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SUB1A-mediated submergence tolerance response in rice involves differential regulation of the brassinosteroid pathway

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Summary

- *Submergence 1A* (*SUB1A*), is an ethylene response factor (ERF) that confers submergence tolerance in rice (*Oryza sativa*) via limiting shoot elongation during the inundation period. *SUB1A* has been proposed to restrict shoot growth by modulating gibberellic acid (GA) signaling.
- Our transcriptome analysis indicated that *SUB1A* differentially regulates genes associated with brassinosteroid (BR) synthesis during submergence. Consistent with the gene expression data, the *SUB1A* genotype had higher brassinosteroid levels after submergence compared to the intolerant genotype. Tolerance to submergence can be activated in the intolerant genotype by pretreatment with exogenous brassinolide, which results in restricted shoot elongation during submergence.
- BR induced a GA catabolic gene, resulting in lower GA levels in *SUB1A* plants. BR treatment also induced the DELLA protein SLR1, a known repressor of GA responses such as shoot elongation. We propose that BR limits GA levels during submergence in the *SUB1A* rice through a GA catabolic enzyme as part of an early response and may repress GA responses by inducing SLR1 after several days of submergence.
- Our results suggest that BR biosynthesis is regulated in a *SUB1A*-dependent manner during submergence and is involved in modulating the GA signaling and homeostasis.

Introduction

Flooding drastically reduces rice yields worldwide. Although rice is adapted to standing water, most rice varieties are susceptible to complete submergence resulting from transient flooding in lowland rice growing regions. During submergence, the diffusion rate of carbon dioxide and oxygen between the plant and its environment is significantly reduced (Armstrong, 1980; Colmer, 2003). The resulting hypoxic condition limits photosynthesis, necessitating a metabolic switch to glycolysis and anaerobic respiration (Setter *et al.*, 1989, 1997; Voesenek *et al.*, 2006). Flood intolerant rice varieties typically exhibit rapid elongation upon prolonged submergence, consuming their carbohydrate reserves during the growth phase, and plants that breach the water surface are usually spindly and prone to lodging once the flood waters recede (Ismail *et al.*, 2009). Most intolerant genotypes do not survive several days of submergence. However, a lowland flood-tolerant landrace, Flood Resistant 13A (FR13A) can survive up to 2 wk of submergence and still recover to resume growth (Mackill *et al.*, 1993). FR13A adopts a quiescent strategy that restricts growth, conserving energy until flood waters recede (Xu *et al.*, 2006; Fukao & Bailey-Serres, 2008).

The quantitative trait locus (QTL) primarily responsible for the tolerance of FR13A was mapped to a small region called *Sub1* (Xu & Mackill, 1996; Xu *et al.*, 2006; Hattori *et al.*, 2009). Flood intolerant rice varieties become tolerant after introgression of the *Sub1* locus, demonstrating that the *Sub1* QTL is sufficient for flood tolerance (Xu & Mackill, 1996). The *Sub1* locus from FR13A and bioengineered tolerant genotypes with the introgressed *Sub1* locus has three Ethylene Response Factor (ERF) genes: *SUB1A*, *SUB1B* and *SUB1C* (Xu *et al.*, 2006). All three genes belong to a large transcription factor family referred to as ERFs and they group within the abiotic stress responding ERF subfamily VII (Nakano *et al.*, 2006; Jung *et al.*, 2010). The intolerant parent variety M202 used in previous submergence studies and in the experiments discussed here has the *SUB1B* and *SUB1C* genes but lacks the *SUB1A* gene. The tolerant near isogenic line used in our experiments is the M202-Sub1 that has the *Sub1* locus from FR13A containing the *SUB1A* gene that confers submergence tolerance (Fukao *et al.*, 2006; Xu *et al.*, 2006). Upon complete submergence, *SUB1A* is rapidly induced within 1 d. Expression of *SUB1C* is induced more strongly in the intolerant M202 when compared to the tolerant M202-Sub1. *SUB1B* transcript abundance was reported to be induced by submergence in both genotypes but its transcript abundance was slightly higher in M202-Sub1 compared to M202 (Fukao *et al.*, 2006). Expression of the ubiquitin promoter driven *SUB1A* transgene (LG (*SUB1A*)) in an intolerant variety, Liaogeng (LG) was sufficient

to introduce the submergence tolerance response, providing conclusive evidence that *SUB1A* was the tolerance conferring gene in the FR13A *Sub1* haplotype (Xu *et al.*, 2006).

In contrast to the quiescent strategy exhibited by FR13A, deep water rice survives season-long flooding by rapidly elongating internodes to maintain contact with air. This strategy is dependent upon a quantitative trait locus encoding two major genes, *SNORKEL1* and *SNORKEL2* (*SK1* and *SK2*) (Hattori *et al.*, 2009). Similar to *Sub1* locus, *SK1* and *SK2* are two ERFs and their expression is induced by submergence stress and the gaseous plant hormone ethylene. Submergence decreases the rate of diffusion of ethylene, which accumulates inside the plants and leads to induction of several members of the ERF family including *SK1*, *SK2*, *SUB1A* and *SUB1C* among others. However, the downstream mechanisms directly affecting both *SUB1A* and *SNORKEL*-mediated tolerance is reported to be dependent upon the plant hormone gibberellin (GA), which is critical for many aspects of plant growth and development as well as stress adaptation (Gao *et al.*, 2011). Deep water rice has elevated GA levels that contribute to the rapid growth of internodes and leaves. Although GA content has not been reported for *SUB1A*-expressing plants, it was suggested that the GA-signaling mechanism is altered due to increased protein concentrations of the GA signal-repressing GRAS family transcription factors SLR1 (SLENDER RICE1) and SLRL1 (SLR1-Like1) during submergence (Fukao & Bailey-Serres, 2008). SLR1 is a DELLA domain protein that is destabilized by GA (Itoh *et al.*, 2002). This occurs when the GA receptor, GID1 (Gibberellin Insensitive Dwarf), binds GA and promotes interaction of GID1-GA with SLR1. Next, the DELLA domain of SLR1 is recognized by the SCF^{SLY1} E3 ubiquitin ligase complex, which results in the poly-ubiquitination and subsequent degradation of SLR1 through the 26S proteasome. GA-mediated derepression of growth can also occur independently of SLR1 degradation (Ariizumi *et al.*, 2008). This may occur in part through DELLA protein modification, or through protein–protein interactions with factors that may block DELLA function (Sun, 2011).

The intolerant variety, M202 and the corresponding tolerant line M202-Sub1 have been used in several studies to identify *SUB1A*-specific gene expression profiles (Fukao *et al.*, 2006; Jung *et al.*, 2010). Expression analyses have shown that genes associated with anaerobic respiration and antioxidant protection were upregulated during submergence in M202-Sub1, consistent with the enhanced ability to adjust to submergence stress. These studies indicated that submergence alters hormone levels as well as transcript abundance of genes associated with hormone homeostasis (both synthesis and catabolic genes). Significant changes were reported for ethylene, ABA and cytokinin pathways. Ethylene levels of both rice lines increase when submerged, but were slightly lower in M202-Sub1 relative to M202 (Fukao *et al.*, 2006). Ethylene can reduce levels of ABA by inducing the expression of an ABA catabolic enzyme (Fukao & Bailey-Serres, 2008). Although M202-Sub1 uniquely exhibits ABA hypersensitivity, ABA content is reduced to comparable concentrations in both genotypes during submergence (Fukao & Bailey-Serres, 2008). The ABA hypersensitivity of M202-Sub1 probably reflects an increase in ABA-responsive genes, which are proposed to prevent dehydration after post-submergence by repressing *SUB1A* expression and inducing genes that prevent water loss (Fukao *et al.*, 2011).

Another hormone regulating cell expansion and plant height is brassinosteroid (BR), but its role in rice submergence tolerance has not been addressed. The plasma membrane-localized receptor Brassinosteroid insensitive (BRI1), a member of the leucine-rich repeat subfamily of receptor-like kinases (LRR-RLK), is primarily responsible for BR recognition in *Arabidopsis thaliana*. Downstream BR-signaling is dependent upon the transcription factors Brassinozole resistant 1 (BZR1) and Bri1 EMS suppressor 1 (BES1), which bind BR-response element (BRRE) or E-box cis elements in the promoters of BR-regulated genes (He *et al.*, 2005; Yin *et al.*, 2005). In the absence of BR, a kinase called Brassinosteroid insensitive 2 (BIN2) negatively regulates BR-signaling through the phosphorylation of BZR1 and BES1, targeting them for degradation. In rice, studies have shown that OsBRI1, OsBZR1,and GSK3-like/BIN2 have similar function to their Arabidopsis orthologs (Yamamuro *et al.*, 2000; Bai *et al.*, 2007; Koh *et al.*, 2007; Nakagawa *et al.*, 2011; Tong *et al.*, 2012). Several BR biosynthesis genes, such as *DWF4* and *CPD*, as well as the *BRI1* receptor are regulated by BR in a negative feedback manner (Nakagawa *et al.*, 2011). BR biosynthesis genes are upregulated in BR signaling mutants and BR biosynthesis mutants become hypersensitive to exogenous BR due to upregulation of *BRI1*. Although BR-deficient mutants have reduced size similar to GA biosynthesis mutants, they are uniquely identifiable by a reduction in lamina leaf bending (Nakagawa *et al.*, 2011). Recent reports suggest that GA and BR pathways coordinate to regulate plant growth through DELLA proteins, BZR1, and other transcription factors (Bai *et al.*, 2012; Hao *et al.*, 2012; Oh *et al.*, 2012; Yang *et al.*, 2012). Given that abundance of SLR1, a rice DELLA protein was positively correlated with *SUB1A* expression, it remains to be determined whether BR could be involved in submergence response in rice (Fukao & Bailey-Serres, 2008).

In this study, we investigated the molecular mechanisms involved in submergence tolerance response in rice. Specifically, our transcriptome analysis revealed a *SUB1A*-dependent BR response during submergence. We present evidence that BR biosynthesis genes and endogenous BR levels are differentially regulated in the *SUB1A* rice line compared to the intolerant line during submergence. Experiments suggest that BR could be negatively affecting GA levels during transient submergence. Based on our experiments, we propose a model for *SUB1A* action during submergence that highlights the role of BR and GA crosstalk in restricting shoot elongation during submergence.

Materials and Methods

Treatment conditions for transcription analysis samples

Rice (*Oryza sativa* L.) seeds were germinated in the dark at 30°C and transferred to the benchtop for several days. Next, seedlings were transplanted into 13 cm pots of soil in a 27°C controlled glasshouse. V3-stage (18 d old) M202 and M202-Sub1 were

submerged in 120 l black plastic tubs filled with tap water in the late afternoon. Nonsubmerged V3-stage LG and LG(*SUB1A*) were used since LG(*SUB1A*) constitutively expresses *SUB1A*. Aerial tissue was collected for RNA isolations.

Real-time PCR analysis

Total RNA was extracted from *c.* 100 mg rice leaf tissue using the trizol method. cDNA was synthesized using VILO (Invitrogen). Gene-specific primers to rice genes *DWF1*, *DWF4*, *GA2ox7*, *SLR1*, *SLRL1* and *IRL3* (used as internal control) were designed and used in real-time PCR (Supporting Information Table S1). All primers were annealed at 60°C and run for 45 cycles for real-time PCR products. PCR reactions were run in a LightCycler (Roche). Gene expression levels were quantified in at least two independent biological samples, each consisting of at least two technical replicates. PCR program and quantification of the relative changes in gene expression were conducted as in (Livak & Schmittgen, 2001).

Hierarchical clustering of BR genes

The microarray data (GSE41103) was analyzed as described in (Walia *et al.*, 2005) and selected BR biosynthesis and signaling genes were used for hierarchical clustering. To perform unsupervised hierarchical clustering we used dChip software (http://www.DChip.org; Li & Wang, 2001). The threshold for calling significant gene clusters was set to a *P* value of 0.001. Before clustering, the algorithm standardized (linearly scaled) the expression value of each of the listed probe sets in all samples to have a mean 0 and SD 1. These standardized values were then used to calculate correlations between probe sets and clusters, and serve as the basis for merging nodes. The distance between two neighboring probe sets was calculated using the Pearson correlation model and expressed as $1 - |r|$, which is the length of the stem connecting two branches where r denoted the Pearson correlation coefficient.

The lamina joint bending assay

Ten-day-old M202 and 11-d-old M202-Sub1 seedlings were used for the leaf bending assay (Wada *et al.*, 1981). One microliter (100 ng μl^{-1}) of 24-epiBrassinolide (an active form of BR) was applied to the lamina joint of each plant. After 3 d of application, the angle of the leaf blade and sheath were measured and photographed.

Immunoblotting

Seedling aerial tissue were frozen and ground to a fine powder in liquid N_2. Then 6X Sample Buffer (0.35 M Tris Cl pH 6.8, 10% SDS, 30% glycerol (v/v), 1% SDS (w/v), 0.6 M DTT, 0.175 mM bromophenol blue) was added (3 $\mu l\ mg^{-1}$ tissue FW) before 5 min of boiling. Proteins were separated by standard SDS–PAGE on 7.5% (w/v) polyacrylamide/Tris–HCl gels and transferred to nitrocellulose membranes (0.22 μM; GE Water & Process Technologies, Trevose, PA, USA). Samples were uniformly loaded based upon tissue FW. Blot blocking and washing were performed as described previously (Stokes *et al.*, 2000) and probed with SLR1 (Itoh *et al.*, 2002) followed by Pierce® goat-anti-rabbit peroxidase-conjugated secondary antibodies (Product #31460; Thermo Scientific, Rockford, IL, USA). Actin antibody (Catalog #A1292; Selleck Chemicals, Houston, TX, USA) probing was followed by Pierce® rabbit-anti-mouse peroxidase-conjugated secondary antibodies (Product #31450; Thermo Scientific). Membranes were developed using SuperSignal® West Pico Chemiluminescent Substrate (Product #34087; Thermo Scientific) and the signal was recorded on autoradiography film.

Brassinosteroid treatments

For the BR height reduction experiment (Fig. S1): M202 and M202-Sub1 seeds were sterilized with 15% bleach for 30 min and placed on ½ MS medium (0.4% phytoagar) containing BL. Seeds were germinated in the dark at 30°C. After 3 d seedlings were exposed to *c.* 16 h day cycle (30°C day : 28°C night) in a growth chamber for an additional 3 d before measuring plant heights.

For pretreatment experiments (Fig. 4): Seedlings germinated in the dark at 30°C were either exposed to *c.* 16 h day cycle (30°C day : 28°C night) in a growth chamber or grown on the benchtop (*c.* 23°C). After 2 d the seedlings were placed for 2 d in sand pre-wet with BL. After pretreatment, beakers were submerged in *c.* 45 l of tap water in clear plastic tubs on the benchtop for 7–8 d.

For time-course experiment (Fig. 5): Seedlings germinated in the dark at 30°C were exposed to *c.* 16 h day cycle (30°C day : 28°C night). After 2 d the seedlings were briefly submerged in 1 μM BL or 0.07% ethanol (mock control) and continuously fed the solution through the roots.

Quantification of hormones

M202 and M202-Sub1 aged 21–22 d old/V3-stage were submerged in the late afternoon as already described. After 1 and 6 d, aerial tissue was pooled from four to seven plants. LG (*SUB1A*) were germinated 2 wk before LG to account for the slow growth of LG(*SUB1A*) enabling collection of LG and LG (*SUB1A*) at V3-stage (four plants pooled each). All samples were flash frozen in liquid nitrogen, freeze-dried and crushed. GA_4 and CS were purified and measured as previously described (Yoshimitsu *et al.*, 2011; Nelissen *et al.*, 2012). See Methods S1 and Tables S2 and S3 for further details.

Results

SUB1A-dependent transcriptome regulation

SUB1A is strongly induced at the transcript level within 1 d of submergence and continues to be highly expressed for up to 10 d after submergence (Fukao *et al.*, 2006; Xu *et al.*, 2006). Although the expression of *SUB1A* is induced as early as 1 d after submergence, differential growth between M202 and M202-Sub1 is evident by day 7 under submergence in our experiments. Intolerant

genotypes are known to start elongating after 2–3 d under complete submergence (Kawano *et al.*, 2008). To elucidate the *SUB1A*-specific responses we compared the transcriptome of M202 and M202-Sub1 at 1 and 6 d after submergence. The rice genome array was used for this analysis (Walia *et al.*, 2005). We also included a published dataset for M202 and M202-Sub1 from control, nonsubmerged plants and 1 d submerged plants (Mustroph *et al.*, 2010; GSE18930) in our analysis. A large number of genes were differentially regulated between M202 and M202-Sub1 during submergence at 1 and 6 d time points consistent with earlier reports by us and others (Jung *et al.*, 2010; GSE41103). Here we focus only on the GA- and BR-responsive genes to determine the role of interhormonal crosstalk between GA and BR during submergence tolerance response.

Brassinosteroid synthesis and brassinosteroid-responsive genes are differentially regulated by *SUB1A*

Transcript abundance of two BR biosynthesis genes, *DWARF 4* (*DWF4*) and *DWARF 1* (*DWF1*), was significantly higher during submergence in M202-Sub1 compared to M202 (Fig. 1a). At the 1 d time point, *DWF4* expression was upregulated by 2.8-fold in M202-Sub1 during submergence, while no significant difference was detected in M202 plants between submerged and control microarray samples (GSE18930). Expression of *DWF4* was higher in M202-Sub1 relative to M202 at 1 and 6 d samples by 3.8- and 2.2-fold, respectively, in our array dataset (GSE41103). *DWF1* expression was also higher in M202-Sub1 compared to M202 plants at 1 and 6 d after submergence by more than two-fold. *DWF4* is feedback regulated by BR in Arabidopsis (Yoshimitsu *et al.*, 2011). However, *DWF1* expression has not been linked with feedback regulation. Higher transcript abundance of both *DWF1* and *DWF4* in M202-Sub1 relative to M202 during submergence suggested that BR biosynthesis pathway could be differentially regulated in a *SUB1A*-dependent manner.

We also found the expression of several BR-related genes such as *OsBZR3*, *BRH1* (Molnar *et al.*, 2002) and *BZS1* (Fan *et al.*, 2012) to be differentially regulated between M202-Sub1 and M202 in the array datasets (Fig. 1a). For instance, the expression of *OsBZR3*, an ortholog of the Arabidopsis BR signaling gene *BZR1*, is positively correlated with *SUB1A* expression. In the

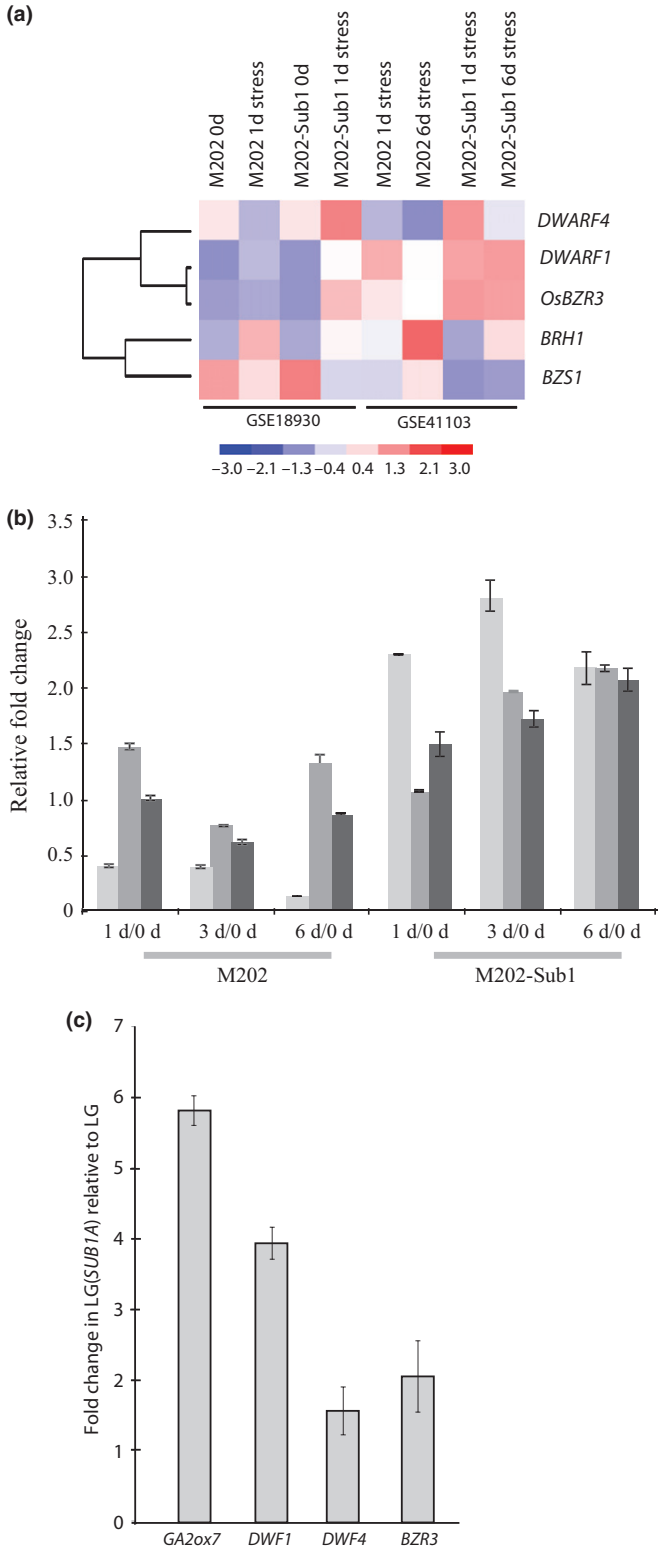

Fig. 1 (a) Expression of brassinosteroid (BR) related genes during submergence. Expression level of BR biosynthesis and BR-responsive genes is shown from two microarray experiments, GSE18930 and GSE41103. GSE18930 includes expression profiles of BR-related genes under nonsubmerged (0 d) and 1 d after submergence in the intolerant M202 and tolerant M202-Sub1. GSE41103 represent expression profiles for both these genotypes at 1 and 6 d after submergence. All expression values were scaled (−3.0 to 3.0). Red color represents higher expression and blue color represents lower expression. Expression of two BR synthesis genes, *DWARF1* and *DWARF4* were induced strongly in M202-Sub1. *OsBZR3* is a putative BR signaling gene and *BRH1* and *BZS1* are known BR-responsive genes. (b) Expression profiles of BR biosynthesis and signaling genes in a time-course experiment. Expression level in control conditions and 1, 3, and 6 d after submergence were measured using real-time PCR assays for *DWF1* (mid gray bars), *DWF4* (light gray bars) and *OsBZR3* (dark gray bars). Notably, expression of *DWF4* was repressed in the intolerant M202 upon submergence in contrast with induction in M202-Sub1. Error bars indicate ± SE. (c) Expression profiles of BR biosynthesis and signaling genes in the *SUB1A*-overexpressing plants. Expression levels in the control (LG) and in the *SUB1A*-overexpression line LG(*SUB1A*) were measured using real-time PCR assays for *GA2ox7*, *DWF1*, *DWF4* and *OsBZR3*. Error bars indicate ± SE.

transcriptome analysis, we found that *OsBZR3* was induced after 1 and 6 d of submergence in M202-Sub1, but there was no significant change in expression during submergence in M202 (Fig. 1a). *OsBZR1* is known to regulate rice BR sensitivity but *OsBZR3* and other orthologs have not yet been characterized (Bai *et al.*, 2007). We did not detect genotypic differences in expression levels between M202 and M202-Sub1 for the other *OsBZR* orthologs.

Expression of the rice ortholog of *BZS1* was downregulated during submergence in M202-Sub1 at 1 and 6 d time points compared to M202 (Fig. 1a). *BZS1* was identified as a suppressor of BR-hypersensitive mutant *bzr1* in Arabidopsis (Fan *et al.*, 2012). *BZS1* encodes a B-box type zinc finger protein that is induced in BR-deficient plants and is repressed by BR treatment in Arabidopsis (Sun *et al.*, 2010). Arabidopsis BZR1 is known to repress *BZS1* expression (Sun *et al.*, 2010). Assuming that rice *BZS1* has a conserved role in determining BR sensitivity as its Arabidopsis ortholog does, these data suggest that repression of *OsBZS1* during submergence could increase BR sensitivity in M202-Sub1. Lower transcript abundance of *BZS1* could be due to higher BR levels resulting from upregulation of *DWF1* and *DWF4* in M202-Sub1 during submergence. Another gene that is repressed by BR treatment in Arabidopsis is *BRH1* (Molnar *et al.*, 2002). The transcript abundance of rice ortholog of BRH1 was lower in M202-Sub1 after 1 and 6 d of submergence compared to M202 (Fig. 1A). These data suggest that BR levels and signaling could be different between the M202-Sub1 and M202 genotypes during submergence.

We validated the expression of a subset of BR synthesis and signaling genes using real-time PCR assays. Samples used in the validation experiment were collected independently from those used for the microarray analysis. We measured transcript abundance of BR biosynthesis genes (*DWF4*, *DWF1*), and the putative BR signaling gene *OsBZR3* in M202 and M202-Sub1 plants at 0 (nonsubmerged control), 1, 3 and 6 d after submergence (Fig. 1b). Consistent with our array data set, we found the expression of *DWF1*, *DWF4* and *OsBZR3* to be higher in M202-Sub1 compared to M202. The expression of the BR receptor gene *OsBRI1* is typically sensitive to changes in BR levels, but was not differentially regulated between M202 and M202-Sub1 in the microarrays. We also examined *OsBRI1* expression in real-time PCR assays. Consistent with the microarray, *OsBRI1* was not significantly altered in submergence conditions (data not shown). Collectively, the expression profiles of BR biosynthesis genes, BR signaling genes and some of the known BR-responsive genes suggested that *SUB1A* could be modulating the BR regulated responses in rice during submergence. To further characterize the *SUB1A*-mediated regulation of BR related genes, we measured the expression level of *DWF1*, *DWF4* and *OsBZR3* in the transgenic line, LG(*SUB1A*) and the wild-type, LG. All three genes had higher expression in the LG(*SUB1A*) relative to the wild-type (Fig. 1c).

SUB1A increases BR sensitivity in rice

Given the differential regulation of BR-associated genes in M202-Sub1 we performed the classical BR leaf lamina bending assay on M202 and M202-Sub1 seedlings (Wada *et al.*, 1981). Greater bending is indicative of increased sensitivity and/or elevated BR levels. We found that M202-Sub1 plants had a greater bending angle of 35.9° ± 4.6 (mean ± SE) compared to the M202 angle of 17.4° ± 3.9 (Fig. 2). We also noted that leaf bending angles for the control (untreated) M202-Sub1 plants were slightly greater than those for M202. Slightly larger leaf angles in M202-Sub1 (26.8° ± 2.8) compared to M202 (8.6° ± 2.2) in control (non-BR treated) plants hinted of higher BR levels in the M202-Sub1. Lamina bending assays and the expression analysis of BR-associated genes indicates that *SUB1A* expression during submergence could be regulating BR homeostasis.

Brassinosteroid levels are altered in a *SUB1A*-dependent manner during submergence

Difference in leaf angles between M202 and M202-Sub1 prompted us to directly measure the endogenous BR content for the two genotypes during submergence. We found that castasterone (CS) levels were significantly higher at 1 and 6 d after submergence in the M202-Sub1 plants compared to M202 plants (Fig. 3). An elevated level of CS in M202-Sub1 relative to M202 as early as 1 d after submergence is consistent with the observed upregulation of BR biosynthesis genes. This also suggests that elevated BR levels are responsible for the BR-hypersensitive phenotype of M202-Sub1 and is consistent with the M202-Sub1-specific increase in *DWF1* and *DWF4* expression levels. Furthermore, we found that the level of CS in LG(*SUB1A*) was elevated relative to LG in seedlings (Fig. 3). This result indicates that *SUB1A* expression increases BR levels and induces BR biosynthesis genes. Based on these data, it is not clear whether higher BR levels in the submerged plants is an essential component of *SUB1A*-mediated submergence tolerance response or simply a consequence of downstream events that are primarily driven by ethylene and gibberellins.

Brassinolide alters the submergence response of the intolerant M202

Exogenous BR has been shown to restrict shoot elongation in rice (Yokota & Takahashi, 1986; Komorisono *et al.*, 2005; Tanabe

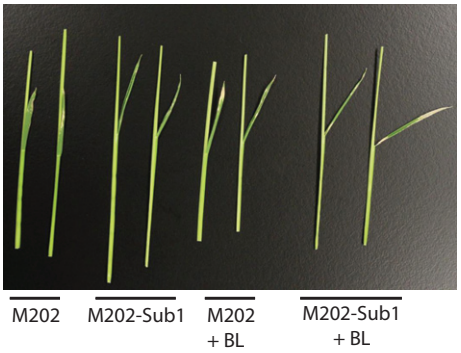

Fig. 2 Lamina leaf angles are increased in M202-Sub1 relative to M202. Leaf lamina joints treated with a 100 ng drop of BL show that the leaf angles were increased for M202-Sub1 relative to M202 in both control (left) and BL-treated plants (right).

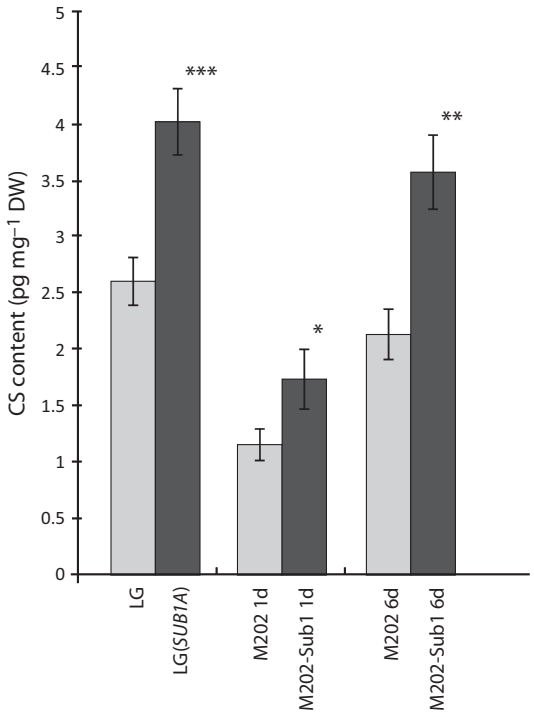

Fig. 3 Endogenous castasterone (CS) hormone content in *SUB1A*-expressing plants. Endogenous CS content was quantified from LG and LG(*SUB1A*), and from 1 and 6 d submerged M202 and M202-Sub1 plants (control genotypes, light gray bars; *SUB1A* genotypes, dark gray bars). Error bars indicate ± SD. The asterisks indicate a significant differences between LG vs LG(*SUB1A*) (***, $P < 0.001$), and M202 and M202-Sub1 genotypes at 1 d (*, $P < 0.05$) and 6 d (**, $P < 0.01$).

et al., 2005; Jeong *et al.*, 2007). We determined that shoot elongation of M202 was also repressed by the presence of brassinolide (BL), an active form of BR, in seedling growth assays (Fig. S1). Growth of M202-Sub1 was similarly repressed. To further explore if BR could have an essential role in *SUB1A*-mediated submergence tolerance in rice, we performed BR-pretreatment experiments. The most tractable phenotype of *SUB1A*-mediated submergence tolerance is restricted shoot elongation upon inundation. We developed an experimental set-up where M202 seedlings reproducibly exhibit significantly higher shoot elongation compared to M202-Sub1 seedlings after submergence. Five-day-old seedlings of M202 and M202-Sub1 were pretreated with BL (10 μM) for 2 d before complete submergence for 1 wk. Final seedling heights of ≥ 24 individuals were measured as an indicator of submergence tolerance (i.e. ability to limit shoot elongation) for the following four treatments: control (nonsubmerged, no BL); BL treated; submerged; and BL pretreated/submerged plants of both M202 and M202-Sub1 (Fig. 4). Pretreated, submerged M202 plants were 28% shorter compared to submerged M202 plants with no BL-pretreatment. Shoot elongation of M202-Sub1 plants was limited by 20% (P-value < 0.001) after submergence when pretreated with BL compared to plants directly submerged. BL treatment did not have a significant effect on either genotype in the absence of submergence. These experiments demonstrate that BR-pretreatment of M202 plants before submergence restricts shoot elongation, partially overcoming the lack of *SUB1A* gene at the *Sub1* locus.

Brassinolide regulates SLR1 protein level

The BR treatment experiments and BR level measurements during submergence suggested that BR could be involved in the *SUB1A*-mediated restriction of shoot elongation during submergence. *SUB1A* was proposed to induce and/or stabilize SLR1 (Fukao & Bailey-Serres, 2008). Because BR limits shoot elongation during submergence, we asked whether BR could be

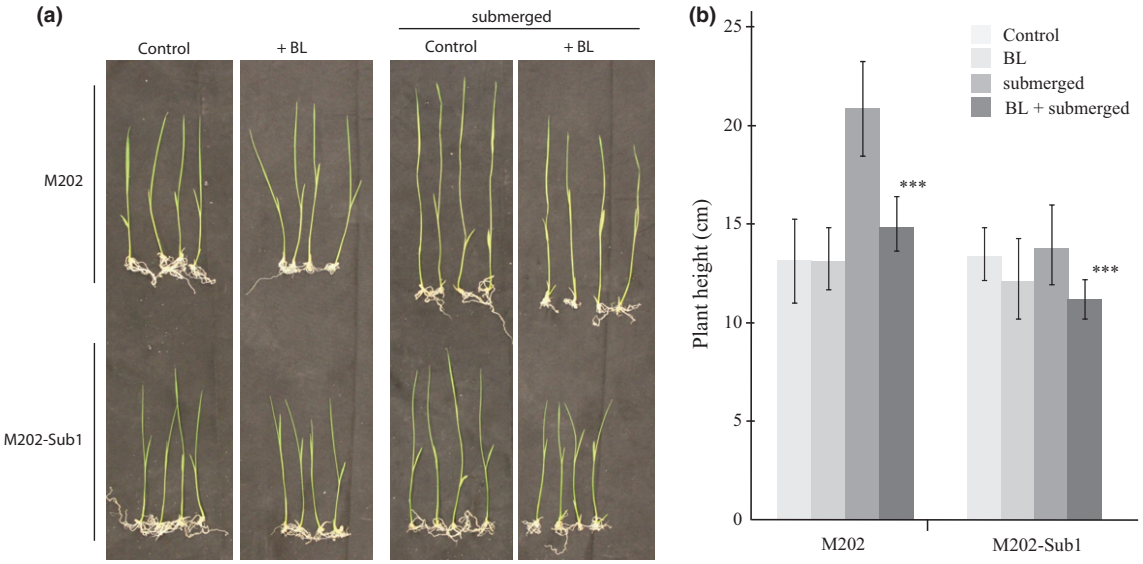

Fig. 4 BL restricts growth of plants after submergence. M202 and M202-Sub1 seedlings pretreated with 10 μM BL and nontreated controls were submerged in tap water for 7 d. (a) Representative individuals after treatment (nonsubmerged with or without BL treatment, submerged with or without BL treatment). (b) Graph of post-treatment seedling heights. The asterisks (***) indicate a significant difference ($P < 0.001$) between submerged M202 vs BL-pretreated submerged M202 and submerged M202-Sub1 vs BL-pretreated submerged M202-Sub1. Error bars represent ± SD.

involved in the *SUB1A*-mediated regulation of SLR1 or if the role of BR is independent of the GA-regulated submergence response. To this end we performed a BR response time series experiment to measure the protein level of SLR1. We treated M202 and M202-Sub1 seedlings with BL and collected shoot tissue at 0 (untreated control), 6, 12, 24, 48 and 72 h after treatment for protein assays. SLR1 protein level was induced at the 6 h time point after BL treatment in both genotypes (Fig. 5). Protein levels of SLR1 decreased in both genotypes at 72 h time point, indicating that effect of BR on SLR1 diminishes after *c.* 2 d. Mock treatment data for SLR1 protein assay is provided as Fig. S2. These data suggest that submergence increases BR levels in M202-Sub1 plants which could induce a negative regulator of shoot elongation, SLR1.

SUB1A expression prevents repression of a GA catabolic gene during submergence

SLR1 was proposed to be involved in repressing GA responses during submergence in M202-Sub1. The most striking difference for SLR1 protein level abundance between M202 and M202-Sub1 was observed at the 14 d time point (Fukao & Bailey-Serres, 2008). Given that shoot elongation typically initiates *c.* 5–7 d of submergence in M202, we asked if SLR1 was the only component mediating the *SUB1A* restriction of GA driven shoot elongation during submergence. We identified a gene from the transcriptome data encoding for a GA catabolic enzyme that could decrease the bioactive levels of GA during submergence. Transcript abundance of *GA2ox7* (*Os02g41954*) was higher by 5.8- and 6.9-fold in M202-Sub1 compared to M202 after 1 and 6 d of submergence, respectively. It is noteworthy that M202 represses *GA2ox7*, but M202-Sub1, which restricts elongation during submergence, increases its expression. We performed a real-time PCR assay for the *GA2ox7* on aerial tissue from 1, 3 and 6 d submerged M202 and M202-Sub1 plants (Fig. 6). *GA2ox7* was repressed during submergence by more than five-fold in M202 at all three time points. By contrast, we found the transcript levels of *GA2ox7* to be more abundant by 16-fold in M202-Sub1 relative to M202 at 1 d and by > 13-fold at 6 d. Notably, we found the expression pattern of the *SUB1A* gene during submergence to positively correlate with *GA2ox7* expression in M202-Sub1 (Fig. 7a). *GA2ox7* also had higher expression in LG(*SUB1A*) relative to the wild-type (Fig. 1c). Collectively, these data suggest that higher expression of *GA2ox7* during submergence could be responsible for decreased bioactive GA levels in M202-Sub1 (see below), thus limiting GA responses including shoot elongation.

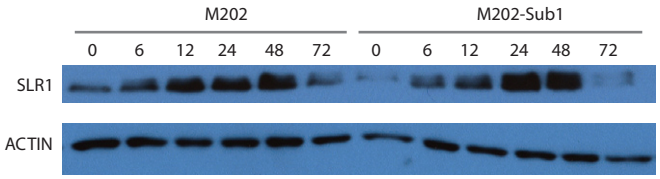

Fig. 5 Time course of SLR1 induction by exogenous BL. M202 and M202-Sub1 seedlings were root-fed 1 μM BL and protein extracts were collected from individuals at 0, 6, 12, 24, 48 and 72 h. Immunoblotting was performed with αSLR1 (top panel). αActin (lower panel) was performed as a loading control.

GA levels differ between M202 and M202-Sub1 during submergence

GA-dependent degradation of SLR1 in rice is an important component in GA signaling and involves GID1, a GA receptor that associates with SLR1 for quantitative regulation of GA signaling in rice (Ueguchi-Tanaka *et al.*, 2007). GA4 is most effective among the GAs in establishing an interaction between SLR1 and GID1 (Ueguchi-Tanaka *et al.*, 2007). Further, GA4 is the most sensitive to GA-inactivating enzymes (Ueguchi-Tanaka *et al.*, 2007). Notably, GA4 levels were significantly elevated in mutants with reduced expression of *AtGA2ox7* and *AtGA2ox8,* the two Arabidopsis orthologs of *OsGA2ox7* (Schomburg *et al.*, 2003; Magome *et al.*, 2008). We measured the GA4 content in shoot tissue and found that GA4 levels in M202 were higher compared to M202-Sub1 after 1 and 6 d of submergence (Fig. 7b). Lower GA4 levels in M202-Sub1 plants relative to M202 during submergence could possibly be due to higher transcript abundance of the *GA2ox7* in M202-Sub1 during submergence (Fig. 6). A difference in GA4 level between the two genotypes is evident early during submergence consistent with the differential regulation of *GA2ox7* within 1 d of submergence.

Brassinolide treatment induces *SUB1A* and *GA2ox7*

Both *GA2ox7* and the BR biosynthesis gene *DWF4* are upregulated in M202-Sub1 but repressed in M202 during submergence. We asked if increased endogenous BR levels in the M202-Sub1 plants during submergence could regulate *GA2ox7*. To examine this possibility, we performed real-time PCR assays for *GA2ox7* after 3 h of exogenous BR application on M202 and M202-Sub1 from control (nontreated), and plants treated with 10 μM BL. Transcript abundance increased by 3.9 ± 0.7-fold in M202 and by 2.7 ± 0.2-fold in M202-Sub1 compared to corresponding untreated seedlings from both genotypes. Next we addressed if *SUB1A* is induced by BL. In nonsubmerged, BL-treated controls of the BL-pretreatment experiments (Fig. 4), *SUB1A* was induced by 5.8 ± 0.9-fold in M202-Sub1 seedlings. These results suggest that increased BR levels in plants may directly induce *GA2ox7* transcript abundance potentially decreasing levels of bioactive GA. Induction of *SUB1A* expression by BL treatment could augment the gene expression of *GA2ox7*. Higher BR levels in M202-Sub1 during submergence positively correlate with increased transcript abundance of *GA2ox7* and reduced GA levels relative to M202.

Discussion

A key feature of the *SUB1A*-mediated response is restricting rice shoot growth during complete submergence to conserve energy until flood waters recede. Shoot growth is driven by cell elongation and/or cell division and responds to environmental factors

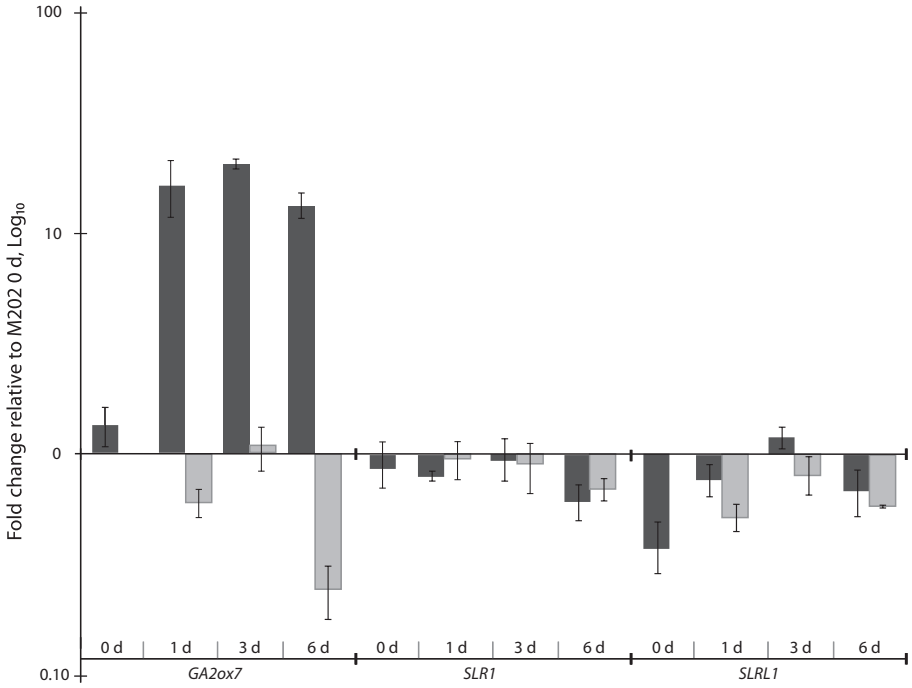

Fig. 6 Expression profiles of gibberellic acid (GA) catabolic and signaling genes in a time-course experiment. Expression levels for *GA2ox7*, *SLR1* and *SLRL1* at 0, 1, 3, and 6 d after submergence in M202 (light gray bars) and M202-Sub1 (dark gray bars) were measured using real-time PCR assays. Values are relative to M202 0 d. Error bars indicate ± SE. Notably, expression of *GA2ox7* was repressed in the intolerant M202 upon submergence in contrast with induction in M202-Sub1.

such as light, temperature, and hormones such as gibberellins, auxin and brassinosteroids. The *SUB1A* response was linked to GA signaling resulting in decreased shoot elongation when submerged (Fukao & Bailey-Serres, 2008). Our results suggest that brassinosteroids could be involved in *SUB1A*-mediated dampening of the GA responses during submergence. BR significantly restricts shoot elongation in M202 plants during submergence, an effect similar to the *SUB1A*-restricted shoot elongation in M202-Sub1 plants (Fig. 4). Consistent with the pretreatment submergence phenotype and lamina leaf bending, we found that BR levels were higher in M202-Sub1 plants during submergence relative to M202. BR treatment increased the expression of a GA catabolic gene (*GA2ox7*), which is induced in a *SUB1A*-dependent manner during submergence (Fig. 1c; Jung *et al.*, 2010). Our observations are consistent with another study where BR treatment strongly induced the transcript abundance of another GA catabolic gene, *GA2ox3* (De Vleesschauwer *et al.*, 2012). We postulate that BR could decrease bioactive levels of GA by inducing *GA2ox7* as part of an early submergence response. Increased bioactive GA, especially GA4, is known to promote SLR1 protein degradation in rice (Ueguchi-Tanaka *et al.*, 2007). These findings suggest that *SUB1A*-mediated submergence tolerance likely involves crosstalk between the BR and GA pathways.

In Arabidopsis, GA and BR regulate growth interdependently via direct interaction between the BR activated downstream protein BZR1 and the GA degraded DELLA protein (Bai *et al.*, 2012; Gallego-Bartolomé *et al.*, 2012). *BZR1* was required for GA-promoted hypocotyl elongation (Bai *et al.*, 2012). Although existence of these interactions remains to be examined in rice, it is likely that some interhormonal crosstalk will be conserved across species. SLR1 induction by BR treatment was observed in both genotypes and is independent of the *SUB1A* presence (Fig. 5). Although, the SLR1 induction was not different between M202 and M202-Sub1 plants, it is pertinent to point out that during submergence M202-Sub1 plants have higher BR levels compared to M202. Therefore, it is possible that higher SLR1 levels observed by others are a consequence of increased endogenous BR in M202-Sub1 plants. Based on our data we cannot directly associate SLR1 with the crosstalk between BR and GA in the M202-Sub1 plants. Although the exact mechanism remains to be determined, it is likely that BR repression of GA responses is via SLR1 protein induction and/or stability rather than transcript level induction of *SLR1*. For instance, in the study on the role of JA in repressing GA regulated growth, JA treatment increased the protein stability and had no effect on *SLR1* transcript abundance (Yang *et al.*, 2012). In our array analysis and quantitative PCR assays, we also did not detect a significant difference in *SLR1* transcript levels between M202 and M202-Sub1 during submergence. We observed that the SLR1 protein levels decrease after 3 d of BR treatment in our time-series experiment. During submergence, BR levels begin to increase within 1 d in M202-Sub1, whereas SLR1 levels increase at later stages of the 2-wk submergence treatment (Fukao & Bailey-Serres, 2008). It is possible that a specific concentration of endogenous BR needs to accumulate during submergence before SLR1 is induced in M202-Sub1 plants. Although we did not observe a differential transcript regulation for *SLRL1* between M202 and M202-Sub1, it could still be an important regulator of *SUB1A*-mediated submergence tolerance with higher protein induction and/or stabilization in M202-Sub1.

Model for *SUB1A* action

Our results suggest that BR homeostasis is differentially regulated in the *SUB1A* plants during submergence compared to M202. A model for *SUB1A* action that integrates our experimental

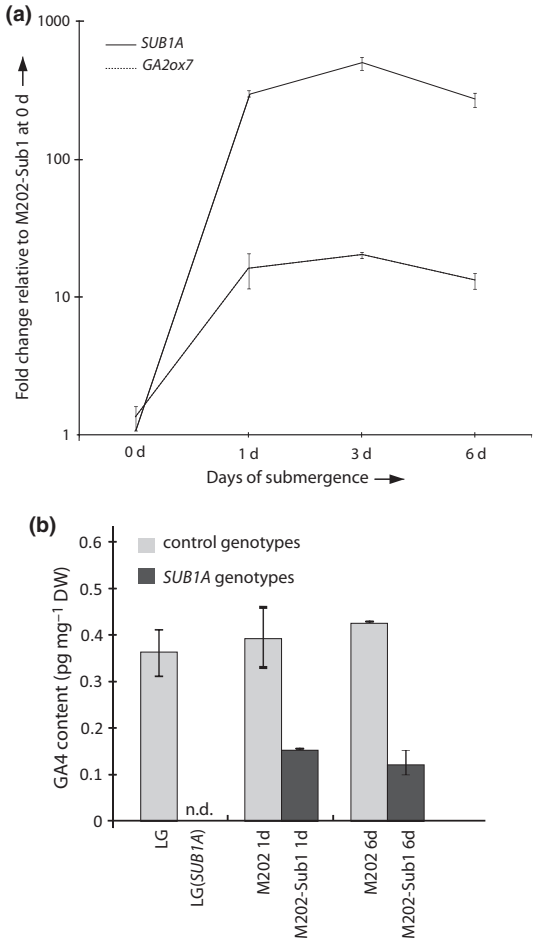

Fig. 7 *SUB1A*-expressing plants have increased *GA2ox7* expression and reduced endogenous GA4 hormone levels. (a) Submergence induced expression of *SUB1A* and of *GA2ox7* in M202-Sub1 was measured at 1, 3, and 6 d using real-time PCR assays and values (relative to M202-Sub1 day 0) were graphed on a $\log_{10}$ scale. Error bars indicate ± SE. (b) Endogenous GA4 content was quantified from LG and LG(*SUB1A*), and from 1 and 6 d submerged M202 and M202-Sub1 plants. Error bars ± SD.

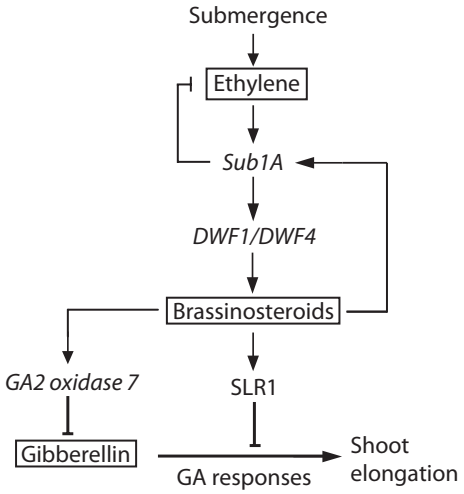

Fig. 8 Model for *SUB1A* action. Based on our experimental data, we propose a model for *SUB1A* action in rice during submergence. Increased ethylene concentration during submergence induces *SUB1A* (an ethylene response factor) within 1 d of submergence. *SUB1A* induction under submerged conditions increases the level of brassinosteroid (BR). Increased BR levels can potentially induce and/or stabilize SLR1, the negative regulator of gibberellic acid (GA) responses such as shoot elongation. Increased BR levels in the *SUB1A* plants also increase the expression of a GA catabolic gene, *GA2ox7*. Expression of *GA2ox7* inversely correlates with the GA level during submergence in the *SUB1A* plants. In summary, we hypothesize that increased BR levels in *SUB1A* genotype could restrict shoot elongation via repressing GA signaling responses and by decreasing GA levels.

observations with those by others is shown in Fig. 8 (Fukao & Bailey-Serres, 2008; Jung *et al.*, 2010). Increased ethylene levels in submerged plants trigger *SUB1A* gene expression in M202-Sub1 (Fukao *et al.*, 2006). Higher *SUB1A* expression dampens ethylene production (Fukao *et al.*, 2006; Fukao & Bailey-Serres, 2008). Within 1 d of submergence, transcript abundance of two BR biosynthesis genes *DWF4* and *DWF1* is increased, resulting in higher BR levels in M202-Sub1 plants. In striking contrast, BR biosynthesis gene expression is repressed during submergence in M202. Higher BR levels could further augment *SUB1A* expression during submergence. We propose that increased BR levels during submergence may modulate the GA responses in a temporal manner during transient submergence events. As part of an early response (within 1 d after submergence), BR promotes the expression of *GA2ox7*, which catabolizes GA thus reducing the endogenous GA4 levels in M202-Sub1 plants. Upon continued submergence, gradual accumulation of BR induces SLR1 at the protein level. Because *GA2ox7* reduces GA levels as an early response to submergence, reduced GA levels diminish the GID1-SLR1 interaction required for SLR1 degradation. Given, that differential elongation is observed fairly early during submergence, it may be that the BR-induced SLR1 induction and stabilization in M202-Sub1 is a downstream effect of altered GA and BR homeostasis occurring as early as 1 d after submergence (Fukao & Bailey-Serres, 2008). Based on previous report, the protein levels of SLRL1, which is resistant to GA-mediated degradation (Itoh *et al.*, 2005), could also be important for *SUB1A* action (Fukao & Bailey-Serres, 2008). The relationship between BR and SLRL1 induction during submergence needs to be explored in future studies.

In our experiments, we focused on shoot elongation during submergence as the key phenotype for submergence tolerance. However, submergence tolerance involves several components critical for submergence recovery such as optimal carbohydrate level regulation, scavenging of reactive oxygen species and minimizing chlorophyll degradation among others. Future studies will focus on hormonal regulation of some of these components. Besides creating a hypoxic condition, complete inundation also alters the light, temperature and carbon status of the rice plants. Based on recent reports from Arabidopsis, it is likely that changes in light status would be a critical component in the crosstalk between BR and GA pathways that restricts growth of M202-Sub1 plants during submergence. In fact, the Arabidopsis ortholog (At4 g21200) of the GA catabolic gene *GA2ox7* is induced by low light and dark conditions based on transcriptome data from multiple independent experiments catalogued in Genevestigator

(Zimmermann *et al.*, 2008). Future experiments will elucidate the possible role of light in mediating BR and GA crosstalk in *SUB1A* genotypes during submergence.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Exogenous BR restricts seedling growth.

Fig. S2 Mock controls for time course of SLR1 induction by exogenous BL.

Table S1 Primers used in this study

Table S2 LC conditions by using LC-ESI-MS/MS

Table S3 Parameters of CS quantification by using LC-ESI-MS/MS

Methods S1 Quantification of BR content.

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