; Yue et al., 2006; Venuprasad et al., 2009). However, no major genes that regulate these traits have been identified because of the weak effect and low mapping resolution of the quantitative trait loci regions. Through genomic and molecular approaches, a number of transcription factors involved in drought tolerance have been identified in Arabidopsis thaliana. Among these factors, members of DEHYDRATION RESPONSIVE ELEMENT BINDING PROTEIN/C-REPEAT BINDING FACTOR (DREB/CFB), ABSCISIC ACID RESPONSIVE ELEMENT (ABRE) BINDING PROTEIN/ABRE BINDING FACTOR (AREB/ABF), NAM ATAF CUC2 (NAC), and ERF serve functional roles in transcriptional regulation of acclimation responses and tolerance to drought (Nakashima et al., 2009; Hirayama and Shinozaki, 2010). Drought stress induces mRNA accumulation of these transcription factors in an abscisic acid (ABA)-dependent or -independent manner, regulating expression of their direct target genes that encode late embryogenesis abundant (LEA) proteins, antioxidant enzymes, osmolyte biosynthesis enzymes, chaperones, and transporters. Rice (Os) orthologs of these Arabidopsis transcription factors, such as Os DREB1A, Os DREB2B, Os ABF1, and Os NAC6, induce similar stress-inducible genes, and their ectopic expression increases drought tolerance in rice (Dubouzet et al., 2003; Nakashima et al., 2007; Amir Hossain et al., 2010; Matsukura et al., 2010), indicating that Arabidopsis and rice share common transcriptional networks that coordinate drought response and tolerance mechanisms.

Plants encounter multiple abiotic stresses simultaneously or sequentially in a natural or agricultural environment. To survive extreme conditions, plants modulate adaptive responses through complex signaling pathways, which are integrated at various levels. ABA plays a crucial role in the adaptive responses to drought, high salt, and freezing, all of which induce a cellular osmotic stress. Each stress stimulates accumulation of ABA in vegetative tissue, resulting in stomatal closure, stress-inducible gene expression, and metabolic adjustment (Zhu, 2002; Seki et al., 2007). It is well known that transcription factors such as DREB1/CFB, DREB2, AREB/ABF, and NAC regulate expression of genes associated with acclimation to osmotic stress (Dubouzet et al., 2003; Nakashima et al., 2007; Amir Hossain et al., 2010; Matsukura et al., 2010). Overexpression of each of these genes enhances tolerance to multiple stresses, including drought, salinity, and low temperature in Arabidopsis and rice.

Despite the significance of submergence and drought to rice production in rain-fed farmlands, the molecular crosstalk between the two stress responses has not been investigated. Here, we evaluated the influence of the submergence tolerance regulator, SUB1A, on acclimation responses to water deficit during the vegetative growth phase of rice. Comparative analyses revealed a pivotal role of SUB1A in water relations, detoxification of reactive oxygen species (ROS), and stress-inducible gene expression during drought. In addition to conditions where water is limited, rice experiences dehydration stress following desubmergence due to reduced hydraulic conductivity in leaf sheaths (Setter et al., 2010). Through the SUB1A-mediated responses to drought, submerged plants overcome this inevitable stress following desubmergence. Thus, SUB1A serves as a convergence point between submergence and drought response pathways, allowing rice plants to survive both extremes of precipitation.

**RESULTS**

**SUB1A Confers Drought Tolerance to Rice**

Although exposure of rice plants to prolonged submergence results in severe leaf senescence, genotypes containing SUB1A are able to recover after desubmergence through formation of new leaves (Fukao et al., 2006; Fukao and Bailey-Serres, 2008). This indicates that SUB1A contributes to protection of meristematic cells from submergence and reoxygenation stress. We considered that the protective role of SUB1A may aid endurance of other abiotic stresses, such as drought. To evaluate the contribution of SUB1A to water deficit tolerance, a japonica inbred line, M202, and a near isogenic SUB1 introgression line, M202(Sub1), were subjected to comparative analysis at physiological and molecular levels. These lines differ in an ~182-kb interval of chromosome 9 containing the polygenic SUB1 locus (Xu et al., 2006). M202(Sub1) possesses all three SUB1 genes, SUB1A, SUB1B, and SUB1C, whereas M202 lacks SUB1A. Both M202 and M202(Sub1) plants were grown in the same pot for 14 d and uniformly exposed to dehydration stress by withholding water for 8 d (Figure 1A). Following the drought, pots were placed in a shallow tray filled with water for complete soil rehydration. In both genotypes, all of the fully expanded leaves were severely wilted after 8 d of drought and did not recover with rehydration.

However, there was a significant difference in the establishment of new leaves by the two genotypes after 14 d of recovery; 71.7% of M202(Sub1) plants recommenced leaf development as compared with only 11.7% of the M202 plants (Figure 1B). Drought stress reduced the fresh weight of aerial tissue similarly in the two genotypes, but recovery of M202(Sub1) plants was distinguished by vigorous leaf formation and fresh weight accumulation (Figure 1C). When leaf relative water content (RWC) was monitored during 7 d of drought, we found it declined similarly in the two genotypes for the first 4 d, after which the decline was significantly less severe in M202(Sub1) leaves (Figure 1D). This indicates that SUB1A contributes to limiting leaf water loss during drought. We also investigated two Sub1 introgression lines in indica backgrounds, IR64(Sub1) and Samba Mahsuri (Sub1), to verify that the influence of SUB1A on drought tolerance was robust (see Supplemental Figure 1 online). The results of viability tests and fresh weight measurements in these Sub1 lines were in accordance with those in M202(Sub1), indicating that
The introgression of SUB1A significantly improves recovery from prolonged dehydration stress in both japonica and indica cultivars with varying drought tolerance. A variety of environmental stresses, including drought, stimulate the excessive accumulation of ROS, which results in oxidative damage of cellular components, such as lipids, proteins, and DNA. To determine whether SUB1A influences ROS accumulation in leaves, lipid peroxidation was monitored by measuring a product of the peroxidation events, malondialdehyde (MDA) (Figure 1E). MDA levels were elevated in response to drought stress in both genotypes but were significantly suppressed in M202(Sub1) from day 3 to 6. These observations indicate that SUB1A diminishes the ROS accumulation triggered by water deficit.

The SUB1 locus encodes up to three ERF transcription factors, SUB1A, SUB1B, and SUB1C, all of which are induced by submergence. To obtain the expression profile of SUB1 genes during drought, we monitored transcripts of these three genes in
aerial tissue of M202 and M202(Sub1) by quantitative RT-PCR (qRT-PCR) (Figure 1F). Accumulation of SUB1A mRNA was highly enhanced in response to drought stress. SUB1B and SUB1C mRNAs were slightly induced by drought, with lower SUB1C mRNA levels in M202(Sub1). This trend is consistent with previous reports that SUB1A dampens accumulation of SUB1C mRNA (Fukao et al., 2006; Xu et al., 2006; Fukao and Bailey-Serres, 2008).

To evaluate if SUB1A expression also aids in tolerance to osmotic stress, M202 and M202(Sub1) seedlings were exposed to constant osmotic stress by use of polyethylene glycol (see Supplemental Figure 2 online). Osmotic stress (∼0.6 MPa) considerably reduced the elongation growth of shoots in both genotypes and roots in M202 (see Supplemental Figures 2A and 2B online). Inhibition of shoot and root growth was less severe in M202(Sub1), indicating that SUB1A enhances tolerance to osmotic stress. To confirm that osmotic stress induces expression of SUB1 genes, mRNA levels were monitored in coleoptiles and roots of seedlings treated with polyethylene glycol (see Supplemental Figure 2C online). The SUB1A transcript was highly elevated 6 h after osmotic stress treatment and became more abundant at later time points. The rapid accumulation of SUB1A mRNA was observed in both shoots and roots, but the fold change value was much greater in shoots. Levels of SUB1B and SUB1C transcripts also increased in response to osmotic stress. As seen under drought, the accumulation of SUB1C mRNA was restricted in M202(Sub1) under osmotic stress.

**SUB1A Upregulates Genes Involved in Acclimation to Drought**

Microarray analyses revealed that SUB1A, encoding an ERF transcription factor, mediates regulation of transcripts encoding other transcription factors, including AP2/ERF genes, which are involved in stress response and acclimation (Jung et al., 2010; Mustroph et al., 2010). To examine the overlap of differentially regulated genes during submergence and dehydration, publicly available microarray data sets were analyzed. Direct comparison of significantly induced genes (fold change ≥2; adjusted P value ≤ 0.05) demonstrated that 25.5% of submergence-inducible gene transcripts are also upregulated by dehydration (see Supplemental Data Set 1 and Supplemental Figure 3A online). Among 793 genes positively regulated by SUB1A (fold change ≥ 1.5; adjusted P value ≤ 0.05), 226 (28.5%) are upregulated in response to water deficit (see Supplemental Data Set 2 and Supplemental Figure 3B online). This suggests that SUB1A regulates drought-responsive genes, several of which were monitored at the level of mRNA accumulation over a time course of water deficit in M202 and M202(Sub1) (Figure 2). DREB1/CFB encodes group Ic AP2/ERFs, which regulate expression of genes associated with tolerance to drought, salinity, and low temperature in *Arabidopsis* and rice (Nakashima et al., 2009). It has been reported that overexpression of rice DREB1A and DREB1E significantly increases survival of water deficit (Ito et al., 2006; Chen et al., 2008). We found that drought stress induced the accumulation of DREB1A mRNA in aerial tissue of M202 and M202(Sub1), but the level was significantly higher in M202(Sub1) at days 3 and 4 (Figure 2). The level of DREB1E transcripts decreased in response to water deficit in both lines, but M202 (Sub1) maintained significantly more transcript during drought stress. AP37 (ERF3) and AP59 belong to the AP2/ERF groups VIIIa and IXa, respectively, and constitutive expression of each enhances recovery from water deficit during vegetative development (Oh et al., 2009). Consistently, we found that both were upregulated by drought stress, with significantly more transcript in M202(Sub1) (Figure 2).

**Figure 2. SUB1A Enhances mRNA Accumulation of Genes Associated with Acclimation to Drought.**

mRNA levels were measured in leaves of plants subjected to 0, 3, 4, or 5 d of drought and are expressed relative to the level in M202 at day 0 (set at 1.0). Data represent mean ± SE from three independent biological replicates, and an asterisk indicates a significant difference between M202 and M202(Sub1) (**P < 0.05; ***P < 0.01).
Overexpression of genes encoding LEAs increases survivability, biomass production, and grain yield in barley (Hordeum vulgare), wheat (Triticum aestivum), and rice under water deficit conditions (Valliyodan and Nguyen, 2006). The rice LEAs, RAB16A (RAB21), LEA3, and LIP9 (DHN1/DIP1), are strongly induced by drought and ABA treatment. Constitutive and conditional expression of LEA3 results in increased spikelet fertility and grain production during drought (Xiao et al., 2007). SalT is a jacalin-like lectin protein and was originally identified as one of the most prominent proteins induced by high salt in rice roots (Claes et al., 1990). Drought stress highly induced the accumulation of RAB16B, LEA3, LIP9, and SalT mRNAs in M202 and M202(Sub1) (Figure 2). Among these genes, the transcripts of RAB16B, LIP9, and SalT were significantly more abundant in M202(Sub1) under dehydrated conditions, indicating that SUB1A acts upstream of these genes.

**SUB1A Increases ABA Responsiveness**

ABA is a key signaling molecule that coordinates water balance, expression of stress-inducible genes, and metabolic adjustment under water deficit conditions (Zhu, 2002; Seki et al., 2007). ABA-deficient and ABA-insensitive mutants of Arabidopsis exhibit disturbed water relations and poor acclimation to water deficit (Koornneef et al., 1998; Zhu, 2002). By contrast, ABA hypersensitive mutants and ABA-overproducing transgenics show restricted water loss and increased tolerance under dehydrated conditions in Arabidopsis and Nicotiana plumbaginifolia (Pei et al., 1998; Iuchi et al., 2001; Qin and Zeevaart, 2002). To determine whether ABA regulates accumulation of SUB1 gene transcripts, genotypes with and without SUB1A were treated with two concentrations of ABA (5 or 50 μM) and the aerial tissue used for RNA analyses. Application of ABA decreased levels of all three SUB1 transcripts in M202 and M202(Sub1) (Figure 3A). No significant difference in SUB1C transcript level was observed between the two genotypes, indicating that the expression of SUB1A mRNA was not sufficient to repress the accumulation of SUB1C mRNA in ABA-treated M202(Sub1) plants. The transgenic Ubi:SUB1A-3 and nontransformed LG control (O. sativa cv Liaoeng) were also evaluated. The constitutive accumulation of SUB1A mRNA was not altered by ABA treatment in Ubi:SUB1A-3 (Figure 3B). This suggests that ABA does not influence turnover of SUB1A mRNA, but it rather restricts SUB1A transcription. Consistent with results from the M202 and M202(Sub1) genotypes, ABA downregulated SUB1B and SUB1C mRNA accumulation in the overexpression line and the control. Overexpression of SUB1A decreased the transcript levels of SUB1B as well as SUB1C under nontreated conditions.

Although ABA reduces the abundance of the SUB1A transcript in aerial tissue under nonstress conditions, the transcript is highly induced by drought and osmotic stress (Figure 1F; see Supplemental Figure 2C online). To determine the influence of SUB1A on ABA responsiveness, seeds of LG and two independent Ubi: SUB1A lines were incubated in a series of ABA solutions (Figure 3C). Application of 10 μM ABA had little effect on germination repression in LG seeds but significantly suppressed germination of the two Ubi:SUB1A lines. Overexpression of SUB1A further enhanced the inhibition of seed germination at higher ABA concentrations. ABA responsiveness of seedling shoots was also evaluated in the three genotypes (Figure 3D). Consistent with the recalcitrance in seed germination, shoot growth was more severely restricted by ABA in the two Ubi:SUB1A lines. These results indicate that SUB1A increases responsiveness to ABA, in addition to the decrease in GA responsiveness shown previously (Fukao and Bailey-Serres, 2008).

We reported that seeds of the SUB1A overexpression lines exhibit a dormancy phenotype (Fukao and Bailey-Serres, 2008). Application of fluridone, an ABA biosynthesis inhibitor, stimulates germination of dormant seeds of N. plumbaginifolia, Arabidopsis, and red rice (Oryza sativa f. spontanea), emphasizing the critical role of ABA in seed dormancy (Grappin et al., 2000; Cadman et al., 2006; Gianinetti and Vernieri, 2007). To evaluate the significance of ABA biosynthesis in SUB1A-mediated seed dormancy, we incubated seeds of LG and the two Ubi:SUB1A transgenics in a series of fluridone solutions (Figure 3E; see Supplemental Figure 4 online). Application of fluridone slightly accelerated the rate of seed germination in LG. However, the inhibitor did not rescue the phenotype of the SUB1A overexpression lines. It appears that ABA production is not involved in the seed dormancy caused by ectopic expression of SUB1A.

To discern if SUB1A increases ABA responsiveness at the gene expression level, we analyzed ABA-regulated genes that are associated with drought tolerance by qRT-PCR (Figure 4). Application of ABA decreased the levels of DREB1A, DREB1E, and AP59 mRNAs in aerial tissue of LG and Ubi:SUB1A-3. However, these three transcripts constitutively accumulated to higher levels in the SUB1A overexpression line under mock conditions, and the higher relative accumulation was maintained at 5 and 50 μM ABA. AP37 was upregulated by ABA, with significantly higher induction in the SUB1A overexpression line under mock and ABA-treated conditions. These results demonstrate that SUB1A coordinates transcription of other AP2/ERF transcription factors that are key regulators of dehydration tolerance. Representative ABA-responsive genes that have been well characterized, such as RAB16A, LEA3, LIP9, and SaIt, were analyzed by qRT-PCR (Figure 4). Interestingly, constitutive expression of SUB1A considerably elevated these ABA-inducible transcripts under mock conditions and even further with ABA treatment. These results reveal that SUB1A enhances responsiveness to ABA at the mRNA accumulation level, which is in accordance with ABA-mediated effects on seed germination and shoot elongation (Figures 3C and 3D).

**SUB1A Moderates Leaf Dehydration Triggered upon Desubmergence**

Plants encounter cellular dehydration as a consequence of a variety of environmental stress, such as drought, high salt, and low temperature, all of which affect osmotic adjustment and water balance. Interestingly, we noted that upper leaves of submerged plants were rolled 1 h after desubmergence/reoxygenation following 7 d of submergence (Figure 5A), indicating that desubmerged plants encounter dehydration stress. Although this duration of submergence is sublethal to both genotypes (Fukao et al., 2006), it appeared that desubmerged M202 plants...
were more severely wilted than M202(Sub1) plants. To evaluate the contribution of SUB1A to leaf water management during the early recovery period, RWC was monitored in the upper leaves of M202 and M202(Sub1) plants after reoxygenation (Figure 5B). Upon desubmergence, the RWC decreased rapidly in both genotypes, but M202(Sub1) significantly moderated this water loss. Thus, SUB1A functions to maintain leaf water content following reoxygenation as observed under drought (Figure 1D).

Reoxygenation injury commonly occurs primarily due to the overproduction of ROS upon sudden reexposure to atmospheric...
mock-treated LG = 1.0). Data represent mean ± SE from three independent biological replicates, and an asterisk indicates a significant difference between LG and Ubi:SUB1A-3 (\( *P < 0.05; **P < 0.01 \)).

![Figure 4. SUB1A Increases ABA Responsiveness at the mRNA Accumulation Level.](image)

Developmentally matched plants 14-d-old LG and 21-d-old Ubi:SUB1A-3 were treated with mock (0.1% [v/v] DMSO) or ABA solution (5 or 50 \( \mu \)M in 0.1% [v/v] DMSO) for 24 h, and aerial tissue was analyzed by qRT-PCR (mock-treated LG = 1.0). Data represent mean ± SE from three independent biological replicates, and an asterisk indicates a significant difference between LG and Ubi:SUB1A-3 (\( *P < 0.05; **P < 0.01 \)).

SUB1A Enhances Oxidative Stress Tolerance

The data presented above indicate that both drought and reoxygenation trigger accumulation of ROS, which results in cellular damage by oxidative stress (Figures 1E, 5C, and 5D). We hypothesized that SUB1A may improve tolerance to oxidative stress, leading to enhanced acclimation to drought and submergence. To examine this, we incubated M202 and M202(Sub1) seedlings with methyl viologen (paraquat), which stimulates formation of ROS within chloroplasts (Figure 7A). Application of methyl viologen dramatically repressed seedling growth in both genotypes, but the reduction in shoot fresh weight was more severe in M202 at all concentrations tested (Figure 7B). Methyl viologen also reduced chlorophyll content, presumably as a secondary consequence of oxidative stress (Figure 7C). Notably, M202(Sub1) restricted the degradation of chlorophyll \( \alpha \) and \( \beta \), as observed in submerged plants (Fukao et al., 2006). To confirm that SUB1A moderates the breakdown of chlorophylls by oxidative stress, leaf segments of M202 and M202(Sub1) were treated with methyl viologen or hydrogen peroxide in the light (see Supplemental Figures 5A and 5B online). Both chemical treatments decreased the content of chlorophyll \( \alpha \) and \( \beta \) in the two genotypes, but to a significantly greater extent in M202.
These results confirm that SUB1A can diminish cellular oxidative damage caused by excessive ROS production.

These observations led us to consider whether oxidative stress might regulate levels of SUB1 gene transcripts. Indeed, methyl viologen upregulated the accumulation of SUB1A mRNA in M202(Sub1), whereas SUB1B and SUB1C mRNAs slightly decreased in both lines (Figure 7D). The level of SUB1A induction was far less than that observed in response to submergence (Figure 6A). Consistent with SUB1A-mediated repression, the level of SUB1C transcript was significantly lower in M202(Sub1) compared with M202.

The abundance of intercellular ROS is tightly regulated through complex antioxidant systems in diverse subcellular compartments. Among these ROS-scavenging pathways, ascorbate peroxidase (APX), superoxide dismutase (SOD), and catalase (CAT) are major enzymes that detoxify superoxide and hydrogen peroxide under stressed conditions in plants (Mittler et al., 2004). The rice (japonica) genome has eight annotated genes that encode APX isoforms, two of which (APX1 and APX2) are localized at the cytosol (Teixeira et al., 2006). Molecular analysis of Arabidopsis apx knockout mutants revealed that cytosolic APX plays a central role in regulation of the ROS scavenging
systems, which modulates protection from high light and a combination of drought and heat (Davletova et al., 2005a; Koussevitzky et al., 2008). Overexpression of rice APX1 and APX2 in Arabidopsis reduces the accumulation of hydrogen peroxide, restricts chlorophyll degradation, and enhances survival under salinity stress (Lu et al., 2007). We determined that the accumulation of both APX1 and APX2 mRNAs was upregulated by methyl viologen treatment in M202 and M202(Sub1), but the induction was more pronounced in the SUB1 genotype (Figure 8). Of the five SOD genes characterized in rice, mitochondrial SodA1 (mitochondrial Mn-SOD) and SodB (Fe-SOD) are significantly induced by osmotic stress and severe low oxygen, respectively (Kaminaka et al., 1999; Magnesi and Perata, 2009). We found that SodA1 mRNA was elevated by oxidative stress, with stronger induction in M202(Sub1), whereas SodB was not regulated by the stress (Figure 8). Three catalase genes have been recognized in rice (Iwamoto et al., 2000). We found that CatA and CatB were oxidative stress inducible and more prominently induced in M202(Sub1), whereas CatC was downregulated by the stress in both genotypes. Based on these directed transcript studies, in planta ROS detection, and lipid peroxidation assays (Figures 1E, 5C, 5D, and 6), we suggest that SUB1A augments the capability of antioxidant systems, thereby improving tolerance to oxidative stress.

DISCUSSION

The results presented here demonstrate that SUB1A, the submergence tolerance regulator found in a limited number of rice accessions, properly coordinates physiological and molecular responses to cellular water deficit when it occurs independently or following desubmergence. ABA is a crucial signaling molecule that affects acclimation responses to both extremes in precipitation, drought, and submergence. Drought increases endogenous ABA levels in aerial tissue, modulating leaf water balance and the expression of genes associated with dehydration tolerance (Zhu, 2002; Seki et al., 2007). In the case of deepwater rice, the decline in ABA augments GA-mediated internode elongation and ethylene-dependent adventitious root formation during submergence (Hoffmann-Benning and Kende, 1992; Steffens et al., 2006). Recently, it was reported that modulation of internal ABA levels is a key determinant in the rate of underwater petiole elongation in submergence-tolerant Rumex palustris ecotypes, allowing escape of leaves from floodwaters (Chen et al., 2010). It appears that submergence-induced ABA degradation is a prerequisite for escape of submergence through rapid shoot elongation. Interestingly, exogenous application of ABA increases survival of oxygen deprivation in maize (Zea mays) seedlings and Arabidopsis roots (Hwang and Vantoai, 1991; Ellis et al., 1999). Thus, it is plausible that ABA may also act as a positive regulator for submergence tolerance, presumably through restriction of energy-consuming development and metabolism.

We previously demonstrated that the rapid breakdown of ABA occurs in a SUB1A-independent manner in aerial tissue of submerged rice using the same genotypes evaluated in this study (Fukao and Bailey-Serres, 2008). In accordance with this, application of fluridone, an ABA biosynthesis inhibitor, did not rescue the slow germination phenotype of SUB1A overexpression lines (Figure 3E; see Supplemental Figure 4 online). For these reasons, it is unlikely that SUB1A regulates the production or turnover of endogenous ABA. On the other hand, our data show that SUB1A intensifies responsiveness to ABA, leading to prevention of leaf water loss and additional induction of stress responsive genes (Figures 1D, 3C, 3D, and 4).

Acclimation responses to drought are properly coordinated through integrated regulatory networks that consist of ABA-dependent and -independent pathways (Nakashima et al., 2009; Hirayama and Shinozaki, 2010). Besides ABA-regulated genes, SUB1A enhances mRNA accumulation of representative drought tolerance regulators, DREB1A, DREB1E, and AP59, which are
This suggests that SUB1A-mediated dehydration tolerance is mediated through impacts on both ABA-dependent and -independent pathways.

ABA and GA antagonistically regulate plant growth, development, and stress responses. We previously reported that SUB1A restricts GA responsiveness through increased accumulation of the central suppressors for GA signaling, SLR1 and SLRL1, during submergence (Fukao and Bailey-Serres, 2008). SLR1 is a DELLA domain-containing protein in the GRAS transcription factor family. Rice has only one DELLA protein (SLR1), whereas Arabidopsis possesses five DELLAs that exhibit functional redundancy (Ueguchi-Tanaka et al., 2007). Arabidopsis quadruple-DELLA mutants display reduced responsiveness to ABA in the GA-deficient ga1-3 backgrounds (Achard et al., 2006). In addition, comparative analysis of wild type, ga1-3, and ga1-3 quadruple-DELLA mutants demonstrated that DELLAs activate expression of genes encoding antioxidant enzymes and diminish ROS accumulation under high salinity in seedlings (Achard et al., 2008). The demonstration that SUB1A-containing genotypes maintain higher levels of SLR1 and SLRL1 is consistent with their increased ABA responsiveness and reduced ROS accumulation.

Figure 7. SUB1A Enhances Tolerance to Oxidative Stress.

(A) Photo of rice seedlings treated with an oxidative herbicide. Seven-day-old seedlings were transferred onto 0.5× MS media containing methyl viologen (0, 1, 5, or 20 μM) and incubated for 14 d (16 h light/8 h dark; light level, 50 μmol m⁻² s⁻¹). Bars = 3 cm. (B) Growth inhibition of aerial tissue by oxidative stress. Relative fresh weight was calculated by comparison to the nontreated seedlings of individual genotypes. Data represent mean ± SD (n = 12) from three independent biological replicates, and an asterisk indicates a significant difference between M202 and M202(Sub1) (*P < 0.05; **P < 0.01). (C) Chlorophyll (Chl) contents in aerial tissue of plants exposed to oxidative stress. Data represent mean ± SD from three independent biological replicates, and an asterisk indicates a significant difference between M202 and M202(Sub1) (*P < 0.05; **P < 0.01). (D) Relative mRNA levels of SUB1 genes in aerial tissue of plants treated with oxidative stress. Fourteen-day-old plants were exposed to methyl viologen (0, 1, or 20 μM) for 24 h in the light (100 μmol m⁻² s⁻¹), and the aerial tissue was subjected to qRT-PCR analysis. Data represent mean ± SE from three independent biological replicates, and an asterisk indicates a significant difference between M202 versus M202(Sub1) (*P < 0.05; **P < 0.01).
Fourteen-day-old plants were exposed to methyl viologen (0, 1, or 20 µM) for 24 h in the light (100 µmol m⁻² s⁻¹), and the aerial tissue was subjected to qRT-PCR analysis. Data represent mean ± SE from three independent biological replicates, and an asterisk indicates a significant difference between M202 versus M202(Sub1) (*P < 0.05; **P < 0.01).

The reaeration of aerial tissue is a phase in the natural progression of a sublethal flooding event. We observed that upper leaves of M202 and M202(Sub1) plants rapidly rolled and wilted upon desubmergence (Figure 5A). Accompanying this, representative dehydration-responsive genes were rapidly induced in aerial tissue (Figure 6B). These observations are consistent with visible symptoms of leaf water deficit observed in rice fields that had experienced 10 d of complete submergence (Setter et al., 2010), strongly implicating dehydration stress as a component of a transient submergence event.

Paradoxically, the desiccation of leaves after desubmergence occurs even though the soil typically contains sufficient water. Physiological analysis of the submergence-intolerant rice cultivar IR42 revealed that leaf water loss upon desubmergence is restricted by stomatal closure, but water transport to leaf blades was hampered by low hydraulic conductivity in the leaf sheaths (Setter et al., 2010). We found that SUB1A moderates desiccation of leaves following desubmergence (Figures 5A and 5B). One possible explanation is that SUB1A manages water transport from the root system through regulation of leaf solute potential. Submergence dramatically promotes consumption of energy reserves, but SUB1A restricts overall soluble carbohydrate consumption in aerial tissue (Fukao et al., 2006). Preservation of soluble carbohydrates may be of benefit for maintenance of a water potential gradient as well as avoidance of carbohydrate starvation. Future evaluation of metabolite dynamics should shed additional light on the relevant processes associated with water relations during submergence and desubmergence.

Both drought and submergence promote rapid accumulation of ROS, which function as signaling molecules to trigger diverse acclimation responses to the stresses (Blokhina and Fagerstedt, 2010; Miller et al., 2010). However, excessive accumulation of ROS results in cellular damage by oxidative stress, mandating the potential for ROS detoxification to enable survival of water deficit and submergence. Indeed, several studies successfully increased tolerance to dehydration through manipulation of genes encoding ROS detoxification enzymes and transcriptional regulators in Arabidopsis, rice, and tobacco (Nicotiana tabacum) (Badawi et al., 2004; Wang et al., 2005; Davletova et al., 2005b; Mittler et al., 2006; Lu et al., 2007; Wu et al., 2008). In addition to dehydration tolerance, the ability to manage ROS detoxification can influence survival of a submergence event. The specific activity of SOD is more significantly induced upon reoxygenation in rhizomes of submergence-tolerant Iris pseudacorus than intolerant Iris germanica (Monk et al., 1987), which is negatively correlated with lipid peroxidation (Blokhina et al., 1999). Similarly, submergence-tolerant FR13A rice, the donor of SUB1A in this study, restricts lipid peroxidation and chlorophyll degradation following desubmergence compared with intolerant IR42 (Ella et al., 2003). The data presented here and previous analysis of microarray data reveal that SUB1A upregulates transcripts of genes associated with antioxidant enzymes and metabolites, thereby dampening oxidative damage during drought and reoxygenation (Figures 1E, 5C, 5D, and 8) (Jung et al., 2010; Mustroph et al., 2010). The future characterization of the direct targets of SUB1A may expose the regulatory mechanisms underlying the relevant ROS detoxification network.

We propose a model for SUB1A-mediated responses to a natural progression of abiotic stresses wrought by a flash-flooding event (Figure 9). During submergence, SUB1A restricts ethylene production and GA responsiveness, resulting in the suppression of shoot elongation and carbohydrate consumption (Fukao et al., 2006; Fukao and Bailey-Serres, 2008). This quiescence response enables the avoidance of carbohydrate starvation, allowing plants to endure submergence for up to 2 weeks. Maintenance of carbohydrate reserves during submergence could also support the rapid production of osmolytes during desiccation and recommencement of leaf development upon desubmergence. As floodwaters subside, reoxygenated aerial tissue encounters an oxidative stress followed by a rapid cellular dehydration. Under reoxygenation and drought, SUB1A restricts ROS accumulation, which diminishes oxidative damage. The induction of SUB1A leads to elevation of ERF mRNAs (e.g., DREB1s and AP59), which regulate genes associated with adaptation to dehydration. Subsequently, the enhancement of
ABA responsiveness by SUB1A triggers expression of LEA mRNAs and dampens leaf water loss. Interestingly, SUB1A mRNA levels rise in response to submergence, drought, oxidative stress, and ethylene to varying levels (3.5- to 900-fold) but are downregulated by ABA (Figure 3; Fukao et al., 2006; Fukao and Bailey-Serres, 2008). ABA might serve as a negative regulator of SUB1A mRNA accumulation to properly manage the magnitude and duration of the diverse acclimation responses to submergence, reoxygenation, and dehydration. Although ethylene upregulates SUB1A mRNA accumulation during submergence, it is not known if ethylene or another signal is responsible for elevation of this transcript in response to drought. The relationship between ethylene production and dehydration stress is species specific, and the role of ethylene in drought response remains unclear in rice (Acharya and Assmann, 2009). Further analysis of SUB1A target genes and the downstream networks will aid the elucidation of the molecular mechanisms underlying water extreme tolerances that contribute to natural variation in semiaquatic plant species.

METHODS

Plant Materials and Growth Conditions

Rice (Oryza sativa) cv M202, cv IR64, cv Samba Mahsuri, cv Liaogeng (LG), the Sub1 introgression lines M202(Sub1) and IR64(Sub1), Samba Mahsuri(Sub1), and SUB1A overexpression lines Ubi:SUB1A-1 and Ubi: SUB1A-3 (background genotype LG) were analyzed in this study (Fukao et al., 2006; Xu et al., 2006; Sepliningsih et al., 2009). Rice plants were grown under the conditions described by Fukao et al. (2006). Sterilized seeds were placed on wet filter paper for 3 d at 25°C in the light. Germinated seeds were transplanted into soil-containing pots (W:L:H, 10 × 10 × 10 cm) and grown in a greenhouse (30°C day, 20°C night) for 14 d under natural light conditions.

Submergence, Drought, and Chemical Treatments

All submergence, drought, and chemical treatments were replicated in at least three independent biological experiments. Submergence and ABA responsiveness by SUB1A expression inhibits the increased GA responsiveness during submergence through elevation of the GA signaling repressors, SLR1 and SLRL1. Negative regulation of GA responses aids in avoidance of carbohydrate starvation, allowing plants to endure submergence. Upon reaeration, leaf dehydration occurs, SUB1A-mediated enhancement of ROS amelioration limits oxidative damage and chlorophyll degradation during reoxygenation. Increased ABA responsiveness by SUB1A induces expression of LEA mRNAs and suppresses leaf water loss. SUB1A also upregulates transcripts encoding ERFs associated with acclimation to drought (e.g., DREB1s and AP59). The SUB1A-mediated responses to water deficit can independently enhance recovery from submergence or provide protection during drought, both of which induce cellular dehydration.
treatments were performed following the methods of Fukao et al. (2006) and Fukao and Bailey-Serres (2008), respectively. Briefly, 14-d-old plants in soil-containing pots were completely submerged in a plastic tank (W:L:H, 65 × 65 × 95 cm) filled with 90 cm of water in a greenhouse. Following 7 d of submergence, plants were desubmerged on noon on day 7 and placed in air in the light (50 μmol m⁻² s⁻¹) for up to 4 h of reoxygenation. The light levels were similar under submerged and recovery conditions. For ABA treatment, aerial tissue of 14-d-old [M202, M202(Sub1), and LG] or 21-d-old (Ub:SUB1A-3) plants was excised at the base of the stem and immediately placed into 20 mL of mock (0.1% [v/v] DMSO) or ABA solution (5 or 50 μM in 0.1% DMSO) in a 250-mL glass beaker for 24 h in the light (50 μmol m⁻² s⁻¹). For oxidative stress treatment, aerial tissue of 14-d-old plants was incubated in water or methyl viologen solution (1 or 20 μM) for 24 h in the light (100 μmol m⁻² s⁻¹) as described above. For osmotic stress treatment, sterilized seeds were grown on wet filter paper in a Petri dish for 7 d at 25°C in the light (100 μmol m⁻² s⁻¹). Seedlings were placed onto filter paper containing 20% (w/v) polyethylene glycol-8000 for up to 24 h in the light. For drought treatment, 40 plants (20 plants per genotype) grown in a 2.5-liter pot for 14 d were exposed to dehydration stress by withholding water for up to 7 d in a greenhouse (30°C day, 20°C night). Aerial tissue was harvested at noon on the day specified. After each treatment, sampled tissue was immediately frozen in liquid nitrogen and stored in −80°C until use.

RNA Extraction and qRT-PCR
RNA extraction and cDNA synthesis were performed as described by Fukao and Bailey-Serres (2008). qRT-PCR was performed in a 20-μL reaction using iQ SYBR Green supermix (Bio-Rad) in a MyiQ real-time PCR detection system (Bio-Rad). All primers were used at 0.5 μM. Melting curve analysis was performed at the end of PCR reactions to validate amplification specificity. Amplification efficiency was verified following the method of Schmittgen and Livak (2008). Relative transcript abundance was calculated by the comparative CT method (Livak and Schmittgen, 2001). ACTIN1 was used as a normalization control. Sequences of primer pairs are listed in Supplemental Table 1 online.

Detection and Quantification of ROS
Fourteen-day-old plants were submerged for 7 d as described by Fukao et al. (2006). Plants grown under normal conditions were used as a control. To visualize superoxide accumulation, aerial tissue of desubmerged or nonsubmerged control plants was excised and immediately placed in a 0.5 mg/mL NBT solution in 10 mM potassium phosphate buffer, pH 7.6, at 25°C for 3 h in the dark. For hydrogen peroxide detection, excised aerial tissue was treated with 1 mg/mL DAB in 50 mM Tris acetate buffer, pH 5.0, at 25°C for 24 h in the dark. After staining, the uppermost leaf of each plant was boiled in 95% (v/v) ethanol for 20 min to remove chlorophyll and rehydrated in 40% (v/v) glycerol for 16 h at 25°C. Each experiment was repeated on at least seven different plants, and representative images are shown.

Lipid Peroxidation
Lipid peroxidation was analyzed with the thiobarbituric acid test, which determines MDA as an end product of lipid peroxidation (Hodges et al., 1999). Briefly, 50 mg of aerial tissue was homogenized in 1 mL of 80% (v/v) ethanol on ice. Following centrifugation at 16,000g for 20 min at 4°C, the supernatant (0.5 mL) was mixed with 0.5 mL of 20% (w/v) trichloroacetic acid containing 0.65% (w/v) thiobarbituric acid. The mixture was incubated at 95°C for 30 min and then immediately cooled in an ice bath. After centrifugation at 10,000g for 10 min, the absorbance of the supernatant was measured at 532 nm, subtracting the value for nonspecific absorption at 600 nm. The MDA concentration was calculated from the extinction coefficient 155 mM⁻¹ cm⁻¹ (Hodges et al., 1999).

Oxidative Stress Tolerance Analysis
To evaluate whole plant tolerance to oxidative stress, dehulled seeds (25 seeds) were grown in Magenta vessels (Sigma-Aldrich; GA-7) containing 80 mL 0.5 × Murashige and Skoog (MS) media at 23°C for 7 d (16 h light/8 h dark; light level of 50 μmol m⁻² s⁻¹). Seedlings were transferred onto 0.5 × MS media including methyl viologen (Sigma-Aldrich) (0, 1, 5, or 20 μM) and incubated for 14 d under the conditions described above. To examine oxidative tolerance in leaves, the fully expanded uppermost leaf of 14-d-old plants was cut into pieces (L × W: 8 × 6 mm), and the leaf segments were floated on a 1 μM methyl viologen or 10 mM H2O2 solution at 25°C in the light (100 μmol m⁻² s⁻¹) for 40 or 24 h, respectively. This experiment was repeated three times and representative images are shown. Chlorophyll a and b contents of aerial tissue and leaf pieces were quantified following the method of Fukao et al. (2006).

Drought Tolerance Analysis
Germinated seeds were transplanted into 2.5-liter soil-containing pots and grown for 14 d as described above. Drought stress was applied by withholding water for 8 d in a greenhouse (30°C day, 20°C night), and recovery was initiated by placing pots in a shallow tray of water. Plant viability was evaluated 14 d after initiation of recovery under normal growth conditions. Plants were scored as viable if one or more new leaves appeared during the recovery period. Leaf RWC was monitored in the fully expanded upper leaves (third leaf) of 14-d-old plants during treatments. Each leaf blade was detached from a plant at noon, and the fresh weight (FW) was immediately measured. After measurement, leaf blades were floated on deionized water for 24 h, and rehydrated leaves were reweighed to determine turgid weight (TW). Finally, leaf blades were oven dried at 65°C for 3 d, and dry weight (DW) of each leaf was measured. RWC was calculated using this equation: RWC (%) = (FW − DW)/(TW − DW) × 100. For the osmotic stress tolerance test, dehulled sterilized seeds were grown on 0.5 × MS medium equilibrated with polyethylene glycol-8000 (final concentration: 15% [w/v]) at 23°C (16 h light/8 h dark; light level, 50 μmol m⁻² s⁻¹). After a 10-d incubation, the entire shoot and root length was recorded.

ABA Response Analysis
Sterilized seeds were soaked in 100 μM fluridone dissolved in 0.1% (v/v) ethanol for 48 h at 4°C. Following the pretreatment, seeds were incubated on wet filter paper with mock (0.1% [v/v] DMSO) or ABA solutions (10 to 100 μM of + and −ABA in 0.1% [v/v] DMSO) for 8 d at 25°C in the light (50 μmol m⁻² s⁻¹). Seeds that formed a shoot or root (≥1 mm) were scored as germinated; shoot length was also recorded. All shoots remained white for 8 d, indicating fluridone was effective during the entire experimental period. For the fluridone sensitivity test, sterilized seeds were placed on wet filter paper with mock (0.1% [v/v] ethanol) or fluridone solutions (0 to 100 μM in 0.1% [v/v] ethanol) for 6 d at 25°C in the light (50 μmol m⁻² s⁻¹). Seed germination was scored each day.

Microarray Data Analysis
Publicly available microarray data (Jain et al., 2007; Mustroph et al., 2010) were reanalyzed by use of the R program (http://cran.at.r-project.org) and Biocondutor package (http://www.biocondutor.org). Affymetrix CEL files (accession numbers GSE18930 and GSE6901) were downloaded from Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/gds). Raw data were normalized by the GC robust multiarray average (GCRMA) method. Differential expression analyses [pairwise comparisons of stress
Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. SUB1A Enhances Drought Tolerance in Rice.

Supplemental Figure 2. SUB1A Increases Tolerance to Osmotic Stress.

Supplemental Figure 3. A Subset of Submergence-Responsive Genes is Upregulated by Drought.

Supplemental Figure 4. Fluridone, an ABA Biosynthesis Inhibitor, Does Not Affect Germination of Ubi:SUB1A-1 Seeds.

Supplemental Figure 5. SUB1A Moderates Chlorophyll Degradation by Oxidative Stress.

Supplemental Table 1. Primers Used for Quantitative RT-PCR.

Supplemental Data Set 1. Gene Transcripts That Are Upregulated by Submergence in M202 and Dehydration in IR64.

Supplemental Data Set 2. Gene Transcripts That Are Upregulated by SUB1A during Submergence and by Dehydration.

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The Submergence Tolerance Regulator SUB1A Mediates Crosstalk between Submergence and Drought Tolerance in Rice
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