

## Full Length Research Paper

# Identification of a novel submergence response gene regulated by the *Sub1A* gene

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Submergence is one of the major constraints to rice production in many rice growing areas in the world. The *Sub1A* gene has been demonstrated to dramatically improve submergence tolerance in rice. Here, we report the identification of a novel submergence response (*RS1*) gene that is specifically induced in the *Sub1A*-mediated submergence tolerance response. Under submergence, *RS1* was upregulated in M202 (*Sub1A*) but downregulated in M202 in RNA-seq and microarray assays. Expression analyses of various tissues and developmental stages show that *RS1* mRNA levels are high in leaves and sheaths, but low in roots, stems, and panicles. Our results also show that *RS1* is highly expressed under submergence, drought, and NaCl stresses, but not under cold or dehydration stress. Hormone ABA treatment induces, whereas GA treatment decreases, *RS1* expression. The *RS1* and *Sub1A* genes are co-regulated under submergence. Overexpression of *RS1* in transgenic Kitaake (without *Sub1A*) and M202(*Sub1A*)×Kitaake do not result in enhanced submergence tolerance. Conversely, down-regulation of *RS1* in M202(*Sub1A*)×Kitaake lead to weaken submergence tolerance. We hypothesize that *RS1* may play a role in the *Sub1A*-mediated submergence tolerance pathway.

**Key word:** Rice (*Oryza sativa* L.), submergence, RNA-seq, *Sub1A*, abiotic stress.

## INTRODUCTION

Submergence is one of main environmental stresses to rice growth and productivity in large rice-growing areas, especially in the flood-prone rainfed lowlands in South and Southeast Asia, where it regularly affects about 15 to 20 million hectares of rice land. Although rice is well adapted to aquatic environments, submergence causes annual losses of over US \$1 billion in Asia (Xu et al.,

2006). Fortunately, some indica cultivars, such as FR13A, can survive 10 to 14 days of complete submergence and renew growth after the floods recede. *Sub1A* was identified as the major gene conferring submergence tolerance in FR13A (Xu et al., 2006). *Sub1A*, an AP2/ERF transcription factor, was mapped on chromosome 9 and subsequently isolated from FR13A. Two alleles of *Sub1A*,

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*Sub1A-1* and *Sub1A-2*, exist that differ only by a SNP variation at position 556. Allele *Sub1A-1* confers submergence tolerance while null allele *Sub1A-2* loses the function (Xu et al., 2006). *Sub1A* limits ethylene-promoted GA responsiveness during submergence by augmenting accumulation of the GA signaling repressors *SLR1* and *SLRL1* (Fukao and Bailey-Serres, 2008). *Sub1A* also enhances the expression levels of *PDC* and *ADH1*, thus providing the ATP needed for rice survival under submergence (Perata and Voesenek, 2007).

Studies on *Sub1A* have resulted in significant advancement in understanding submergence tolerance in rice. However, submergence tolerance is a complex trait orchestrated by many genes (Xu et al., 2006; Fukao and Bailey-Serres, 2008). The differences in submergence tolerance between different rice cultivars are only partly resolved since the identification of the *Sub1A* gene (Perata and Voesenek, 2007). For example, FR13A and Goda Heenati are two indica cultivars that carry the *Sub1A-1* gene. FR13A is a submergence tolerant cultivar, which can survive approximately two weeks under submergence whereas Goda Heenati does not (Xiong et al., 2012). The difference of submergence tolerance between FR13A and Goda Heenati indicate that other genes may interact with *Sub1A-1* that are necessary for *Sub1A-1* to confer submergence tolerance. Towards this goal, genes differentially expressed between FR13A and Goda Heenati under submergence, including two novel submergence responsive genes (Os09g0269900 and Os12g0202700), were identified using suppression subtractive hybridization and microarray approaches (Xiong et al., 2012). In addition, Seo et al. (2011) identified 24 proteins that interacted with *Sub1A-1* through yeast two-hybrid (Y2H) screens. However, the *Sub1A-1* gene is not sufficient for rice to tolerate more than two weeks under submergence (Septiningsih et al., 2012). Therefore, it is necessary to identify novel genes involved in *Sub1A*-mediated tolerance in order to investigate the molecular mechanism and further improve submergence tolerance.

RNA-seq technology has provided an effective way to obtain transcript sequences and abundance at the genomic scale, facilitating the identification of genes differentially expressed between different varieties (Grabherr et al., 2011; Trapnell et al., 2010). M202 (Sub1A) is an inbred rice line carrying the *Sub1A* gene, which leads to enhanced tolerance to submergence. Here, we performed RNA-seq to identify differentially expressed genes in M202(Sub1A) under submergence. We selected 38 increased genes and further tested their differential expression patterns under submergence both in M202 (a rice variety without *Sub1A* gene, and intolerance to submergence) and M202(Sub1A) by qRT-PCR. We identified a novel gene responsive to submergence, called *RS1*. The expression patterns of *RS1* in various tissues were also determined. The responses of *RS1* to various abiotic stresses, such as

submergence, drought, cold, dehydration, and high NaCl, and to hormones, including ACC, GA, ABA, were assessed. However, overexpression of *RS1* did not result in significant improvement of submergence tolerance in transgenic Kitaake (a rice inbred line without *Sub1A-1* gene). In M202(Sub1A)×Kitaake background, the seedling height was significantly elongated with the down-regulation of *RS1*, indicating that the down-regulation of *RS1* resulted in weakened waterlogging tolerance. These results will help to explore the molecular mechanism of submergence tolerance in rice and facilitate breeding of submergence tolerant varieties.

## MATERIALS AND METHODS

### Plant growth and submergence treatment

Dehusked seeds were treated with 70% ethanol for 1 min, soaked in 1% sodium hypochlorite for 15 min, and rinsed with sterile water 4 to 5 times. Sterilized seeds were transferred to MS medium and grown in a chamber at 28°C for one week. Six one-week-old seedlings were planted at a silica sand pot (18 cm×16 cm), and were grown in the greenhouse at 25 to 28°C in a 16 h/8 h light-dark cycle. Three-week-old M202(Sub1A) seedlings were subjected to submergence for 0 or 3 days. Approximately 0.5 g leaves from six seedlings were detached. The leaf samples were ground to a fine powder in the presence of liquid nitrogen. Total RNA was isolated using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). All the experiments were performed with three biological replications. In total, 6 RNA samples were used for RNA-seq at BGI.

In order to accurately measure the increased seedling height under submergence, six sterilized seeds were planted into the tube (30 cm×2 cm). Six tubes each genotype were selected, and grown in the growth chamber at 25 to 28°C in a 16 h/ 8 h light-dark cycles. All experiments were performed with three biological replications. Seedlings of approximately 3 to 4 cm height were subjected to submergence treatment. Seedling heights were recorded every 24 h during submergence.

### Identification of *RS1*

Under submergence, the identification of differentially expressed genes in M202 (Sub1A) was conducted according to Reiner et al. (2002). In previous research, our groups conducted microarray assays to identify the differentially expressed genes between M202 and M202 (Sub1A) under submergence (Jung et al., 2010). We combined the RNA-seq and microarray results and selected 38 differentially expressed genes that were upregulated in M202 (Sub1A) compared to M202 upon submergence treatment. The expression levels of these 38 genes under submergence were determined by qRT-PCR for both M202 and M202(Sub1A). Under submergence, the expression level of *RS1* was increased in M202 (Sub1A) but decreased in M202. Therefore, *RS1* was chosen as a candidate gene responsive to submergence.

### Various environmental stresses and hormone treatment

All submergence, drought and hormone treatments were conducted with at least 3 independent biological replications. For gibberellin (GA), abscisic acid (ABA) and 1-aminocyclopropane-1-carboxylic acid (ACC) treatments, M202 and M202 (Sub1A) seedlings of 14 days old grown in germination paper were transferred into solutions

containing mock (0.1% DMSO), GA (10  $\mu$ M), ABA (10  $\mu$ M), and ACC (10  $\mu$ M), respectively, and incubated for 6 h. Submergence treatment was carried out as described by Fukao and Bailey-Serres (2008). M202 and M202 (Sub1A) of 35 days old were subject to 0 or 5 days of drought stress in greenhouse. Seedlings of 14 days old were treated with stresses including dehydration (the intact seedlings were exposed in the air without water supply), salinity stress (using 150 mM NaCl solution), and cold exposure at 4°C (Xiong et al., 2014).

### RNA isolation and expression analysis

All experiments were performed with three biological replications. Approximately 0.2 g tissues selected from six seedlings were used for RNA isolation. Leaves were selected from various treatments, including submergence, drought, cold, dehydration, NaCl, GA, ACC, and ABA. In addition, various tissues, including leaves, roots, shoots, stems, and panicles at different growth stages were also collected. First strand cDNA was synthesized using SuperScript-II reverse transcriptase according to the manufacturer's instructions (Invitrogen). The *actin 1* gene (Os03g50890.1) was used as an endogenous control to normalize expression data (Supplement Table 1). The qRT-PCR primers specific for the *RS1* and *Sub1A* genes are listed on Supplementary Table 1. Real-time PCR was conducted using the SYBR real-time PCR kit (Takara Japan) with IQTM SYBR® Green Supermixture according to the manufacturer's instructions (Bio-Rad USA). The reaction conditions were as follows: 94°C for 1 min; followed by 40 cycles of 95°C for 10 s, and then 55°C for 10 s.

### Binary construct and rice transformation

The full coding sequence (CDS) of the *RS1* gene (Os02g0134200) encoding 255 amino acids (aa) derived from M202(Sub1A) was isolated by RT-PCR and transferred into the pCAMBIA1300 binary vector under control of the ubiquitin (Ubi-1) promoter to generate the *RS1* overexpression construct (*RS1ox*). The *RS1* RNAi construct (part sequence of *RS1* cDNA) under control of the CaMV35s promoter was transferred into the pBWA(V)HS vector by Wuhan Biorun Biotechnology company. The *RS1ox* and *RS1* RNAi constructs were transformed into Kitaake (a rice inbred line), respectively. Partial maps of the constructs including the loop sequence and the *RS1* sequence in the RNAi construct are shown in Supplementary Figure 1. Rice transformation was also conducted by Wuhan Biorun Biotechnology Company.

## RESULTS

### Differentially expressed genes specific to *Sub1A*-mediated submergence tolerance were identified

Differentially expressed genes were identified through comparing their expression levels based on RPKM (reads per kilobase of exon model per million mapped reads). The transcriptomic profiles of M202 (Sub1A) were determined by comparing their gene expression levels under normal condition and submergence treatment. Under submergence, 3712 genes were differentially expressed in M202 (Sub1A); 1696 of the 3712 genes were decreased and 2016 genes were increased (Figure 1). Our previous study using microarray has identified genes differentially expressed between M202 and

M202 (Sub1A) under submergence (Jung et al., 2010). Here, we combined the RNA-seq and microarray results and selected 38 genes that increased in M202(Sub1A). Subsequently, their expression levels both in M202 (Sub1A) and M202 under submergence were determined by qRT-PCR (data not shown). Of particular interest, the expression level of *RS1* increased in M202 (Sub1A) but decreased in M202 (Figure 2) upon submergence. Under 1, 3, and 6 days submergence, the expression level of *RS1* in M202 (Sub1A) is approximate 2, 6, and 5 folds compared with that in M202 separately (Figure 2). Therefore, we chose *RS1* as a candidate gene for possible involvement in *Sub1A*-mediated submergence tolerance.

### The *RS1* gene responds to drought and high salt stresses

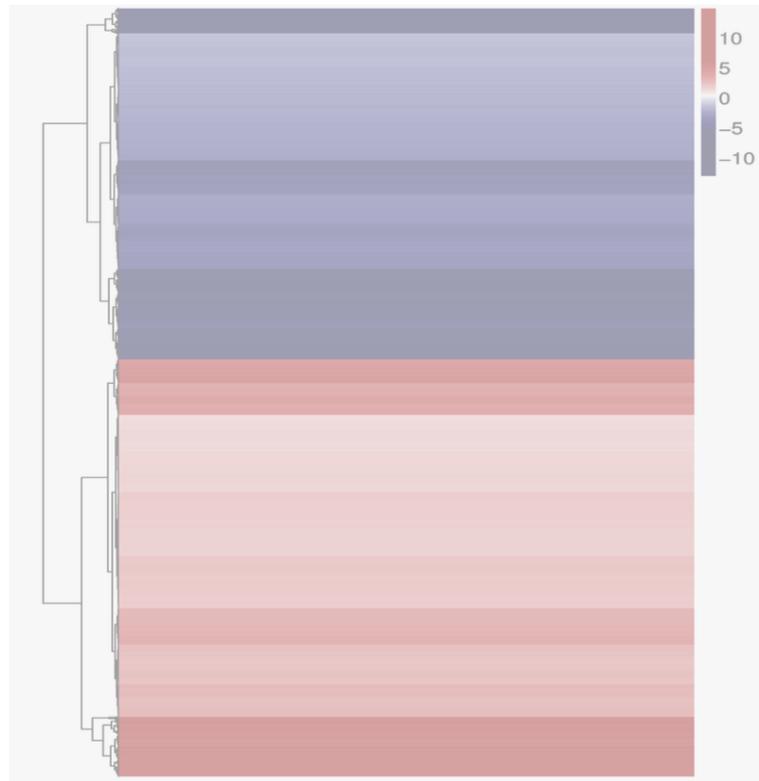
We assessed changes of *RS1* expression levels by qRT-PCR when subject to various abiotic stresses, including drought, cold, dehydration, and high NaCl. The results show that the *RS1* expression levels both in M202 and M202(Sub1A) increased upon drought treatment, peaking at day 7 (Figure 3a). Under cold and dehydration stresses, the *RS1* expression levels remained unchanged (Figure 3b), suggesting that *RS1* does not respond to cold or dehydration stress. Under high concentration of NaCl, the *RS1* levels were significantly higher than those of untreated controls (Figure 3b). Therefore, the *RS1* gene responds to drought and high salt stresses.

### *RS1* is responsive to ACC and ABA treatments

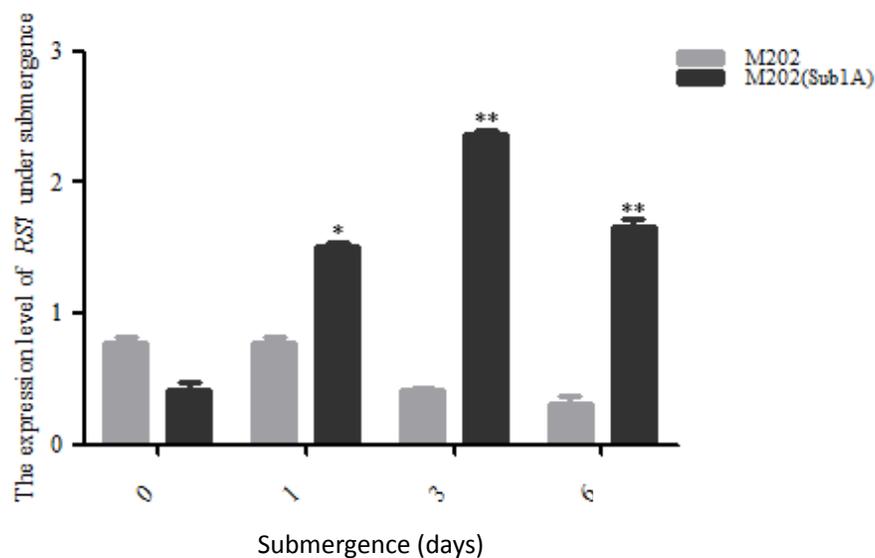
Hormones play important roles in plant responses to abiotic stresses. Thus, we treated M202 and M202 (Sub1A) seedlings of 14 days old with ABA, GA, and ACC (a precursor of ethylene). The *RS1* expression levels were also determined by qRT-PCR. The results show that the *RS1* levels were significantly increased both in M202 and M202 (Sub1A) upon ACC treatment compared to untreated controls (Figure 4a). Under GA treatment, the *RS1* levels were increased both in M202 and M202 (Sub1A), but not statistically significant (Figure 4b). ABA treatment significantly increased the *RS1* levels both in M202 and M202 (Sub1A) (Figure 4c). In summary, the results show that ACC and ABA, but not GA, treatments induce *RS1* expression, indicating that the *RS1* gene is responsive to ACC and ABA hormones.

### *RS1* is mainly expressed in leaves and sheaths

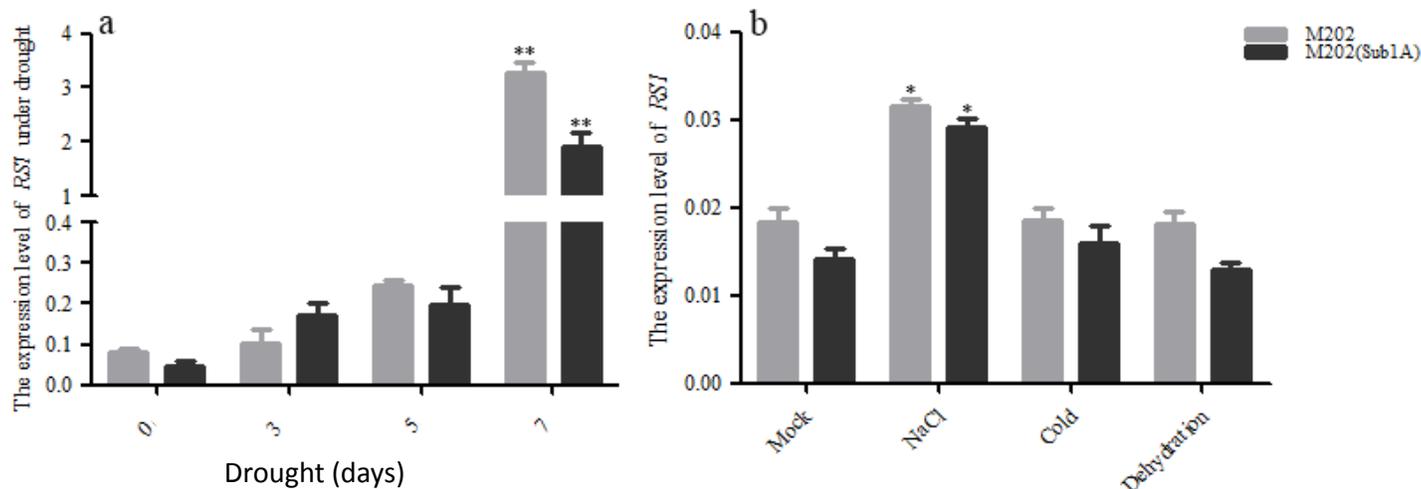
The *RS1* expression levels in various tissues were determined using qRT-PCR. The transcript levels of *RS1* were low in shoots and roots of one-week and two-week old seedlings both in M202 and M202 (Sub1A). In one-



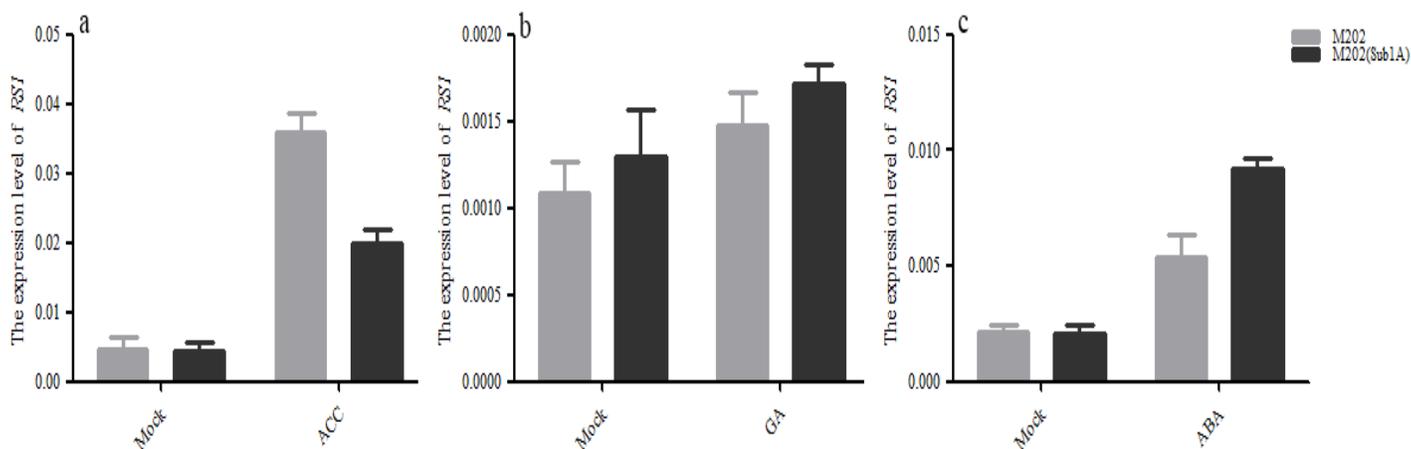
**Figure 1.** The clustering analysis of differentially expressed genes in M202(Sub1A) in response to submergence. Heat map of Pearson's correlation across 3712 genes differentially expressed under submergence for 2 days. The dendrogram of correlation between genes was displayed at the left of the heatmap.



**Figure 2.** Expression levels of the *RS1* gene under submergence in M202 and M202(Sub1A). M202 and M202(Sub1A) seedlings of fourteen days old were subject to submergence treatment for 0, 1, 3, and 6 days. The leaves were chosen for total RNA extraction and qRT-PCR experiments. The expression level of *RS1* was calculated using the  $2^{-\Delta\Delta Ct}$  value. Each data point represents the mean  $\pm$  SD of 3 independent biological replicates.



**Figure 3.** Expression levels of the *RS1* gene under drought, high NaCl, dehydration, and cold treatments. a. The *RS1* expression level under drought; b. The *RS1* expression levels under treatments with high NaCl, dehydration, and cold. All experiments were performed with three biological replications. Plants of 35 days old were subject to drought treatment. Fourteen-day old seedlings were treated with high NaCl, dehydration, or cold. Approximately 0.2 g leaves selected from six plants were used for RNA isolation. The expression levels of *RS1* under various stresses were determined by qRT-PCR. Each data point represents the mean $\pm$ SD of 3 independent biological replicates. The student's t-test was performed. \* indicates  $P < 0.05$ ; \*\* indicates  $P < 0.01$ .

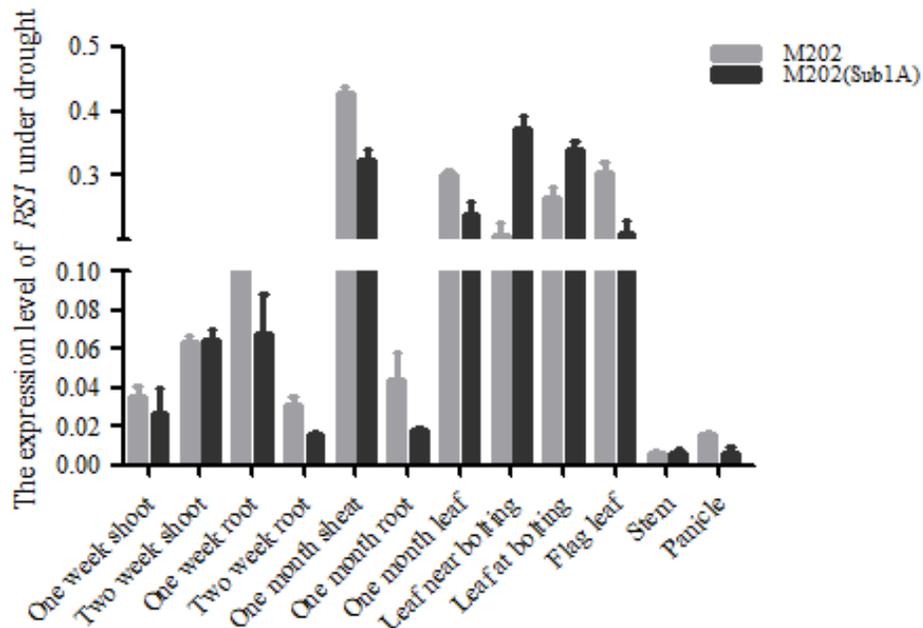


**Figure 4.** Expression levels of *RS1* under GA, ABA, and ACC treatments. Fourteen-day old seedlings were treated with GA (10  $\mu$ M), ABA (10  $\mu$ M), or ACC (10  $\mu$ M) solution for 6 h. a. Expression level of *RS1* under ACC treatment; b. Expression level of *RS1* under GA treatment; c. Expression level of *RS1* under ABA treatment. Three biological replications were carried out. Approximate 0.2 g leaves collected from six seedlings each genotype was used for RNA extraction and qRT-PCR. The expression level of *RS1* was calculated using the  $2^{-\Delta\Delta Ct}$  value. Each data point represents the mean $\pm$ SD of 3 independent biological replicates. The student's t-test was performed. \* indicates  $P < 0.05$ ; \*\* indicates  $P < 0.01$ .

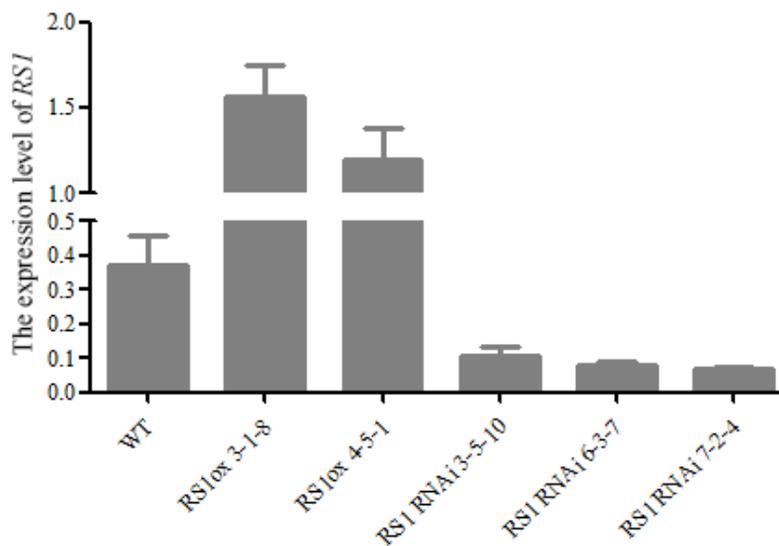
month old plants, the *RS1* levels were high in leaves and sheaths, but remained low in roots. At the reproductive stage, the *RS1* levels were high in leaf tissues, including the flag leaf, leaves near bolting, and leaves at bolting. The *RS1* levels remained low in stems and panicles (Figure 5). These results show that *RS1* is mainly expressed at high levels in leaves and sheaths, while only expressed at low levels in shoot, root, stem, and panicle tissues.

### Overexpression of *RS1* in Kitaake did not enhance submergence tolerance

Constructs designed to overexpress (*RS1ox*) or to knockdown (RNAi) the *RS1* gene have been transformed into Kitaake separately. Twenty transgenic *RS1ox* and 16 transgenic *RS1* RNAi independent lines were obtained. The homozygous transgenic lines were chosen in the  $T_3$  generation, and their *RS1* expression levels were



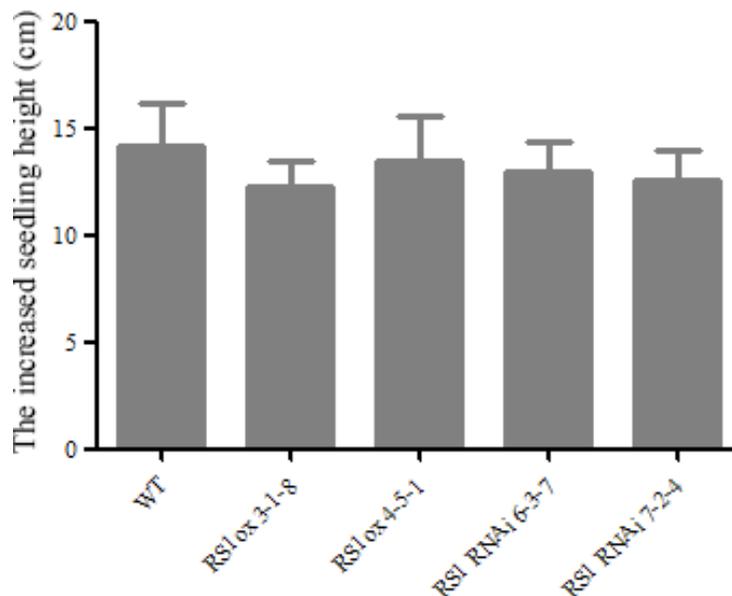
**Figure 5.** *RS1* expression levels in various tissues at different developmental stages. Approximately 0.2 g tissues were collected at various growth stages. The expression level of *RS1* was calculated using the  $2^{-\Delta\Delta Ct}$  value. Each data point represents the mean $\pm$ SD of 3 independent biological replicates. The student's t-test was performed. \* indicates  $P < 0.05$ ; \*\* indicates  $P < 0.01$ .



**Figure 6.** Expression levels of *RS1* in *RS1ox* and *RS1 RNAi* transgenic plants. For each transgenic line, approximately 0.2 g mature leaves collected from six plants were used for total RNA isolation. *RS1* expression levels were determined by qRT-PCR. Expression level was calculated using the rice *actin1* gene as the internal reference. Each bar represents the mean $\pm$ SD of three independent biological replicates. Student's t-test was performed. \* indicates  $p < 0.05$ ; \*\* indicates  $p < 0.01$ .

measured by qRT-PCR. The results showed that the *RS1* expression levels in the transgenic *RS1ox* plants (# 3-1-

8 and 4-5-1) were higher than others (Figure 6). Conversely, the *RS1* expression levels in the transgenic



**Figure 7.** Increases in seedling height under submergence for 10 days. Seedlings of approximately 3 to 4 cm grown in tubes were subject to submergence. The increases in seedling height were measured every 24 h. Each bar represents the mean $\pm$ SE of three independent biological replicates. Student's t-test was performed. \* indicates  $p < 0.05$ ; \*\* indicates  $p < 0.01$ .

*RS1* RNAi plants (# 3-5-10, 6-3-7, and 7-2-4) were lower than WT (Figure 7), indicating that the endogenous *RS1* was suppressed in the presence of the *RS1* RNAi construct.

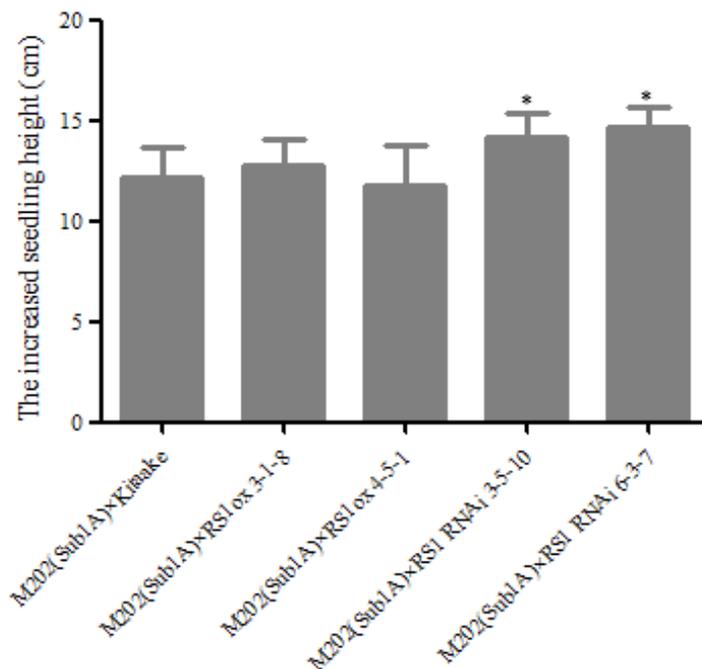
Submergence-tolerant rice plants show stunted growth upon submergence and restart growth after the flood has receded (Xu et al., 2006). Thus, we subjected their seedlings planted in tubes to submergence tests, and measured their increased seedling heights. Under submergence for 10 days, transgenic *RS1ox* seedlings elongated (12.26 cm for line #3-1-8 and 13.47 cm for #4-1-5) slightly less than the WT (14.17 cm) (Figure 7), but not statistically significant. The similar results were also observed in transgenic *RS1* RNAi seedling (Figure 7). We did not observe other differences in submergence tolerance between *RS1ox* and WT plants, including seedling survival rates (data not shown). We conclude that the seedling elongation in transgenic *RS1ox* plants was not significantly different than that in WT upon submergence, indicating that *RS1ox* in rice did not result in significant improvement of submergence tolerance.

To measure the interaction between *Sub1A* and *RS1*, M202(*Sub1A*) was crossed with transgenic *RS1ox* and RNAi lines (Kitaake background), separately. The seedlings in  $F_1$  generation were subjected to submergence. The increased seedling height was measured during submergence. With overexpression of *RS1*, the increased seedling height was unchanged compared with that of M202 (*Sub1A*) $\times$ Kitaake.

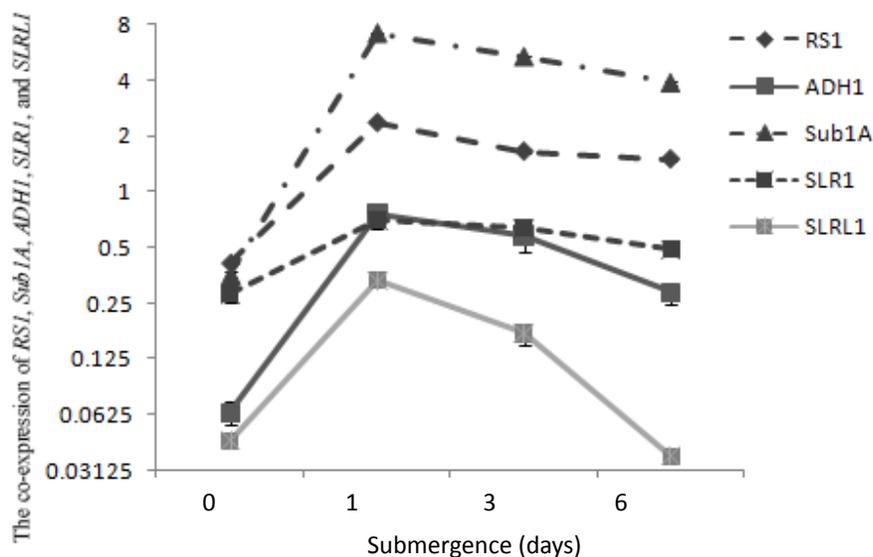
Conversely, the seedling height was significantly elongated with the down-regulation of *RS1* compared with the control even with the presence of *Sub1A* (Figure 8). Above results indicate that *RS1* may play the role in *Sub1A* pathway to confer submergence tolerance in rice.

## DISCUSSION

Reverse genetics approaches are widely used to identify genes of interest. In our previous and current studies, RNA-seq and microarray have been conducted to identify the candidate genes responsive to submergence in rice. *RS1*, a gene differentially expressed between M202 and M202(*Sub1A*) under submergence, was identified by both RNA-seq and microarray assays. M202(*Sub1A*), carrying the *Sub1A* gene, is a submergence tolerant line. Previous studies suggest that *Sub1A* limits ethylene-promoted GA responsiveness during submergence by augmenting accumulation of the GA signaling repressors *SLR1* and *SLRL1* (Perata and Voesenek, 2007; Fukao and Bailey-Serres, 2008). *ADH1* is a submergence tolerance marker gene, whose levels in M202(*Sub1A*) increased during submergence (Xu et al., 2006). Here we show that *RS1*, *Sub1A*, *ADH1*, *SLR1*, and *SLRL1* genes are co-induced upon submergence, reaching a peak within 24 h (Figure 9), indicating that *RS1* is tightly co-regulated under submergence with these well-characterized submergence-associated genes. The contents of GA and



**Figure 8.** The increased seedling height in M202(Sub1A) x Kitaake background under submergence for 10 days. M202(Sub1A) was crossed with transgenic *RS1* or RNAi lines, respectively. In the F<sub>1</sub> generation, seedlings of approximately 3 to 4 cm were subjected to submergence. The increases in seedling height were measured every 24 h. Each bar represents the mean  $\pm$  SE of three independent biological replicates. Student's t-test was performed. \* indicates  $p < 0.05$ ; \*\* indicates  $p < 0.01$ .



**Figure 9.** Co-expression of *RS1*, *Sub1A*, *ADH1*, *SLR1*, and *SLRL1* in M202(Sub1A) during submergence. Fourteen-day old seedlings were subjected to submergence for 0, 1, 3 and 6 days. All experiments were performed with three biological replications. The leaves from six seedlings collected from different time points during submergence were used for RNA isolation. Expression levels of *RS1*, *Sub1A*, *ADH1*, *SLR1*, and *SLRL1* were determined by qRT-PCR, and were calculated using the  $2^{-\Delta\Delta Ct}$  value. Each data point represents the mean  $\pm$  SD of 3 independent biological replicates.

ABA play a role to suppress shoot elongation during submergence (Fukao and Bailey-Serres, 2008). The *RS1* expression level was changed by treatments with GA and ABA, indicating that *RS1* is regulated by GA and ABA. Together, these results suggest that *RS1* is associated with adaptation to submergence stress in rice.

Although *Agrobacterium*-mediated transformation technique has been widely applied to *japonica* rice, several *japonica* rice, including M202 (Sub1A), remain difficult to transformation (Roy et al., 2000). We subsequently used the Kitaake rice variety for *RS1* transformation because of its transformability and abundant genetic resources (Li et al., 2016). Although analysis of *RS1* suggests that *RS1* is responsive to submergence stress in M202 (Sub1A), overexpression of *RS1* did not result in significantly enhanced submergence tolerance in Kitaake. The different genetic backgrounds of M202 (Sub1A) and Kitaake, which does not contain *Sub1A*, may have contributed to these results. M202 (Sub1A), a near isogenic line of M202, possesses a 182 kb genomic fragment carrying the *Sub1A* gene derived from FR13A (Xu et al., 2006). When subject to submergence, the expression of *RS1* in M202 was greatly increased in the presence of *Sub1A*, but decreased in the absence of *Sub1A*. Therefore, we conclude that *RS1* positively responds to submergence stress regulated by *Sub1A*. To further pursue this issue, transgenic *RS1ox* and RNAi lines were crossed with M202 (Sub1A), respectively. With overexpression of *RS1* both in Kitaake or M202 (Sub1A)×Kitaake, the increased seedling height do not significant decreased compared with their controls, indicating that overexpression of *RS1* do not result in enhanced submergence tolerance in rice. Conversely, with the presence of *Sub1A*, down-regulation of *RS1* in M202 (Sub1A)×Kitaake lead to weaken submergence tolerance. Therefore, we conclude that *RS1* may be interacted with *Sub1A* to confer submergence tolerance in rice.

### Conflicts of interests

The authors have not declared any conflict of interests.

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### Abbreviations

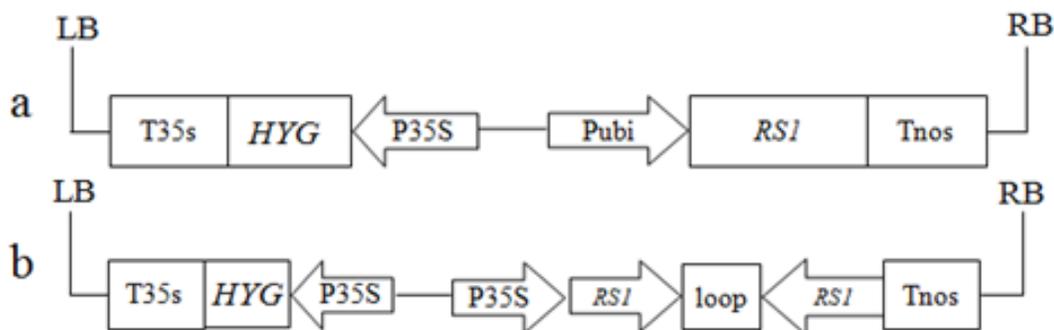
**RS1**, Submergence response; **PDC**, pyruvate decarboxylase; **ABA**, abscisic acid; **GA**, gibberellin; **QTL**, quantitative trait locus; **ACC**, 1-aminocyclopropane-1-carboxylic acid; **ADH**, Alcohol dehydrogenase; **SLR1**, Slender rice-1; **SLRL1**, SLR1 like-1; **ERF**, ethylene response factors; **RPKM**, reads per kilobase of exon model per million mapped reads; **qRT-PCR**, quantitative reverse transcription-PCR; **SNP**, single nucleotide polymorphism.

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**Supplementary Table 1.** Primers used in this study.

Primers	Sequence (5'-3')	Usage
<i>RS1</i> forward	CGCCGCCATCATCCAAACT	Real-time PCR
<i>RS1</i> reverse	TCGGTGACCACGCCCTTCT	Real-time PCR
Actin1 forward	ACAGGTATTGTGTTGGACTCTGG	Real-time PCR
Actin1 reverse	AGTAACCACGCTCCGTCAGG	Real-time PCR
<i>Sub1A</i> forward	CGGCCTCATCACAATCGGAG	Real-time PCR
<i>Sub1A</i> reverse	ATGTCCATGTCCATATGTCGTCG	Real-time PCR
<i>ADH1</i> forward	TGTTGGAGAGGGTGTGACTG	Real-time PCR
<i>ADH1</i> reverse	GCCATCACCAATCATCACAC	Real-time PCR
<i>SLR1</i> forward	GATCGTCACCGTGGTAGAGC	Real-time PCR
<i>SLR1</i> reverse	GAGGGAATCGAACATGGTGG	Real-time PCR
<i>SLRL1</i> forward	GGCGGCGACAATAACAACAACAGT	Real-time PCR
<i>SLRL1</i> reverse	TACAAACACACGCTGCTACCATCC	Real-time PCR



**Supplementary Figure 1.** Schematic diagrams of *RS1* constructs. a The *RS1ox* construct in the pCAMBIA1300 vector (not to scale). b The *RS1* RNAi construct in the pBWA(V)HS vector. LB and RB, T-DNA left and right borders, respectively; Pubi, ubiquitin gene promoter; Tnos, nopaline synthase gene terminator; HYG, hygromycin resistance gene; T35s, CaMV 35S terminator; and P35s: CaMV35S promoter. The full-length cDNA of *RS1* was used in the *RS1ox* construct.