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SHORT COMMUNICATION

Rice ASR1 has Function in Abiotic Stress Tolerance during Early Growth Stages of Rice

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Abstract *OsASR1* expression was induced through Abscisic acid (ABA) and stress treatments in leaves. The constitutive overexpression of *OsASR1* in rice reduced ABA sensitivity, and increased high salinity and osmotic stress tolerance in early growth stages. These results indicated that *OsASR1* has function in abiotic stress tolerance during early growth stages of rice.

Keywords abiotic stress · ASR1 · rice

Abiotic stresses lead to an average yield loss of >50% for 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 during mild water stress (Hirasawa, 1999). Abscisic acid (ABA) is a key regulator of the abiotic stress response (Verslues and Zhu, 2005). Numerous genes are regulated by ABA during the abiotic stress response. One of them, the *ASR* (abscisic acid-, stress- and ripening- induced) gene, was first described in tomato (Iusem et al., 1993) and was cloned from a salt-stressed rice cDNA library (Vaidyanathan et al., 1999). *ASR* is induced by ripening, drought stress, salt stress, and ABA (Carrari et al., 2004). The promoter of *ASR* has been shown to be responsive to ABA (Rossi et al., 1998; Rom et al., 2006). ASR proteins are plant-specific proteins. They are considered to act as a transcription factors, because they have zinc-dependent DNA-binding activity (Carrari et al., 2004). Tomato ASR1 protein expressed in *Arabidopsis* showed DNA binding competition with ABI4 (Shkolnik and Bar-Zvi, 2008). The N-terminal conserved region, thought to be associated with a Zn-binding motif (Kalifa et al., 2004a), and the C-terminal conserved domain, including a putative nuclear localization signal (Cakir et al., 2003), are well-conserved in OsASR1. Additionally, C-terminal PEHAHKHK residues, which may be involved in the zinc-dependent DNA-binding activity of ASR (Rom et al., 2006) are also well-conserved in OsASR1.

ASR genes have been implicated in plant responses to environmental signals, and they are typically up-regulated in response to ABA and abiotic stress (Jeanneau et al., 2002; Cakir et al., 2003; Yang et al., 2005). To evaluate the relationship between *OsASR1* gene and abiotic stress in the rice plant, the expression of *OsASR1* gene was analyzed under ABA, drought, and high salinity treatments (Fig. 1). Distinct transient increases in *OsASR1* transcript levels were observed in response to the ABA, high salinity, and drought treatments. These results coincide with those of other plant *ASR* genes report that rice *Asr1* (*OsASR1* in this study) levels in shoots are up-regulated by the exogenous application of ABA and osmotic stress imposed by mannitol and salt (Vaidyanathan et al., 1999; Kawasaki et al., 2001).

To examine the role of *OsASR1* in rice plants, we constructed plasmids for rice transformation in which the *OsASR1* coding sequence was expressed under the control of the constitutive rice *cytochrome c* promoter (Jang et al., 2002). A full-length clone of *OsASR1* was obtained from GreenGene Biotech, Korea. Transgenic rice plants overexpressing *OsASR1* (*OsCc1:OsASR1*) was obtained via the *Agrobacterium*-mediated transformation method. Three independent lines for *OsASR1* overexpressing transgenic rice were obtained (Fig. 1B). T_1 to T_4 seeds were collected from individual transgenic plants, and three independent homozygous T_4 lines for

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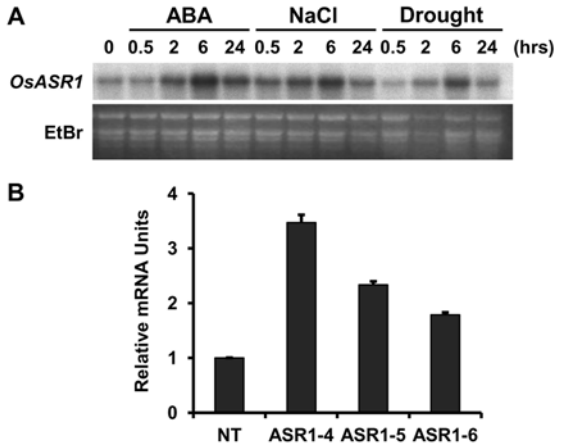

Fig. 1 Expression patterns of *OsASR1* genes under stress conditions. (A) 14-day-old plants grown on soil were hydroponically adapted in water for 3 days and then transferred to fresh water containing 100 μM ABA or 200 mM NaCl for 24 h in the greenhouse. Drought-stressed plants were removed from water and then air-dried in the same time course (Drought). Total RNA prepared from each condition at the indicated time point. Ethidium bromide (EtBr) staining was used to determine equal loading of total RNA. (B) The quantitative real-time RT-PCR analysis of *OsASR1* overexpressing transgenic rice using the primer ASR1-F (5'-CCATGGCGGAGGAGAAGCAC) and ASR1-R (5'-GTTGTGATGAGG TCGATCAG). *OsUbi1* was used as a reference gene, and ratios were normalized against the control. Data presented are means ± SE (n =3).

each construct were used for further analysis. To evaluate the response of *OsCc1:OsASR1* plants to abiotic stress during germination stage, seeds of nontransgenic (NT) and three independent homozygous *OsCc1:OsASR1* plants were germinated in a one-half strength Murashige and Skoog (MS) solid medium containing various concentrations of ABA, NaCl or mannitol (Fig. 2A). Under high salinity condition, the germination rates of both NT and *OsCc1:OsASR1* seeds decreased. In other conditions, they germinated within 2 days. There were no significant differences of the germination rates between NT and *OsCc1:OsASR1* seeds under all tested conditions by Student´s *t* test. The result of the germination experiments suggested that OsASR1 does not play a role in germination in rice.

To further examine the response of *OsCc1:OsASR1* plants to abiotic stress during early growth stage, two independent homozygous T_4 lines of *OsCc1*:*OsASR1* transgenic and NT seeds were germinated on MS solid medium for 2 days. After germination, germinants of equal size were selected and then transferred to MS solid medium containing various concentrations of ABA, NaCl or mannitol. The selected germinated seedlings were incubated with 16 h light/8 h dark cycles at 28°C in a growth chamber. The shoot and root growths of NT seedlings were more severely inhibited by ABA and stresses than *OsCc1:OsASR1* seedlings (Fig. 2B). The results of the stress experiments suggested that the overexpression of *OsASR1* in transgenic rice increases tolerance to abiotic stress during the early growth stage. It has been reported that the overexpression of ASRs increases abiotic stress tolerance (Kalifa et al., 2004b; Yang et al., 2005; Shkolnik and Bar-Zvi, 2008; Kim et al., 2009;). Like other ASRs, rice transgenic lines constitutively overexpressed *OsASR1* under control of the *OsCc1* promoter, thereby improving abiotic stress tolerance (Fig. 2B). Lily *ASR*-overexpressed *Arabidopsis* seeds are able to germinate under unfavorable conditions (Yang et al., 2005), whereas rice *ASR*-overexpressed seeds showed similar germination rates compared to NT seeds under comparative stress conditions (Fig. 2A). These results indicated that rice ASR1 does not function during the seed germination stage.

To identify genes that are up- or down-regulated by *OsASR1* overexpression, global expression profiling was performed on the *OsCc1*:*OsASR1* plants in comparison with the NT plants grown under normal growth conditions. Profiling was conducted, using the Rice 3'-Tiling Microarray (GreenGene Biotech, Korea) on total RNA samples from leaf tissues of 2-week-old transgenic and NT plants. Microarray data sets were obtained from two biological replicates. Statistical analyses of each data set, using one-way ANOVA, identified 49 and 25 genes that were up- or down-regulated by *OsASR1* overexpression at levels of two-fold or greater in the transgenic plants compared to the NT plants ($p < 0.05$). Up-regulated genes included 7 genes encoding members of a disease resistance protein family, 5 genes encoding leucine-rich repeat-containing proteins, 4 genes encoding protein kinase domain-containing proteins and 2 genes coding for cyclin-like F-box domain-containing proteins. Down-regulated genes included cytochrome P450 family protein, Rp1-like protein, Piwi domain containing protein, and NB-ARC domain containing protein, rust resistance-like protein RP1 and 15 hypothetical proteins. Based on the microarray data, 11 genes (8 up-regulated and 3 down-regulated genes) were selected, and their expression patterns under normal conditions were verified by reverse transcription polymerase chain reaction (RT-PCR) (Fig. 3). To assess whether their expression levels changed under stress conditions, the transcript levels of these genes were also measured under drought and high-salinity conditions. Only AK100303 (disease resistance protein family protein) was up-regulated and AK065971 (cytochrome P450 family protein) was down-regulated by stress treatments in the NT plants. These two genes were similarly regulated by *OsASR1* overexpression and abiotic stresses. Unlike other stress-related genes, whose overexpression in transgenic rice show increased stress tolerance, reported rice stress-responsive genes such as *Dip1*, *SalT*, and *LEA3* are not up-regulated in *OsCc1:OsASR1* transgenic plants. Nine of the twenty-nine up-regulated genes belong to the leucine-rich repeat-containing protein family (5 genes) and protein kinase family (4 genes). Therefore, the stress tolerance brought about by OsASR1 is not directly related to known stress induced genes expression.

ASR proteins are charged, low-molecular weight, plant-specific proteins whose expressions are induced by abiotic stress and are shown to possess zinc-dependent DNA-binding activity, suggesting they may act as a transcription factors (Carrari et al., 2004). Tomato ASR1 competed with ABI4 for DNA binding in

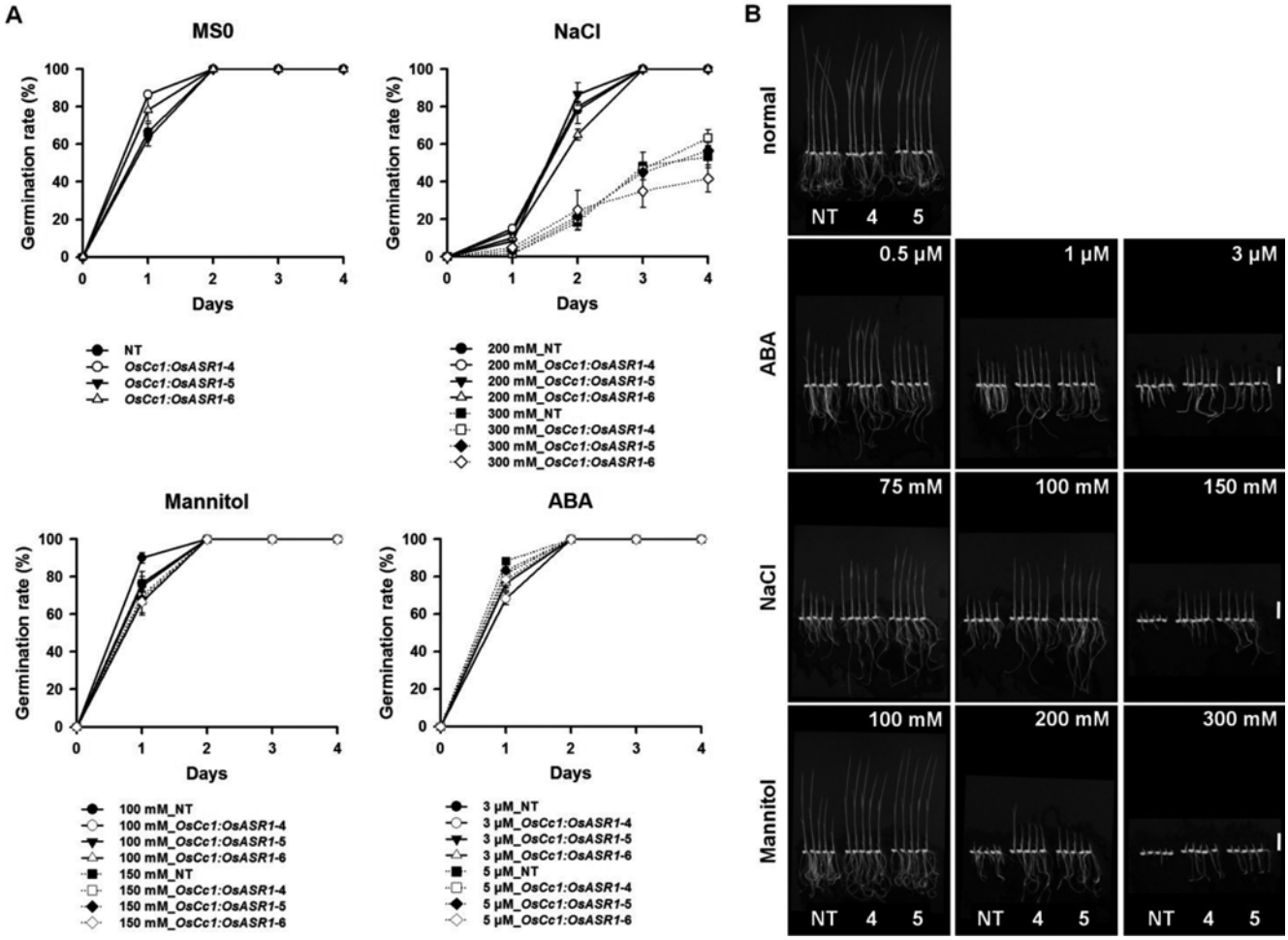

Fig. 2 Effect of stress treatment during germination and early growth of *OsCc1*:*OsASR1* transgenic rice. (A) Germination rate of *OsCc1*:*OsASR1* transgenic seeds under stress conditions. Thirty seeds of three independent homozygous T_4 lines of *OsCc1*:*OsASR1* transgenic and NT were germinated on MS solid medium as a control experiment. For stress conditions, seeds were germinated on MS solid medium, containing NaCl (200, 300 mM), mannitol (100, 150 mM) or ABA (3, 5 μM). Germination was scored every day for 4 days. Data are presented are mean ± SE (n = 13) of two independent experiments. (B) Early growth of *OsCc1*:*OsASR1* transgenic seedlings under stress conditions. Ten seedlings of two independent homozygous T_4 lines of *OsCc1*:*OsASR1* transgenic and NT seeds were germinated on MS solid medium for 2 days. After germination, germinants of equal size were selected and then transferred to MS solid medium containing ABA (0.5, 1, 3 μM), NaCl (75, 100, 150 mM) or mannitol (100, 200, 300 mM). Photographs were taken 5 days after transfer to stress medium. Bar indicates 2 cm. Two independent experiments showed similar results. One experiment was represented.

Arabidopsis (Shkolnik and Bar-Zvi, 2008). Furthermore, rice ASR5 (OsASR1 in this study) was found in the nuclear fraction and cytosolic fraction (Takasaki et al., 2008). Therefore, OsASR1 could act as a transcription factor to directly or indirectly regulate stress response. From accumulated data, we postulate that the function of rice OsASR1 is related to the abiotic stress response. Recently, it has been reported that plantain *Asr* over-expressed transgenic *Arabidopsis* showed increased soluble sugars (Dai et al., 2011). Strawberry FaASR may be involved in fruit ripening (Chen et al., 2011). *ASR1* overexpressed maize exhibited alteration of the branched-chain amino acid biosynthesis (Virlouvet et al., 2011).

In conclusion, *OsASR1* expression was induced through ABA and stress treatments in leaves. The constitutive overexpression of *OsASR1* in rice reduced ABA sensitivity, and increased high salinity and osmotic stress tolerance in early growth stages, whereas overexpression of *OsASR1* has no effect on the germination rate by ABA and stress treatments.

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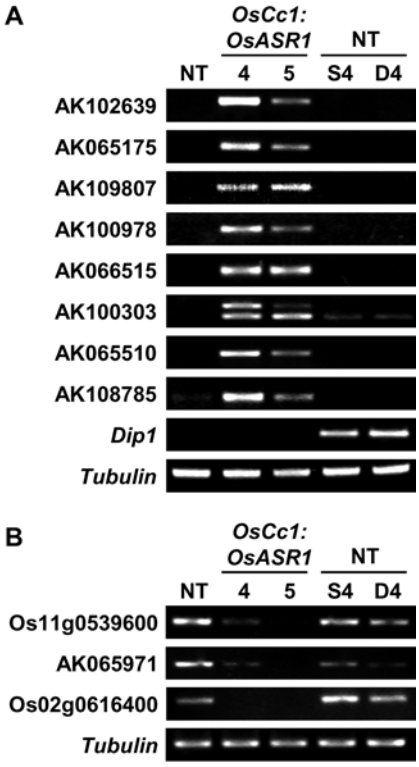

Fig. 3 RT-PCR for up- or down-regulated genes in *OsCc1*:*OsASR1* transgenic rice plants. (A) Up-regulated genes in *OsCc1*:*OsASR1* transgenic rice plants. (B) Down-regulated genes in *OsCc1*:*OsASR1* transgenic rice plants. Total RNA extracted from 14-day-old seedlings of NT and *OsCc1*:*OsASR1* transgenic rice plants. Transcript levels of up- or down-regulated genes in *OsCc1*:*OsASR1* transgenic rice was determined by RT-PCR. For stress treatment, NT plants were subjected in 300 mM NaCl solution for 4 h (S4) or air-dried for 4 h (D4) in a growth chamber. *Dip1* was used as a marker for the up-regulation of key genes following stress treatments. The rice *tubulin* gene was used as an equal loading control.

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