Abiotic Stress Responsive Rice *ASR1* and *ASR3* Exhibit Different Tissue-Dependent Sugar and Hormone-Sensitivities

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The expression of the six rice *ASR* genes is differentially regulated in a tissue-dependent manner according to environmental conditions and reproductive stages. *OsASR1* and *OsASR3* are the most abundant and are found in most tissues; they are enriched in the leaves and roots, respectively. Coexpression analysis of *OsASR1* and *OsASR3* and a comparison of the *cis*-acting elements upstream of *OsASR1* and *OsASR3* suggested that their expression is regulated in common by abiotic stresses but differently regulated by hormone and sugar signals. The results of quantitative real-time PCR analyses of *OsASR1* and *OsASR3* expression under various conditions further support this model. The expression of both *OsASR1* and *OsASR3* was induced by drought stress, which is a major regulator of the expression of all *ASR* genes in rice. In contrast, ABA is not a common regulator of the expression of these genes. *OsASR1* transcription was highly induced by ABA, whereas *OsASR3* transcription was strongly induced by GA. In addition, *OsASR1* and *OsASR3* expression was significantly induced by sucrose and sucrose/glucose treatments, respectively. The induction of gene expression in response to these specific hormone and sugar signals was primarily observed in the major target tissues of these genes (i.e., *OsASR1* in leaves and *OsASR3* in roots). Our data also showed that the overexpression of either *OsASR1* or *OsASR3* in transgenic rice plants increased their tolerance to drought and cold stress. Taken together, our results revealed that the transcriptional control of different rice *ASR* genes exhibit different tissue-dependent sugar and hormone-sensitivities.

INTRODUCTION

Plants are frequently exposed to various stresses under natural conditions. Drought, high salinity and low temperature are the most common abiotic stresses that adversely affect growth and productivity in plants. The perception of abiotic stresses evokes a response that involves the regulation of gene expression at the transcriptional level (Ingram and Bartels, 1996). Absciscic

acid (ABA) is a key regulator of the signal transduction that modulates gene expression in stress adaptation and sugar sensing (Verslues and Zhu, 2005). Some drought-responsive and low temperature-responsive genes are not induced by ABA treatment (Shinozaki and Yamaguchi-Shinozaki, 1997), suggesting that both ABA-dependent and ABA-independent pathways regulate the transcription of stress-responsive genes. ASR, a novel protein induced by ABA during stress and ripening, was initially isolated in the tomato (Iusem et al., 1993). The *ASR* promoter was shown to be responsive to ABA (Rossi et al., 1998) and to contain *cis*-acting elements (Hong et al., 2002).

Multigene families in various plant species encode ASR proteins (Frankel et al., 2006). These species include dicots, such as the tomato and potato, and monocots, such as pine, rice and maize. However, no *ASR*-like genes have been identified in *Arabidopsis*. In different species, distinct members of one ASR family might be expressed in different organs under different conditions and with different expression patterns (Canel et al., 1995; Maskin et al., 2001). The functions of ASRs are not evident based on sequence homology, but these proteins exhibit certain characteristics that are consistent with transcription factor activity. These 13–15.4 kDa proteins are hydrophilic, with many charged residues (Amitai-Zeigerson et al., 1994; Iusem et al., 1993; Silhavy et al., 1995). Most ASR proteins, such as the grape, pine, lily and melon ASRs, contain a putative nuclear localization signal at their C termini (Cakir et al., 2003; Hong et al., 2002; Huang et al., 2000; Padmanabhan et al., 1997). Tomato ASR1 homodimers were found in both the cytosol and the nucleus (Ricardi et al., 2012). Moreover, a tomato ASR binds DNA in a Zn- and sequence-dependent manner (Kalifa et al., 2004a; Rom et al., 2006) and competes with AB14 for DNA binding in *Arabidopsis* (Shkolnik and Bar-Zvi, 2008). The transcriptional activity of ASR was further supported by evidence that the unfolded ASR protein in the cytosol becomes folded upon zinc-dependent DNA binding (Goldgur et al., 2007).

The increased expression of ASR improves abiotic stress tolerance in plants. Tobacco plants overexpressing tomato ASR1 showed decreased germination inhibition, water loss from the leaves and NaCl accumulation, and increased proline accumulation after exposure to NaCl (Kalifa et al., 2004b). To-

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mato *ASR1*-overexpressing *Arabidopsis* plants exhibited ABA, glucose and NaCl insensitivity (Shkolnik and Bar-Zvi, 2008). Over-expression of wheat *ASR1* gene in tobacco increased drought stress tolerance (Hu et al., 2013). The expression of a lily *ASR* in *Arabidopsis* facilitated germination in salt and reduced dormancy, water loss from detached leaves, damage from abiotic stresses and stomatal closure (Yang et al., 2005). Lily *ASR* over-expressed *Arabidopsis* increased cold and freezing tolerance (Hsu et al., 2011). Plantain *Asr* over-expressed *Arabidopsis* increased osmotic stress tolerance (Dai et al., 2011). Interestingly, a grape *ASR* mediates the activation of sugar-responsive genes (Cakir et al., 2003). In potatoes, the insertion of the *Asr1* antisense gene resulted in decreased tuber fresh weight, whereas *Asr1* overexpression reduced the number of tubers (Frankel et al., 2007). The *ASR* protein acts as a downstream component of a common signal transduction pathway that is shared by sugar and ABA signals (Cakir et al., 2003; Shkolnik and Bar-Zvi 2008). Recently, it is reported that tobacco *ASR1* mediated glucose-hormone crosstalk (Dominguez et al., 2013). However, the function of *ASR* proteins in abiotic stress and sugar signaling is not well understood. Most studies concerning *ASR* function have been associated with ABA signaling, and some *ASR* genes do not respond to ABA (Virilouvet et al., 2011). This finding indicates that an ABA-independent pathway is able to regulate *ASR* expression.

The six *ASR* genes in rice are up-regulated by abiotic stresses (Kawasaki et al., 2001; Vaidyanathan et al., 1999; Yang et al., 2004). Rice *ASR5* silenced lines were highly aluminium sensitive, and *ASR5* expression did not respond to aluminium exposure in highly aluminium sensitive clutiva (Arenhart et al., 2013). However, data regarding the expression and regulatory roles of these genes are scarce. Although *ASR* genes have been reported to play an important role in various abiotic stresses and to be transcriptionally regulated by ABA and sugars, the physiological role and control mechanism of *ASR* genes has proven elusive. Coexpression analysis and comparison of *cis*-acting element of *ASR* genes will provide essential information. In this study, we described the expression of the members of the rice *ASR* gene family in various organs at different development stages and under various abiotic stresses. Our data showed that *OsASR1* and *OsASR3* were the dominantly expressed isoforms in rice and that these genes contain functional *cis*-acting elements to respond to various factors. These different *cis*-acting elements conferred different sensitivities to stress, hormones and sugar. We also used a transgenic approach to examine the functions of *ASR* genes and to evaluate the role of the *ASR* family in the stress response. The results showed that rice *ASR1* and *ASR3* exhibit different tissue-dependent sugar and hormone sensitivities and have common functions in drought stress tolerance.

MATERIALS AND METHODS

The identification of rice *ASR* genes

Rice genes encoding *ASR* proteins were identified using BLAST searches of the *Oryza sativa* DNA sequences in the National Center for Biotechnology Information database with the amino acid sequence of tomato *ASR1* as a query. Clones AK119547 (*OsASR1*), AK105960 (*OsASR2*), AK066415 (*OsASR3*), AK104613 (*OsASR4*), AK063053 (*OsASR5*) and AK318549 (*OsASR6*) from the KOME full-length cDNA library were selected from among the matches. The sequences of these clones were compared with sequences in the GGB EST database (GreenGene BioTech, Korea; <http://www.ggbio.com>). The

matching clones 14ETL-06-A16 (*OsASR1*) and 14ROOT-01-K14 (*OsASR3*) were obtained from GGB.

Plant materials and growth conditions

Transgenic and non-transgenic (NT) rice plants with an *Oryza sativa* subsp. *japonica* cv. Nakdong background were used. The husked seeds were washed with 70% (v/v) ethanol for 5 min and sterilized with 50% (v/v) commercial bleach for 15 min with gentle shaking. The sterilized seeds were rinsed several times with sterile water and germinated on solid one-half strength MS medium (with or without 4 µg/L of phosphinotricin for selection) in a growth chamber. After 3 days at 28°C in the dark, the germinated seedlings were incubated with a 16 h light/ 8 h dark cycle for 2 days at the same temperature. Finally, the seedlings were transplanted into soil pots and grown in the greenhouse until further use. The samples of each rice organ were prepared as described below. Embryogenic calli were induced from mature seeds in 2N6 solid medium containing 2 µg/L of 2,4-dichlorophenoxyacetic acid in the dark at 28°C for 4 weeks. The germinants (germinating shoot and root) were obtained from the seedlings, which were germinated on solid MS medium in a growth chamber at 28°C in the dark for 3 days and then in the light for 1 day. After germination, the seedlings were grown in the greenhouse for 2 weeks (to obtain young leaves and roots) or 4 weeks (to obtain mature leaves, roots and internodes). Rice panicles at different developmental stages were obtained from field-grown rice plants. The young panicles were harvested from the sheath, measured and categorized into three groups (P3, 3-5 cm; P4, 10-15 cm, and P5, 15-20 cm) based on the length of the panicle and landmark developmental events (Itoh et al., 2005). The rice seeds were tagged on the day of pollination (0 DAP) and collected every day from 0 to 29 DAP (S1, 0-2 DAP; S2, 3-4 DAP; S3, 5-10 DAP; S4, 11-20 DAP; and S5, 25-29 DAP).

RNA gel blot and quantitative real-time PCR analysis

Total RNA was isolated from the rice tissue samples using the TRI REAGENT® (Molecular Research Center) according to the manufacturer's instructions. RNA gel blot analysis was performed with 10 µg of total RNA per lane as previously reported (Jang et al., 2002). The hybridization signals were measured using a phosphorimager analyzer (FLA 3000, Fuji, Japan). For quantitative real-time PCR (qRT-PCR), first strand cDNA was synthesized from 5 µg of total RNA as a template using oligo (dT)₁₈ primers according to the manufacturer's instructions (RevertAid™ First Strand cDNA Synthesis Kit, Fermentas). A one-third dilution of the cDNA synthesis reaction mixture was prepared, and 1 µl of the diluted cDNA mixture was used as a template for subsequent real-time PCR analyses with 2X real-time PCR Pre-Mix containing EvaGreen (SolGent). Thermocycling and fluorescence detection were performed in an Mx3000p Real-Time PCR instrument (Stratagene). The PCR reaction was conducted at 95°C for 10 min, followed by 40 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s. Each qRT-PCR was performed in triplicate, and each experiment was repeated three times. The expression data were normalized to the *Actin* gene as a control. The primers used for the RNA gel blot and qRT-PCR analyses are listed in Supplementary Table 7.

RiceArrayNet and promoter analysis

The genes that are coexpressed with *OsASR1* and *OsASR3* were identified using the RiceArrayNet (RAN) database (Lee et al., 2009). The GO terms that were enriched among the top-

ranked 5% of the coexpressed genes in each database were tested. These genes represented approximately 1,700 of the 33,689 genes identified in RAN. To determine which GO terms were enriched, these genes were subjected to analysis using the GoMiner program (Zeeberg et al., 2003). Among the 60,000 rice genes, 17,962 matched TAIR8 *Arabidopsis* genes with a score of at least 100 in BLASTP analyses. These 17,962 genes were subsequently used as a total gene set in the GoMiner analysis for rice, and the P-values were calculated using one-sided Fisher's exact tests for the number of total categorized GO terms. The false discovery rate (FDR) values were obtained from 100 randomizations, and the GO terms with FDRs of less than 0.05 were collected. For the promoter analysis, 1.0 -kb segments of the 5' regulatory region of the *OsASR1* and *OsASR3* genes were scanned for the presence of putative *cis*-acting elements that were identical or similar to the motifs registered in Plant CARE (<http://bioinformatics.psb.ugent.be/web-tools/plantcare/html/>) and PLACE (<http://www.dna.affrc.go.jp/PLACE/>).

Hormone, sugar, abiotic stress treatments and chlorophyll fluorescence measurement

For the hormone, sugar and abiotic stress treatments, 3-week-old NT plants that had been grown in a greenhouse were washed to remove the soil from the roots and then transferred to a growth chamber (28°C, 16 h light/8 h dark cycles) for 3 days for adaptation to growth in water. After adaptation, the plants were treated with 100 μ M ABA, 100 μ M GA, 100 mM sucrose, 100 mM glucose, or 200 mM NaCl solutions or air-dried. The leaf and root tissues were collected separately at the indicated time points (0, 0.5, 2, 6, and 24 h). To test the drought stress resistance, 4-week-old NT and transgenic plants grown on soil were subjected to 3 days without water, followed by 25 days of watering in a greenhouse. The chlorophyll fluorescence of 3-week-old NT and transgenic plants was measured using a pulse modulation fluorometer (mini-PAM, Walz, Germany). For the leaf disc test, the green portions of approximately 10 seedlings were cut using scissors prior to stress treatments *in vitro*. Under continuous light at 150 μ mol m⁻² s⁻¹, the leaf discs were air-dried for 2 h (to induce drought stress) and treated with a 400 mM NaCl solution for 7 h at 28°C (to induce salt stress). To induce cold stress, the leaf discs were incubated in a 4°C growth chamber for 5 h under the same light conditions. After the stress treatments, the leaf discs were dark-adapted for 10 min, and the minimal fluorescence level (F_0) was measured. Then, a saturating light pulse was applied, and the maximal fluorescence level (F_m) was measured. The ratio of F_v to F_m ($F_v/F_m = F_m - F_0/F_m$), representing the activity of photosystem II, was used to assess the functional damage to the plants (Artus et al., 1996). The statistical significance of differences between groups was assessed using Student's *t*-test.

Plasmid construction and transformation of rice

The overexpression plasmid pMJ101 contained the *bar* gene under the control of the cauliflower mosaic virus 35S promoter for herbicide-based selection and a pair of matrix-attachment region (MAR) sequences from the chicken lysozyme gene for stable transgene expression (Phi-Van and Stratling, 1996). The rice *cytochrome c* promoter was used to drive constitutive expression (Jang et al., 2002). The coding regions of *OsASR1* and *OsASR3* were PCR amplified from full-length cDNA clones using a pair of primers containing the *attB* sequence to introduce a Gateway[®] recombination site. The *attB*-PCR products were inserted into pMJ101 through BP and LR recombination

reactions performed according to the manufacturer's instructions (Invitrogen). The sequences of the plasmids pMJ101-*OsASR1* and pMJ101-*OsASR3* were confirmed by direct sequence analysis. The primers used for the PCR reactions are listed in Supplementary Table 7. The plasmids were introduced into *Agrobacterium tumefaciens* LBA4404 by triparental mating, and embryogenic calli from mature rice seeds were transformed. The callus induction, co-cultivation with *A. tumefaciens* and selection of transformed calli were performed as described previously (Jang et al., 2002).

RESULTS

A multigene family encodes rice ASRs

To identify rice *ASR* genes, we performed BLAST searches against the National Center for Biotechnology Information database using Tomato ASR1 (Gilad et al., 1997) as a query. Using TBLASTN, six rice *ASR* genes were identified to have significant sequence identity to tomato ASR1. The *ASR* genes were named *Oryza sativa* ABA-, stress- and ripening-induced (*OsASR1-6*). There have been several reports describing rice *ASR* genes. However, the nomenclature of *ASR* genes is inconsistent. Therefore, we compared the previously reported rice *ASR* genes to *OsASR1-6* obtained in this study (Table 1). Multiple alignments of the deduced amino acid sequences of OsASRs with other ASR protein sequences showed extensive sequence identity (Supplementary Fig. 1). Two highly conserved regions were identified: namely, an N-terminal domain containing a stretch of histidine residues and a C-terminal domain including a putative nuclear localization signal. All rice *ASR* genes contained two ABA/WDS signatures that are commonly found in ASRs (Canel et al., 1995) and drought stress-induced proteins (Padmanabhan et al., 1997). The *OsASR1* and *OsASR3* genes contained a well-conserved N-terminal His stretch and a C-terminal PEHAHKHK motif, which might be involved in the zinc-dependent DNA-binding activity of ASRs (Rom et al., 2006). All rice *ASR* genes exhibited a common structure, comprising two exons and one intron (Supplementary Fig. 2).

Organ- and developmental stage-specific expression of the *OsASR* genes

An RNA gel blot analysis was conducted to examine the expression of the *OsASR* genes in various organs (Fig. 1A). Transcripts of *OsASR1* and *OsASR3* were detected in all tested organs except the callus, and *OsASR4* was expressed only in the green tissues, such as young and germinated leaves. Interestingly, *OsASR1* and *OsASR3* exhibited the opposite expression patterns in the leaves and roots. *OsASR1* was transcribed at higher levels in the leaves than in the roots, whereas *OsASR3* was transcribed at higher levels in the roots than in the leaves. The other ASR genes, namely, *OsASR2*, *OsASR5* and *OsASR6*, were not detected in the tested organs in our RNA gel blot analyses. To further analyze the expression of the *OsASR* genes, we performed qRT-PCR in the dissected organs, including mature leaves (ML), internodes (I), and mature roots (MR), and in panicles before (BH) and after pollination (AH) (Fig. 1B). The results of the qRT-PCR analysis for *OsASR1*, *OsASR3* and *OsASR4* were similar to those of the RNA gel blot analysis. Additionally, *OsASR1* transcripts were highly expressed in the internode. Although no transcripts of *OsASR2*, *OsASR5* and *OsASR6* were detected using RNA gel blot analysis, amplicons corresponding to these genes were detected in specific organs using qRT-PCR. *OsASR2* was transcribed at higher levels in the internodes, and *OsASR5* and 6 were de-

Fig. 1. Expression analysis of *OsASR* genes in various organs. (A) RNA gel blot analyses of *OsASR1*, *OsASR3* and *OsASR4*. Samples from the embryogenic callus (C), germinating leaves and roots (G), young roots (YR), young leaves (YL) and panicles after heading (AH) are shown. Gene-specific labeled probes were used for hybridization. Ethidium bromide (EtBr) staining was used to determine equal loading. (B) Quantitative real-time PCR analysis of the rice *ASR* genes. Samples from the mature leaves (ML), internodes (I), mature roots (MR), and panicles before (BH) and after heading (AH) are shown. The transcript levels were normalized to rice Actin levels and then compared with the expression in ML. The values represent the means $\pm$ SE of three biological replicates.

tected in the roots. These results suggested that all isoforms of the rice *ASR* gene are expressed in an organ-specific manner and that *OsASR1* and *OsASR3* encode the most abundant *ASR* isoforms in rice.

ASRs are involved in various stages of plant development including fruit ripening and pollen maturation (Chen et al., 2011; Iusem et al., 1993; Yang et al., 2008). To examine the roles of

the different *OsASR* genes in the reproductive organs of rice plants, we performed a qRT-PCR analysis in plants at various stages of panicle and seed development (Fig. 2). The samples were collected according to panicle length (cm) and days after pollination (DAP), as follows: panicle development stages (3-5 cm [P3], meiotic stage; 10-15 cm [P4], young microspore stage; 15-20 cm [P5] panicle length, vacuolated pollen stage); seed development stages (0-2 DAP [S1], early globular embryo; 3-4 DAP [S2], middle and late globular embryo; 5-10 DAP [S3], embryo morphogenesis; 11-20 DAP [S4], embryo maturation; 25-29 DAP [S5], dormancy and desiccation tolerance). These stage specifications were approximated based on information from Itoh et al. (2005) and Jain et al. (2007). The levels of the *OsASR1* transcript increased gradually according to the panicle size and reached a maximum level at the P5 stage of panicle development. Conversely, *OsASR1* expression decreased gradually during seed development and showed relatively lower levels at the S4-S5 stage. *OsASR1* and *OsASR3* showed similar expression patterns during these stages. It has been assumed that *OsASR1* and *OsASR3* play similar roles in reproductive organ development. However, *OsASR2* transcripts were highly expressed at the S1-S3 stage and rapidly declined to low expression at the S4-S5 stage during seed development. The level of the *OsASR5* transcript was lower before pollination than after pollination, was markedly increased at the S2 stage and was maintained at half the expression level of the S2 stage until the S3-S5 stage. Unlike other *OsASR* genes, *OsASR5* was consistently expressed at the S4-S5 stage of seed development. Thus, it has been proposed that *OsASR2* and *OsASR5* have additional functions in the early and late seed development stages, respectively. *OsASR4* was nearly undetectable in the panicle, and the expression of *OsASR6* was similar to that of *OsASR5* (Fig. 1B). Therefore, *OsASR4* and *OsASR6* were excluded from further analysis. Overall, these results indicated that the expression of the rice *ASR* genes was organ- and panicle/seed developmental stage-specific and that each isoform might play different roles in the different organ/developmental stages.

The response of *OsASR* transcript levels to abiotic stress treatments

To evaluate the relationships between the *OsASR* genes and abiotic stress in the rice plant, the expression levels of these genes in response to ABA, drought and high salt treatments were analyzed (Fig. 3). Under all conditions tested, *OsASR1*, *OsASR2* and *OsASR4* expression was detected predominantly in the leaves, but *OsASR3*, *OsASR5* and *OsASR6* expression was detected predominantly in the roots. *OsASR1* and *OsASR2* transcripts were induced in response to ABA, drought and high-salinity treatments in the leaves and significantly increased under drought stress in the roots. *OsASR4* transcripts were induced in response to ABA and drought treatments in the leaves as well as the roots, whereas they were repressed by high salinity in the leaves. *OsASR3*, *OsASR5* and *OsASR6* were highly induced in the roots and leaves in response to drought treatment. However, these genes showed a transient increase in expression in response to ABA treatment (0.5 h) and late induction under salt stress (6 or 12 h) in the roots. All six *OsASR* transcripts were most highly induced by drought treatment in both tissues. These data suggested that each rice *ASR* isoform might be subject to differential spatial and/or temporal regulation in response to various environmental conditions in vegetative tissues. Through a series of expression analyses, *OsASR1* and *OsASR3* were found to encode the most

Fig. 2. Expression analysis of *OsASR* genes at different reproductive stages. The relative expression levels of *OsASR1*, *OsASR2*, *OsASR3* and *OsASR5* were determined using quantitative real-time RT-PCR analysis with gene-specific primers. The young panicles and developmental seeds were divided into three (P3, 3-5 cm; P4, 10-15 cm; P5, 15-20 cm of panicle length) and five groups (S1, 0-2 DAP; S2, 3-4 DAP; S3, 5-10 DAP; S4, 11-20 DAP; S5, 25-29 DAP), respectively. The transcript levels were normalized to rice Actin transcription and then compared with the expression in P3. The values represent the means $\pm$ SE of three biological replicates.

Fig. 3. Analysis of *OsASR* gene expression under abiotic stress conditions. The relative expression of *OsASR* genes in the leaves and roots in response to 100 μ M ABA, 200 mM NaCl or drought stress treatments was determined using quantitative real-time RT-PCR. The transcript levels were normalized to rice Actin transcription and then compared with the expression levels at time zero in the leaves (for *OsASR1*, *OsASR2* and *OsASR4*) or roots (*OsASR3*, *OsASR5* and *OsASR6*). The values represent the means $\pm$ SE of three biological replicates.

Table 1. Comparison of the previously reported rice *ASR* genes with *OsASR1-6* obtained in this study

This study	Philippe et al. (2010)	Frankel et al. (2006)	Vaidyanathan et al. (1999)	Takasaki et al. (2008)	Kim et al. (2009)
<i>OsASR1</i>	Asr5	Asr5	OsAsr1	ASR5	OsAsr1
<i>OsASR2</i>	Asr4	Asr6			
<i>OsASR3</i>	Asr3	Asr1	OsAsr2		
<i>OsASR4</i>	Asr6	Asr2			
<i>OsASR5</i>	Asr1	Asr4			
<i>OsASR6</i>	Asr2	Asr3			

Table 2. GO categories shared among genes coexpressed with *OsASR1* and *OsASR3*

Name space	GO ID	Term	Over ^a or Under ^b		Common genes ^c
			<i>OsASR1</i>	<i>OsASR3</i>	
Biological process	GO:0050896	Response to stimulus	70	120	8
	GO:0006950	Response to stress	44	79	5
	GO:0009628	Response to abiotic stimulus	41	46	5
	GO:0019748	Secondary metabolic process	20	22	3
	GO:0009416	Response to light stimulus	20	18	2
	GO:0009698	Phenylpropanoid metabolic process	10	14	2
	GO:0006955	Immune response	9	14	2
	GO:0009415	Response to water	8	11	2
	GO:0009414	Response to water deprivation	8	11	2
	GO:0065007	Biological regulation	47	110	4
	GO:0050789	Regulation of biological process	43	104	3
	GO:0032502	Developmental process	28	73	5
	GO:0007275	Multicellular organismal development	28	65	5
	GO:0032501	Multicellular organismal process	29	65	5
	GO:0048513	Organ development	14	30	2
	GO:0048731	System development	14	30	2
Cellular component	GO:0044427	Chromosomal part	8	14	-
Molecular function	GO:0003824	Catalytic activity	176	272	25
	GO:0009882	Blue light photoreceptor activity	2	3	1
	GO:0003677	DNA binding	40	84	1
	GO:0030528	Transcription regulator activity	33	82	1
	GO:0003700	Transcription factor activity	27	73	1

^aNumber of genes coinduced with *OsASR1* or *OsASR3* (bold letters).

^bNumber of genes inversely regulated with *OsASR1* or *OsASR3* (italic letters).

^cNumber of genes commonly coregulated with *OsASR1* and *OsASR3*.

abundant ASR isoforms in rice and to be up-regulated in response to drought treatment. Interestingly, *OsASR1* and *OsASR3* exhibited different responses to ABA. To further analyze the roles of the rice ASR genes, we focused on the functions of *OsASR1* and *OsASR3* in this study.

Coexpression analysis of *OsASR1* and *OsASR3*

To further characterize the biological functions of the two major rice ASR isoforms, coexpression analyses based on microarray data were performed for *OsASR1* and *OsASR3* using the RiceArrayNet database (RAN; <http://www.ggbio.com/arraynet>)

(Lee et al., 2009). Using the criterion of correlation coefficient (r) ≥ 0.5 and ≤ -0.5 , we identified 796 and 2206 coexpressed genes for *OsASR1* and *OsASR3*, respectively (Supplementary Tables 1 and 2). These gene sets were subjected to analysis using the GoMiner program (<http://discover.nci.nih.gov/gominer/>) (Zeeberg et al., 2003) to identify Gene Ontology (GO) terms that were enriched in each set. Of the genes that were coexpressed with *OsASR1* and *OsASR3*, 606 and 1350 had TAIR8 counterparts, respectively (Supplementary Tables 3 and 4), and these sets of genes were enriched for 128 and 191 GO categories with a false discovery rate (FDR) cutoff of ≤ 0.05

Table 3. Distinct GO categories of genes coexpressed with *OsASR1* and *OsASR3*

Name space	Subcategory of related GO categories (X ^a /Y ^b)	
	<i>OsASR1</i>	<i>OsASR3</i>
Biological process	Photosynthesis (18/51)	Response to stimulus (23/49)
	Carbohydrate metabolism (11/51)	<i>DNA/RNA process (16/78)</i> <i>Reproductive development (15/78)</i> <i>Gene silencing (9/78)</i> <i>Chromatin/histone modification (8/78)</i>
Cellular component	Plastid/Chloroplast (19/43)	<i>Nucleus (5/12)</i>
	Photosystem (8/43)	<i>Proteasome complex (4/12)</i>
Molecular function	Oxidoreductase activity (3/8)	<i>Related to translation (4/15)</i> <i>DNA polymerization (4/15)</i>

^aNumber of GO categories belonging to this subcategory.

^bNumber of GO categories belonging to name space.

Bold letters indicate GO categories of genes that are coincuded with *OsASR1* or *OsASR3*.

Italic letters indicate GO categories of genes that are inversely regulated with *OsASR1* or *OsASR3*.

(Supplementary Tables 5 and 6). A comparison of the GO categories that were enriched among the genes that were co-expressed with the two rice *ASR* genes revealed that 22 GO categories were coregulated with both *OsASR1* and *OsASR3* (Table 2). The biological processes that were coincuded with *OsASR1* and *OsASR3* included response to abiotic stress and secondary metabolic processes. Additionally, the genes in the GO categories associated with organ development were inversely regulated with *OsASR1* and *OsASR3* expression. Genes that were annotated with the molecular function of “transcription activity” were negatively and positively correlated with *OsASR1* and *OsASR3* expression, respectively. Although many genes in the 22 shared GO categories were coexpressed with *OsASR1* or *OsASR3*, only a few genes were commonly co-regulated with both *OsASR* genes. These results suggest that *OsASR1* and *OsASR3* are both involved in the abiotic stress response but are alternatively regulated and are coexpressed with distinct sets of genes. Interestingly, different GO terms were enriched for the genes coexpressed with *OsASR1* and *OsASR3* (Table 3). *OsASR1* expression was predominantly coincuded with genes that are involved in photosynthesis and carbohydrate metabolism. *OsASR3* expression was inversely regulated with genes that are associated with several nuclear processes. The distinct coexpression patterns for these two *OsASR* genes suggest that *OsASR1* and *OsASR3* might function differently in various biological pathways.

Analysis of the *cis*-acting elements in the 5' regulatory regions of *OsASR1* and *OsASR3*

The characterization of the *cis*-acting region bound by transcription factors (TFs) that control gene expression often provides essential information about gene function. Therefore, the genomic sequences 1.0 kb upstream of the translational start sites of the *OsASR1* and *OsASR3* genes were analyzed for the presence of *cis*-acting elements that could control the expression of the *OsASR1* and *OsASR3* genes using promoter prediction software. Several functionally significant *cis*-acting elements associated with stress response, hormonal regulation, carbon metabolism and development were identified within the promoter regions of the *OsASR1* and *OsASR3* genes, and their predicted functions and frequencies are summarized in Table 4.

Drought-associated *cis*-acting elements, including dehydration-responsive element (DRE), Erd1, MYB and MYC; the temperature-associated *cis*-acting element low temperature response element (LTRE); and the pathogen or insect wound-associated *cis*-acting W-box element were present at varying frequencies in the regulatory regions of both genes. The wound-responsive element (WRE) and RAV1 protein recognition sequence (RAV1) motifs were uniquely detected in *OsASR1* and *OsASR3*, respectively. Interestingly, multiple abscisic acid-responsive element (ABRE) motifs were detected only in the *OsASR1* regulatory region. In contrast, gibberellic acid (GA)-associated *cis*-acting elements, such as gibberellin responsive element (GARE), CAACTC regulatory elements (CARE), the pyrimidine box and the TA/Amy box, were detected only in the *OsASR3* gene regulatory region. The auxin response factor (ARF) binding site and CGCG box were also detected only in *OsASR3*. In addition, the sucrosebox, which is associated with sugar responsive gene expression, was detected in the regulatory regions of both genes. carbohydrate metabolite signal responsive element 1 (CMSRE-1) and sucrose responsive element (SURE) were detected in the *OsASR1* regulatory region. The CGACG element, which is required for rice alpha-amylase Amy3D expression in response to sugar starvation, was detected in the *OsASR3* regulatory region. Most of the *cis*-acting elements involved in seed-specific and meristem expression, such as Skn-1, RY and the TGTCACA-motif, were found within the 5' regulatory region of the *OsASR3* gene; the ACGT motif, which is involved in seed-specific expression, was found upstream of the *OsASR1* gene. Moreover, sulfur responsive element (SuRE), light box element (l-box) and CGCG BOX were detected in the regulatory regions of both genes. GT elements were detected upstream of *OsASR3* only. The analysis of the 5' regulatory regions of *OsASR1* and *OsASR3* revealed that these genes contain common *cis*-acting elements involved in the stress response and different *cis*-acting elements involved in hormone regulation, the sugar response and tissue-specific expression. These results suggest that stress, sugar and hormones are the key regulators of *OsASR1* and *OsASR3* expression. In particular, ABA and GA/auxin might affect different regulatory pathways through *OsASR1* and *OsASR3*, respectively.

As shown in Fig. 3, *OsASR1* and *OsASR3* transcripts were

Table 4. Potential *cis*-acting regulatory elements in the promoters of *OsASR1* and *OsASR3* genes

Class	<i>Cis</i> -acting elements	Sequence	Copy number		Function	References
			<i>OsASR1</i>	<i>OsASR3</i>		
Stress	DRE	A/GCCGAC, RYCGAC, GTCGAC	3	4	Salt/drought responsive element	Dubouzet et al. (2003); Xue (2002)
	LTRECORE	CCGAC	2	2	C-repeat/dehydration responsive element	Kim et al. (2002)
	RAV1AAT	CAACA		4	RAV1 protein recognition sequence	Kagaya et al. (1999)
	Erd1	ACGT	4	2	Required for early response to dehydration	Simpson et al. (2003)
	WRE	AAWGTATCSA	1		Wound-responsive element	Palm et al. (1990)
	MYB	WAACCA, TAACTG, CNGTTR, YAACKG, GGATA, CAACTG	7	13	Involved in regulation of drought inducible gene expression	Abe et al. (2003); Shinozaki and Yamaguchi-Shinozaki (2000)
	MYC	CATGTG, CACATG	2	1	Involved in early response to drought and ABA induction	Abe et al. (2003); Shinozaki and Yamaguchi-Shinozaki (2000)
	W-box	TTGAC, TGAAT, TGACY, TGAC	3	5	Involved in activation of genes involved in response to wounding and defense	Eulgem et al. (2000); Maleck et al. (2000)
ABA	ABRE	(C/A)ACG(T/C)G(T/C/G)	5		Absciscic acid responsive element	Kaplan et al. (2006)
Auxin	ARF (AuRE)	TGTCTC		1	Involved in Auxin responsiveness	Ulmasov et al. (1999)
GA/ Sugar	Pyrimidine box	CCTTTT		1	Partially involved in sugar repression	Washio (2003)
	GARE	TAACAA(G/A), TATCCCA		2	Involved in GA responsiveness	Gubler and Jacobsen (1992); Ogawa et al. (2003)
	TA/Amy box	TATCCA, TATCCAY		2	Involved in tissue-specific sugar sensitivity of alpha-Amylase	Chen et al. (2006); Toyofuku et al. (1998)
	CAREs	CAACTC		1	GA-inducible expression of hydrolase genes	Sutoh and Yamauchi (2003)
	CGACG element	CGACG		1	Required for rice alpha-amylase Amy3D expression during sugar starvation	Hwang et al. (1998)
Sugar	Sucrose box	NNAATCA	6	6	Required for sugar responsive gene expression	Chen et al. (2002); Fillion et al. (1999)
	CMSRE-1	TGGACGG	1		Involved in the sucrose-inducible gene expression	Morikami et al. (2005)
	SURE	AATAGAAAA	1		Sucrose Responsive Element	Grierson et al. (1994)
	ACGT motif	GTACGTG	2		Minimal <i>cis</i> -element requirements for seed-specific expression	Blackwell et al. (1994); Wu et al. (2000)
	Skn-1_motif	GTCAT		2	Required for seed-specific expression	Blackwell et al. (1994)
Tissue	RY-motif	CATGCATG		2	Involved in seed-specific regulation	Ezcurra et al. (1999)
	TGTCACA motif	TGTCACA		1	Involved in fruit-specific expression	Yamagata et al. (2002)
Others	SuRE	GAGAC	1	2	Sulfur responsive element	Maruyama-Nakashita et al. (2005)
	I-box	GATAA	3	4	Light box element	Lopez-Ochoa et al. (2007)
	CGCG BOX	VCGCGB	1	8	Involved in ethylene signaling, absciscic acid signaling, and light signal perception	Yang and Poovaiah (2002)
	GT elements	GGTTAA		1	Involved in cell type-specific transcriptional control	Villain et al. (1996)

Fig. 4. Regulation of *OsASR1* and *OsASR3* expression through GA, sucrose, and glucose treatments. The relative expression levels of the *OsASR1* and *OsASR3* genes in the leaves and roots in response to 100 μ M GA (A), 100 mM sucrose (B) or 100 mM glucose (C) were determined using quantitative real-time RT-PCR. The transcript levels were normalized to rice Actin transcription and then compared with the expression at time zero in the leaves or roots. The values represent the means $\pm$ SE of three biological replicates.

Fig. 5. The overexpression of *OsASR1* and *OsASR3* in transgenic rice plants. (A) The overexpression plasmid contains a constitutive *OsCc1* promoter cloned upstream of the *OsASR1* or *OsASR3* coding region, the 3' region of the potato proteinase inhibitor II gene (3' *pinII*) and a Basta resistance gene expression cassette that contains the 35S promoter, the *bar* coding region and the 3' region of the nopaline synthase gene (3' *nos*). LB, left border; RB, right border. (B) RNA gel blot analysis of *OsASR1*- or *OsASR3*-overexpressing transgenic rice. Total RNA was isolated from the leaves of three homozygous T4 lines of *OsASR1*-OX, *OsASR3*-OX and NT plants. The full-length CDS for *OsASR1* and *OsASR3* and the 3' region of *pinII* (*pinII*-3' UTR) were used as probes for hybridization. EtBr staining was used to confirm equal RNA loading.

significantly up-regulated in both the leaves and roots after drought treatment. These results suggest that the drought-associated *cis*-acting elements DRE, Erd1, MYB and MYC in the regulatory regions of both genes sufficiently affected *OsASR1* and *OsASR3* expression. Moreover, ABA treatment markedly up-regulated the *OsASR1* transcription in the leaves compared with that in the roots. In contrast, *OsASR3* transcription was repressed and transiently increased in response to ABA in the leaves and roots, respectively. This result was consistent with the presence of multiple ABREs in the 5' regulatory region of the *OsASR1* gene but not the *OsASR3* gene. Interestingly, the induction of *OsASR1* transcription through the responses of these ABREs to ABA was more sensitive in the leaves than in the roots. To quantitatively verify the regulation of *OsASR1* and *OsASR3* gene expression by different *cis*-acting elements, we performed qRT-PCR under various conditions. In response to exogenous GA treatment (Fig. 4A), the expression of *OsASR3* was dramatically up-regulated compared with that of *OsASR1* in the roots but not in the leaves. This result was consistent with the fact that GA-associated *cis*-acting elements are present only in the *OsASR3* gene and the fact that *OsASR3* was expressed in response to GA in a root-specific manner. The tis-

sue-specific induction of *OsASR1* and *OsASR3* was also observed in response to sucrose/glucose treatments. The results of the sucrose feeding experiment showed that the *OsASR1* and *OsASR3* transcripts were notably up-regulated in the leaves and roots, respectively (Fig. 4B). *OsASR1* transcription was slowly induced in response to sucrose in the roots, and *OsASR3* transcription was less sensitive in the leaves than in the roots. The results of the glucose feeding experiment showed that *OsASR3* transcription was markedly up-regulated in a root-specific manner, whereas the expression of *OsASR1* was only slightly induced in both tissues (Fig. 4C). These results indicated that the expression of *OsASR1* was predominantly induced in the leaves in response to sucrose, whereas that of *OsASR3* was specifically induced in the roots in response to both sucrose and glucose. Thus, the expression of *OsASR1* and *OsASR3* is controlled via a tissue-dependent sugar and hormone-sensitive regulation mechanism. ABA and sucrose positively regulated the expression of *OsASR1* in the leaves, and GA and sucrose/glucose positively regulated the expression of *OsASR3* in the roots.

Fig. 6. Abiotic stress assays of *OsASR1*-OX and *OsASR3*-OX transgenic rice. (A) Drought stress tolerance of *OsASR1*-OX and *OsASR3*-OX transgenic plants. Three independent homozygous T4 lines of *OsASR1*-OX and *OsASR3*-OX and NT controls were subjected to 3 days of drought stress followed by 25 days of re-watering. Pictures were taken at 0 and 2 days after water draining (D0 and D2) and at 5, 11 and 25 days after re-watering (R5, R11 and R25). (B) Changes in the chlorophyll fluorescence (F_v/F_m) of *OsASR1*-OX and *OsASR3*-OX transgenic plants in response to drought, cold (4°C) or salt (400 mM NaCl) stress. Leaf discs from transgenic and NT plants were used for the experiments. The data represent the means $\pm$ SE ($n = 9$) of three independent experiments. Asterisks indicate statistically significant differences from NT, as calculated using Student's *t*-test. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$.

The ectopic expression of two major *OsASR* genes improved drought stress tolerance

To examine the roles of *OsASR1* and *OsASR3* in rice plants, we constructed rice transformation plasmids (Fig. 5A) in which the *OsASR1* and *OsASR3* coding sequences were expressed under the control of the constitutive rice *cytochrome c* promoter (*OsCc1*; Jang et al., 2002). Transgenic rice plants overexpressing *OsASR1* (*OsASR1*-OX) and *OsASR3* (*OsASR3*-OX) were obtained via the *Agrobacterium*-mediated transformation method. The ectopic expression of the transgenes in *OsASR1*-OX and *OsASR3*-OX plants was confirmed using RNA gel blot analysis (Fig. 5B). The transcript levels of *OsASR1* and *OsASR3* were enhanced in individual transgenic lines relative to the NT control. Endogenous *OsASR1* expression was also detected in NT. To distinguish between the endogenous and exogenous *OsASR1*, a probe recognizing the *PinII* 3' UTR region was used. *OsASR1* transcripts containing *PinII* 3' UTR were clearly detected in all of the transgenic lines, except for NT, and were highly expressed in lines 4 and 5 compared with line 6. T₁ to T₄ seeds were collected from individual transgenic plants, and three independent homozygous T₄ lines for each construct were subjected to further analysis.

To evaluate the responses of the *OsASR1*-OX and *OsASR3*-OX plants to water deficit, 4-week-old NT and T₄ transgenic seedlings were subjected to drought stress for 3 days, followed by re-watering (Fig. 6A). During re-watering, most of the trans-

genic plants showed better recovery from drought stress and more stimulated growth than the severely injured NT plants. In *OsASR1*-OX plants, the ability to recover from drought stress was highest in line 4 and lowest in line 6, consistent with the levels of *OsASR1* expression in these plants (Fig. 5B). The drought-stressed plants exhibited visual symptoms, such as leaf wilting and rolling, with a concomitant loss of chlorophyll. To further verify the stress tolerance of *OsASR1*-OX and *OsASR3*-OX plants, we measured the variations in the chlorophyll fluorescence ratio (F_v/F_m) after abiotic stress treatments. F_v/F_m is a parameter that is widely used to indicate the maximum fluorescence after dark adaptation, representing the maximum quantum yield of PSII. The chlorophyll fluorescence ratio is presented as the ratio of the variable fluorescence (F_v) to the maximum fluorescence value (F_m). Healthy plants typically achieve a maximum F_v/F_m value of approximately 0.85, and lower values are observed in plants that are exposed to biotic or abiotic stress factors. For the stress treatments, leaf discs from 3-week-old transgenic and NT seedlings were exposed to drought, cold (4°C) and salt stress (400 mM NaCl), and the associated reductions in the F_v/F_m values were measured (Fig. 6B). The values of F_v/F_m were approximately 50% and 25% higher in the *OsASR1*-OX and *OsASR3*-OX plants, respectively, than in the NT plants under the drought stress condition. Under cold stress conditions, both transgenic plants showed significantly higher values for F_v/F_m than did the NT plants. However,

OsASR1-OX and *OsASR3*-OX plants showed no difference from the NT plants in response to salt stress. The results of the stress experiments confirmed that the overexpression of *OsASR1* and *OsASR3* in transgenic rice increases the tolerance to drought stress during the vegetative stage.

DISCUSSION

Rice *ASR* genes are a small gene family with a simple structure. There are six *ASR* genes in rice; however, their expression patterns have not yet been analyzed. In the present study, all six identified rice *ASR* genes were isolated and characterized. The two most abundant isoforms, *OsASR1* and *OsASR3*, were selected for further analysis to characterize their expression profiles and regulatory mechanisms. The gene expression analyses performed here revealed the differential expression of *ASR* isoforms associated with distinct plant structural regions and environmental conditions (Fig. 1). The *OsASR* genes are expressed in both the vegetative and reproductive organs (Fig. 2). In the vegetative organs, *OsASR1* and 4 were predominantly expressed in the leaves, while *OsASR3* was expressed mainly in the roots. *OsASR1* and *OsASR3* were also predominantly expressed in the panicles. The expression of *OsASR1* and *OsASR3* was similar during panicle development, increasing gradually with panicle size and rapidly decreasing after the S3 stage. Moreover, similar to other plant *ASRs*, *OsASR1* and *OsASR3* were highly up-regulated in response to drought (Kalifa et al., 2004b; Shkolnik and Bar-Zvi, 2008; Yang et al., 2005). Rice is most susceptible to drought stress at the reproductive stage (Pantuwan et al., 2002). A dramatic reduction in grain yield occurs when drought stress coincides with irreversible reproductive processes (Pantuwan et al., 2002; Price and Courtois, 1999). The lily *ASR* exhibits a marked increase in LLA23 translocation from the cytoplasm to both nuclei of pollen grains in 12-cm buds prior to the commencement of desiccation during anther development (Yang et al., 2008). The expression patterns and functions of the *OsASR* genes suggested that *OsASR1* and *OsASR3* might play roles in drought response during pollen maturation and/or panicle development. However, the expression of *OsASR5* was relatively low before pollination but markedly increased after pollination, and it remained high until the late stage of seed development. In association with an increase in endogenous ABA content, *FaASR* transcripts were markedly induced at the ripening stage, remaining high at the late ripening stages (Chen et al., 2011). *OsASR5* is also expressed in response to ABA, and its expression pattern during seed development is similar to that of *FaASR*. These results suggest that *OsASR5* might be involved in rice seed maturation/desiccation.

ASR genes have been implicated in plant responses to environmental signals, and they are typically up-regulated in response to ABA and abiotic stress (Cakir et al., 2003; Jeanneau et al., 2002; Yang et al., 2005). Recently, it has been reviewed that *ASR* can be used to improve crops and economically important plants against various environmental stresses (Wang et al., 2013). Increases in *OsASR1* transcript levels were also observed in response to ABA and abiotic stress treatments (Fig. 3, *OsASR1*). These results are consistent with the up-regulation of rice *Asr1* (*OsASR1* in this study) in response to osmotic stress and the exogenous application of ABA (Vaidyanathan et al., 1999). Unlike *OsASR1* and other plant *ASRs*, which are up-regulated in response to ABA, the expression of *OsASR3* transcripts only transiently increased or decreased in response to the ABA in the roots and leaves, respectively (Fig. 3, *OsASR3*).

It was recently shown that the expression of *ZmASR5* transcripts was down-regulated in response to ABA treatment. A phylogenetic tree analysis revealed that *ZmASR5* and *OsASR3* belong to the same subclade II-1 (Virlouvet et al., 2011). These results indicated that *OsASR3* and *ZmASR5* expression in response to ABA might be similarly regulated in monocot plants. Our data showed that the expression of *ASRs* could be regulated differently in different tissues and in response to different abiotic stresses, suggesting that members of the rice *ASR* gene family might perform multiple functions, depending on the vegetative and developmental stage, under different environmental conditions. Recently, it has been reported that the overexpression of *ASR1* in maize showed a decrease in branched-chain amino acid biosynthesis and changes in BCAA-related gene expression (Virlouvet et al., 2011). The physiological roles and the developmental and environmental dependence of *ASR* gene expression should be studied further.

The RAN analysis showed that *OsASR1* and *OsASR3* were coexpressed with non-overlapping gene sets with similar process annotation profiles (Table 2) and with different genes in distinct processes (Table 3). The different coexpression patterns of these two genes indicate that their expression is regulated through distinct pathways. To obtain essential information relevant to the regulation of these genes, we analyzed the regions upstream of the *OsASR1* and *OsASR3* genes using Plant CARE and PLACE (Table 4). We uncovered potentially important *cis*-acting elements that are associated with the stress response, hormonal regulation, carbon metabolism and development, specifically DRE, the binding site for AP2/ERF TFs (Dubouzet et al., 2003; Xue, 2002), implying that TFs in this family might participate in the transcriptional regulation of *OsASR1* and *OsASR3*. DRE binding proteins (DREBs) are important plant TFs that regulate the expression of many stress-inducible genes in a primarily ABA-independent manner (Lata and Prasad, 2011). Interestingly, the interaction of grape *ASR* proteins with a DREB was demonstrated using yeast two-hybrid screening and the BiFC approach (Saumonneau et al., 2008). DREBs might function as *trans*-acting elements for the mRNA expression of *ASR* proteins and other proteins involved in the formation of the hetero-protein complex. Additional drought-associated *cis*-acting elements, such as *Erd1*, MYB and MYC, are present in the regulatory regions of both genes. The MYB and MYC TFs, which serve as activators in one of the ABA-dependent regulatory systems in response to drought stress, recognize MYB and MYC sites (Abe et al., 2003). The multiple drought-associated *cis*-acting elements and corresponding binding proteins for the *OsASR1* and *OsASR3* regulatory system are consistent with the finding that *OsASR1* and *OsASR3* transcripts were greatly up-regulated after drought treatment (Fig. 3).

The expression levels of the two most abundant isoforms, *OsASR1* and *OsASR3*, were primarily enriched in the leaves and roots, respectively. The application of different hormones differentially regulated the expression of these genes in their target tissues. *OsASR1* was up-regulated by ABA treatment in the leaves, whereas *OsASR3* was up-regulated by GA treatment in the roots (Figs. 3 and 4A). ABRE is a *cis*-acting element that regulates dehydration-responsive gene expression in *Arabidopsis* and rice (Kang et al., 2002; Uno et al., 2000). ABA-responsive gene expression requires multiple ABREs or an ABRE with a coupling element as a functional promoter (Shen et al., 1996). Multiple ABRE motifs were detected in the regulatory regions of *OsASR1* only. A group of drought and salinity-induced *trans*-acting factors belonging to the bZIP class of pro-

teins interact with ABREs to mediate the ABA-dependent induction of stress response genes (Kang et al., 2002; Uno et al., 2000). Thus, the rice bZIP proteins might participate in the transcriptional regulation of *OsASR1* via ABA-dependent pathways.

GA is an important phytohormone that controls many aspects of plant growth and development. The GARE sequence plays a fundamental role as a mediator of GA activity because changes in this sequence cause a major reduction in GA-driven gene expression (Gubler and Jacobsen, 1992). The GA response complex (GARC) includes the O2S/W box, the pyrimidine box, the GARE sequence and the TA/Amy box (Gubler et al., 1999; Lanahan et al., 1992; Sun and Gubler, 2004; Zhang et al., 2004). The 5' regulatory region of *OsASR3* contained a complete GARC. Additionally, CAREs, which are involved in the GA responsiveness and GAMyb transactivation of a cysteine proteinase (*REP-1*), were detected in the regulatory region of that gene (Sutoh and Yamauchi, 2003), showing that two pairs of GAREs and CAREs were necessary and sufficient to confer the GA inducibility of the *REP-1* gene. GARE and CARE sequences were also detected within the promoters of the rice α -amylase gene *RAmy1A* and the barley proteinase gene *EPB1*, which are both expressed in germinating seeds. Mutations in CARE result in a loss of GA inducibility and GAMyb transactivation, suggesting that CARE is the regulatory element responsible for the GA-inducible expression of hydrolase genes in germinating seeds. The presence of GARE and CARE motifs in the 5' regulatory region of the *OsASR3* gene might be evidence of an association between *OsASR3* and the carbon/energy demand of the cell during the induction of plant growth and development by gibberellins.

Plantain *Asr* over-expressed transgenic *Arabidopsis* showed increased soluble sugars (Dai et al., 2011). *Asr1* silenced *Nicotiana tabacum* plants showed higher levels of leaf glucose, but reduction of transcript levels of *Ht1* ortholog, on the other hand, over-expressed lines showed no variation in sugar contents in leaves (Dominguez et al., 2013). The *in silico* analysis of the *OsASR1* and *OsASR3* gene promoters revealed sucrose boxes, which are associated with sugar-responsive gene expression, in both regulatory regions. In addition, CMSRE-1 (carbohydrate metabolite signal responsive element 1) and SURE (sucrose responsive element) were detected in *OsASR1* only, whereas the CGACG element, which is required for rice α -amylase *Amy3D* expression during sugar starvation, was found in *OsASR3* only. We analyzed the relationship between sugar exposure and the expression of the two *OsASR* genes, and our data showed that their expression in their target tissues was controlled through different sugar signals (Fig. 4). The expression of *OsASR1* transcripts in the leaves was significantly enhanced after sucrose treatment, whereas the expression in the leaves and roots was only slightly increased after glucose treatment. This result suggests that sucrose itself, but not the readily produced hexoses, might be an actual inducer of *OsASR1* expression. *OsASR3* transcripts were strongly up-regulated in the roots after treatment with both sucrose and glucose. Taken together, these results suggest that the expression of *OsASR1* and *OsASR3* was differentially regulated by stress, hormones and sugar via tissue-dependent factors. The relationship to tissue-specific regulation and the functional roles of the two *OsASR* genes requires further study.

Abiotic stresses, such as drought, salinity and extreme temperatures, are among the key factors that determine crop yield and quality. Abiotic stresses cause an average yield loss of >50% in most major crop plants (Boyer, 1982). Therefore, it is

important to understand the abiotic stress response to improve the yield and quality of crops. Rice is a notoriously drought-susceptible crop due in part to its small root system, rapid stomatal closure and reduced cuticular wax production during mild water stress (Hirasawa, 1999). Transgenic rice plants that constitutively overexpress *OsASR1* and *OsASR3* show improved drought stress tolerance (Fig. 6A). Differences in the optimal quantum yields of the transgenic plants further support the increased drought and cold tolerance phenotypes (Fig. 6B). However, there was no difference between NT and transgenic rice plants in the response to salt stress. Although many *ASR* genes were reported to involve in various abiotic and biotic stresses (Dai et al., 2011; Hsu et al., 2011; Jeanneau et al., 2002; Kalifa et al., 2004b; Liu et al., 2010; Shkolnik and Bar-Zvi, 2008; Virlouvet et al., 2011; Yang et al., 2005), the exact molecular mechanism remains unclear. The stress tolerance conferred by the overexpression of rice ASRs might confirm the following assumptions about their activities based on common ASR properties: (1) an enhanced water-retaining ability (Yang et al., 2005), (2) chaperone-like activity (Konrad and Bar-Zvi, 2008), (3) transcription factor activity (Cakir et al., 2003; Frankel et al., 2007; Kalifa et al., 2004a; Saumonneau et al., 2008) and (4) effective ROS scavenging activity (Hu et al., 2013; Kim et al., 2012). Our data showed that *OsASR1* and *OsASR3* are expressed and regulated differentially but have common functions in abiotic stress tolerance.

Plants encounter a wide range of environmental insults, hormonal changes and metabolic demands during cellular growth and different developmental stages during a typical life cycle. This study demonstrated that the six rice *ASR* genes are differentially expressed in various tissues during different developmental stages; in particular, the expression of *OsASR1* and *OsASR3* is differently regulated by stress, hormone and sugar signals in target tissues. The rice *ASR* genes might influence a broad range of plant systems, including stress, hormone and sugar status, at various growth and developmental stages during the plant life cycle.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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