J. Plant Biol. (2016) 59:62-73
DOI 10.1007/s12374-016-0180-7

ORIGINAL ARTICLE

Over-expression of a DUF1644 Protein Gene, *SIDP361*, Enhances Tolerance to Salt Stress in Transgenic Rice

Min Li[1,†], Lijia Guo[2,†], Chiming Guo[1], Liangjiang Wang[3] and Liang Chen[1,*]

[1]*Xiamen Key Laboratory for Plant Genetics, School of Life Sciences, Xiamen University, Xiamen 361005, China*
[2]*Key Laboratory of Integrated Pest Management on Tropical Crops, Ministry of Agriculture, Environment and Plant Protection Institute, Chinese Academy of Tropical Agricultural Sciences, Danzhou, Hainan 571737, China*
[3]*Department of Genetics and Biochemistry, Clemson University, Clemson, SC 29634, USA*

Received: April 13, 2015 / Accepted: November 16, 2015

Abstract Salt stress has adverse effects on the growth and production of rice crops. In this study, we isolated and characterized *SIDP361*, which encodes a DUF1644 family protein. This gene was expressed in various rice tissues and was induced by high salt (200 mM NaCl), dehydration, and abscisic acid (100 μM ABA) treatments. Stable expression of SIDP361-GFP in rice cells suggested that SIDP361 is a cytoplasmic protein. When compared with the untransformed wild-type (WT) control, transgenic plants over-expressing *SIDP361* exhibited significantly improved tolerance to salt stress at both the seedling and heading stages. Under salinity conditions, the transgenics also had elevated amounts of free proline. Moreover, transcript levels for genes encoding proline synthetase enzymes were significantly higher in transformants than in the WT. The transgenic lines were also hypersensitive to exogenous ABA. Quantitative real-time PCR analysis showed that transcription of several stress-related genes was greater in *SIDP361*-overexpressing plants than in the WT under both normal and salt-stressed conditions. These results demonstrate that *SIDP361* has high potential as a tool for genetically improving salt tolerance in rice.

Key words: DUF1644, Salt tolerance, *SIDP361*, Signal transduction

Introduction

Plants encounter numerous biotic and abiotic stresses throughout their life cycles. Adverse environmental conditions, e.g., drought, high salinity, and extreme temperatures (hot or cold), influence the growth, development, and productivity of crop plants in cultivated areas (Ingram and Bartels 1996; Bray 1997; Zhu 2002; Shinozaki et al. 2003). Salt stress is one of the most important detrimental factors affecting global yields. To survive under these environmental conditions, plants utilize sophisticated signaling mechanisms to induce physiological and biochemical changes at the cellular and molecular levels (Cushman and Bohnert 2000; Hasegawa et al. 2000; Zhang et al. 2000; Gao et al. 2008). Environmental stresses can also induce the expression of a variety of plant genes (Ingram and Bartels 1996; Shinozaki and Yamaguchi-Shinozaki 2000). The ability to express such genes in a timely manner is crucial to the survival of stressed plants (Bray 1997; Zhu 2002; Chen and Zhu 2004; Yamaguchi-Shinozaki and Shinozaki 2006; Chinnusamy et al. 2007; Shinozaki and Yamaguchi-Shinozaki 2007). Many stress-related genes have been cloned and characterized. Their products either directly protect cells against abiotic stresses or further control the expression of other target genes to enhance stress tolerance (Martinez-Atienza et al. 2007; Xiao et al. 2007).

For the majority of the world's population, rice (*Oryza sativa*) is one of the most important crops, especially in Asia, and it is also a model plant for the study of monocot species (Khush 1997; Tyagi et al. 1999; Tyagi and Mohanty 2000; Cantrell and Reeves 2002). Soil salinity causes a dramatic reduction in agricultural production (Boyer 1982; Zhu 2001), affecting approximately 30% of the acreage used for growing rice worldwide (Prasad et al. 2000). Complicated tolerance mechanisms are employed to cope with ion toxicity and salinity-related osmotic stress (Zhu 2002). Although genetic manipulation of stress-related genes can enhance salt tolerance

[†]These authors contributed equally to the paper.

*Corresponding author; Liang Chen
Tel : +86-3666087304
E-mail : chenlg@xmu.edu.cn

(Thomashow 1998; Xiong et al. 2002), the molecular basis for the salinity response and tolerance by rice plants is still not fully understood. Thus, a primary step toward improving stress tolerance via genetic engineering must involve an analysis of the functioning of stress-inducible genes.

High salinity can cause increased biosynthesis and accumulations of abscisic acid, or ABA (Xiong et al. 2002). This phytohormone has a role in various aspects of plant growth and development. Because ABA mediates adaptive responses to various environmental stresses, especially drought and high salt (Ingram and Bartels 1996; Shinozaki and Yamaguchi-Shinozaki 2000; Zhu 2002), it is considered an important "stress hormone" (Nambara and Marion-Poll 2005). The pathways involved in these adaptations are categorized as either ABA-dependent or -independent (Shinozaki and Yamaguchi-Shinozaki 2000; Zhu 2001; Finkelstein et al. 2002; Xiong et al. 2002; Himmelbach et al. 2003). Expression of ABA-inducible genes often relies on the existence of *cis*-acting elements in the promoter region, such as ABRE, MYBRS, or MYCRS (Shinozaki and Yamaguchi-Shinozaki 2000; Finkelstein et al. 2002).

Domains of unknown function (DUF) are a large set of uncharacterized protein families within the Pfam database, which currently contains approximately 3000 DUF families (Bateman et al. 2002). Such families are active in many biological processes (Wrzaczek et al. 2010; Kim et al. 2012). In the study described here, we focused on identifying the function of a protein with a DUF1644 motif (Accession Number: PF07800). This motif is approximately 156 amino acids long, has nine highly conserved cysteine residues, and encodes a large and divergent gene family. Sequencing of the rice genome has revealed nine genes encoding DUF1644 proteins. However, none of those proteins has yet been functionally characterized. Therefore, we performed a functional analysis of one gene-*SIDP361*-that contains a DUF1644 motif. We then investigated salt tolerance and ABA sensitivity in transgenic rice plants that over-express this gene. Several stress-related genes in those overexpressing plants were profiled under both normal (unstressed) and stress conditions (200 mM NaCl) to deduce the putative target genes regulated by *SIDP361*. We also monitored *SIDP361* expression and evaluated its possible involvement in ABA-dependent and -independent signaling pathways in rice. Our research objective was to determine whether this gene might have a key role when engineering rice plants with enhanced tolerance to salt stress.

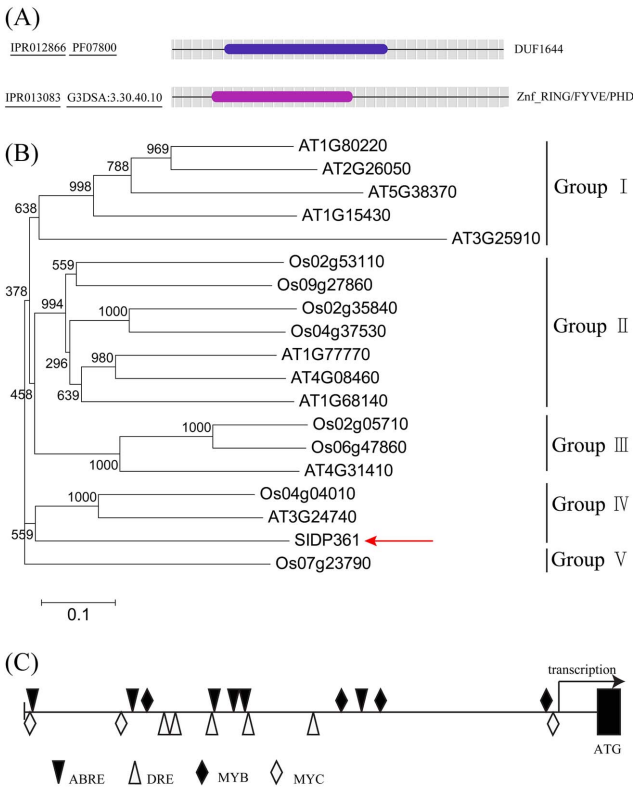

Fig. 1. Sequence analysis of *SIDP361*. (A) Schematic diagram of putative conserved functional domains analyzed with protein–protein BLAST tool from NCBI (http://www.ncbi.nlm.nih.gov). (B) Phylogenetic relationships among DUF1644 proteins of Arabidopsis and rice. SIDP361 is indicated by red arrow. (C) Distribution of major stress-related *cis*-elements in 1.5-kb genomic region upstream of transcriptional start for *SIDP361*.

Results

Isolation of SIDP361 and Sequence Analysis

For functional analysis, we cloned *SIDP361*, which contains a complete open reading frame (ORF) of 981 bp. The predicted SIDP361 protein encodes a peptide of 326 amino acids, with a predicted molecular mass of 36 kDa and a pI of 6. Deduced amino acid sequence analysis revealed that this protein has a conserved motif, DUF1644, in the region between positions 52 and 207 amino acids, as well as a canonical RING/FYVE/PHD zinc finger domain at amino acids 40 to 173 (Fig. 1A). We did not find a sequence for putative nuclear localization signals.

To investigate the evolutionary relationships among plant DUF1644 proteins, we constructed a phylogenetic tree using the ClustalX 2.0 program and full-length amino acid sequences. This produced five groups, with SIDP361 belonging to Group IV (Fig. 1B).

The promoter sequence for *SIDP361* contains several putative stress-responsive *cis*-elements (Fig. 1C). These include ABRE (six hits for the core sequence of ABRE), DRE (five hits), MYC recognition site (three hits), MYB recognition site (four hits), and LTRE (seven hits).

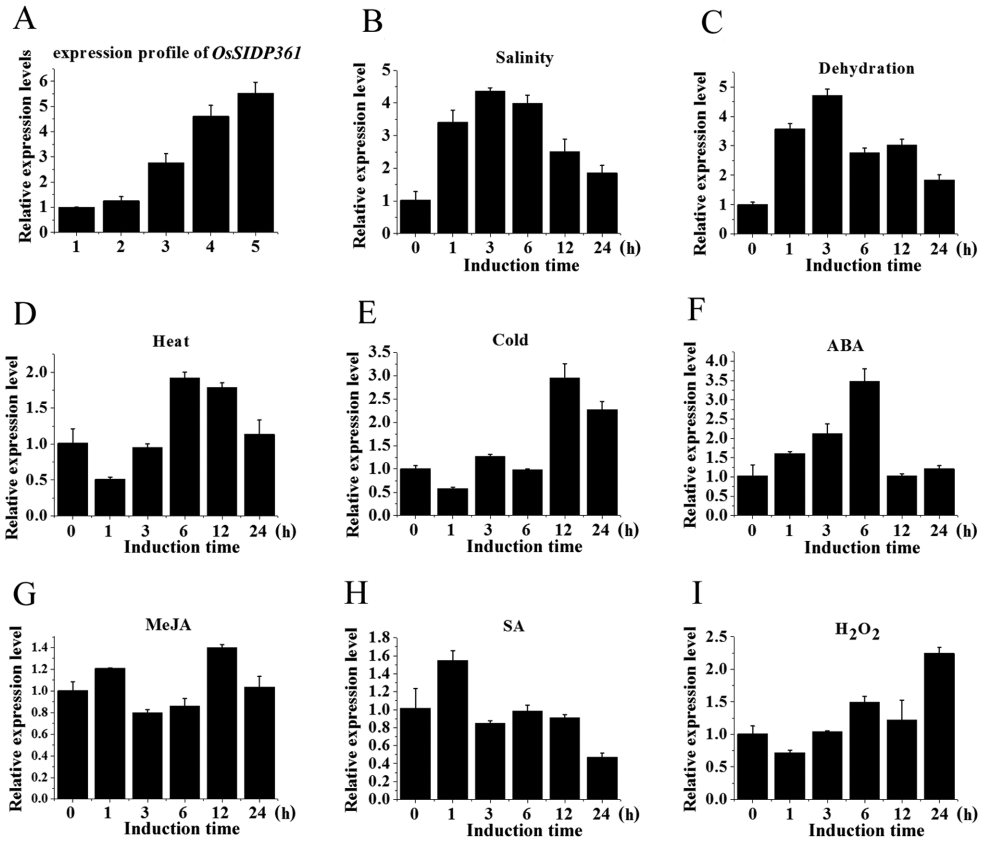

Fig. 2. Expression analysis for *SIDP361* by Q-PCR. (A) Profile of expression in rice flowers (1), immature spikes (2), stems (3), roots (4), and leaves (5). (B-I) Changes in transcript levels over time in response to salinity, dehydration, heat, cold, ABA, MeJA, SA, or oxidative (H_2O_2) treatment. Data represent means and standard errors of 3 replicates.

Tissue Specificity and Induction of *SIDP361* by Abiotic Stresses and Exogenous ABA

We examined tissue specificity for *SIDP361* and detected expression throughout the whole plant. However, this gene was more strongly expressed in the leaves and roots than in the immature spikes, stems, or flowers (Fig. 2A).

The relationship between plant physiology and functioning of *SIDP361* in wild-type (WT) plants was investigated in response to abiotic stresses and chemical treatments (Fig. 2B-I). Under salinity conditions (200 mM NaCl), transcript levels began to increase after 1 h of exposure, then peaked after 3 h before gradually decreasing to a still relatively high level up until 24 h of the treatment period. When seedlings were dehydrated by placing them on dry filter paper to represent drought-stress conditions, *SIDP361* expression was induced within 1 h, then peaked at 3 h before declining. Similar to the results seen from the salinity experiments, however, transcript levels under drought stress were still higher than those measured from the control plants after 24 h.

Plants exposed to 100 μM ABA showed induced expression of *SIDP361* after 1 h. Transcript levels gradually reached a maximum after 6 h before declining slowly.

Compared with the noticeable induction of *SIDP361* by salt, drought, and ABA, expression was not obviously affected by temperature extremes or treatment with various hormones. For example, when cold stress was induced at 7°C, transcript levels increased only for the first 12 h and then decreased. Similar results were obtained following treatment with heat stress (45°C), methyl jasmonate (100 μM JA), salicylic acid (2 mM SA), or oxidative stress (20 mM H_2O_2).

SIDP361 Protein is Possibly Cytoplasmic

To identify the subcellular localization of SIDP361 protein in living cells, we generated transgenic rice that expressed SIDP361–GFP fusion protein. The GFP signal was detected predominantly in the cytoplasm and occasionally in the cytoplasmic foci of SIDP361-GFP-transformed cells (Fig. 3). By comparison, the control (transformation with a GFP construct only) showed ubiquitous distribution of GFP signal throughout the entire cell. This suggested that SIDP361 is mainly located in the cytoplasm.

Transgenic Rice Over-expressing *SIDP361* Shows Enhanced Salt Tolerance

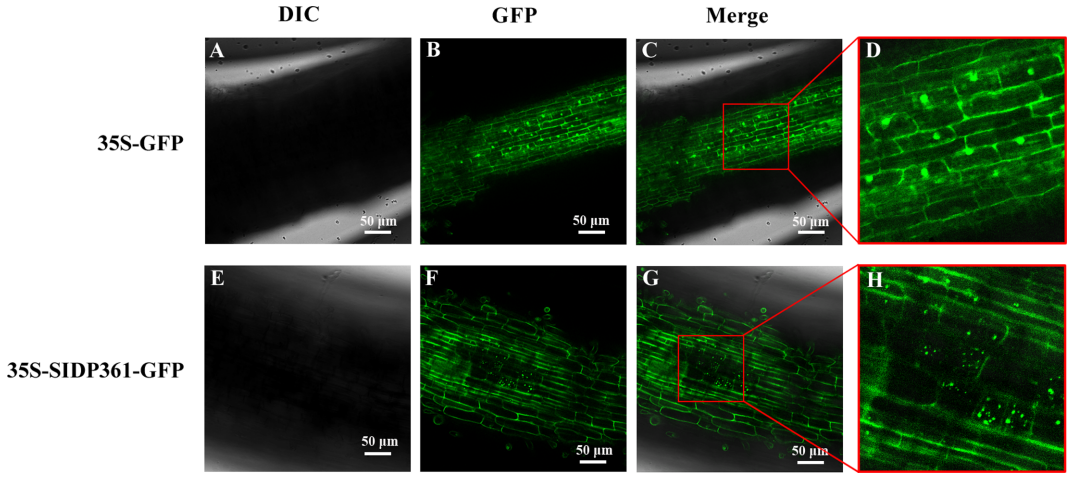

Fig. 3. Subcellular localization of SIDP361 protein in rice root cells. Localization of 35S-GFP control protein (A, B, C, D) and 35S-SIDP361-GFP fusion protein (E, F, G, H). Photographs feature dark field to show green fluorescence (A, E), bright field for cell morphology (B, F), and merged fields (C-D, G-H).

The phenotypes for WT and transgenic rice are presented in Fig. 4A while relative expression levels for *SIDP361* are shown in Fig. 4B. When rice seedlings were grown in media without supplemental NaCl, shoot lengths and fresh weights did not differ significantly between overexpressing transgenics and WT plants. In media containing 200 mM NaCl, average shoot lengths were 2.19 cm for line *S1* and 2.59 cm for *S2* versus only 0.89 for the WT (Fig. 4C). Fresh weights were significantly higher for the transgenics at $P < 0.01$ (averaging 62.87 mg per plant) than for WT plants, which averaged 38.97 mg each (Fig. 4D). These results also demonstrated that salt tolerance was enhanced in SIDP361-overexpressing transgenic rice.

To evaluate how salt stress influences survival rates, plants were irrigated with 200 mM NaCl for 21 d, then returned to normal growing conditions for 7 d of recovery. During the stress period, most of the WT leaves became yellow and wilted completely while leaves from the transgenic plants continued to grow well. After the recovery period, survival rates were significantly higher ($P<0.01$) for the two transgenic lines (50.0-61.0%) than for the WT (0.0-5.6%). This provided further evidence that *SIDP361* confers enhanced salt tolerance.

Overexpression of *SIDP361* Increases Levels of Proline in Transgenic Rice

Plant adaptations to environmental stresses are often associated with metabolic adjustments, such as the accumulation of proline (Abraham et al. 2003). To investigate possible physiological reasons for improved salt tolerance, we calculated the levels of proline, on a fresh-weight (FW) basis, in WT plants and overexpressing lines *S1* and *S2*. Under non-stressed, normal growing conditions, proline concentrations were 52.57 (*S1*), 49.93 (*S2*), and 50.24 μg g^{-1} FW (WT) (Fig. 4F). After the induction of salt stress, proline levels increased in all plant types, albeit more dramatically in *S1* and *S2* (421.78 and 455.11 μg g^{-1} FW, respectively) than in the WT (132.32 μg g^{-1} FW). This suggested that *SIDP361* regulates the accumulation of free proline in rice under salt stress. If so, then the enhancement of salt tolerance in transgenic plants was partially due to their elevated capacity for proline synthesis as directed by *SIDP361*.

SIDP361-overexpressing Plants are Hypersensitive to ABA

During early seedling development, enhanced salt tolerance usually accompanies hypersensitivity to ABA (Hu et al. 2006). Our study showed that *SIDP361* was induced by ABA and transgenic rice plants were more tolerant to salinity. Therefore, we tested whether SIDP361 protein is involved in ABA sensitivity in rice. Growth was significantly inhibited in all plant types when the media were supplemented with ABA. This response was more dramatic for the transgenic lines than for the WT (Fig. 5A). For example, at 5 μM ABA, WT shoots were 1.73 cm long versus 0.55 cm for *S1* and 0.51 cm for *S2* (Fig. 5B). At the same ABA concentration, the average shoot FWs were 49.28 mg for the WT but only 38.93 and 39.13 mg for *S1* and *S2*, respectively (Fig. 5C). At an ABA concentration of 10 μM, WT shoots were 1.13 cm long and they weighed 44.97 mg, which was lower than the FW for WT plants not exposed to ABA. By comparison, *S1* and *S2* shoots were only 0.33 and 0.32 cm long, respectively, and their FW values were also reduced from the ABA-free control, at 37.57 mg (*S1*) and 35.63 mg (*S2*). These results indicated that the overexpressing transgenic plants were more sensitive than the WT to ABA, thereby suggesting a critical role for *SIDP361* in mediating ABA sensitivity by rice.

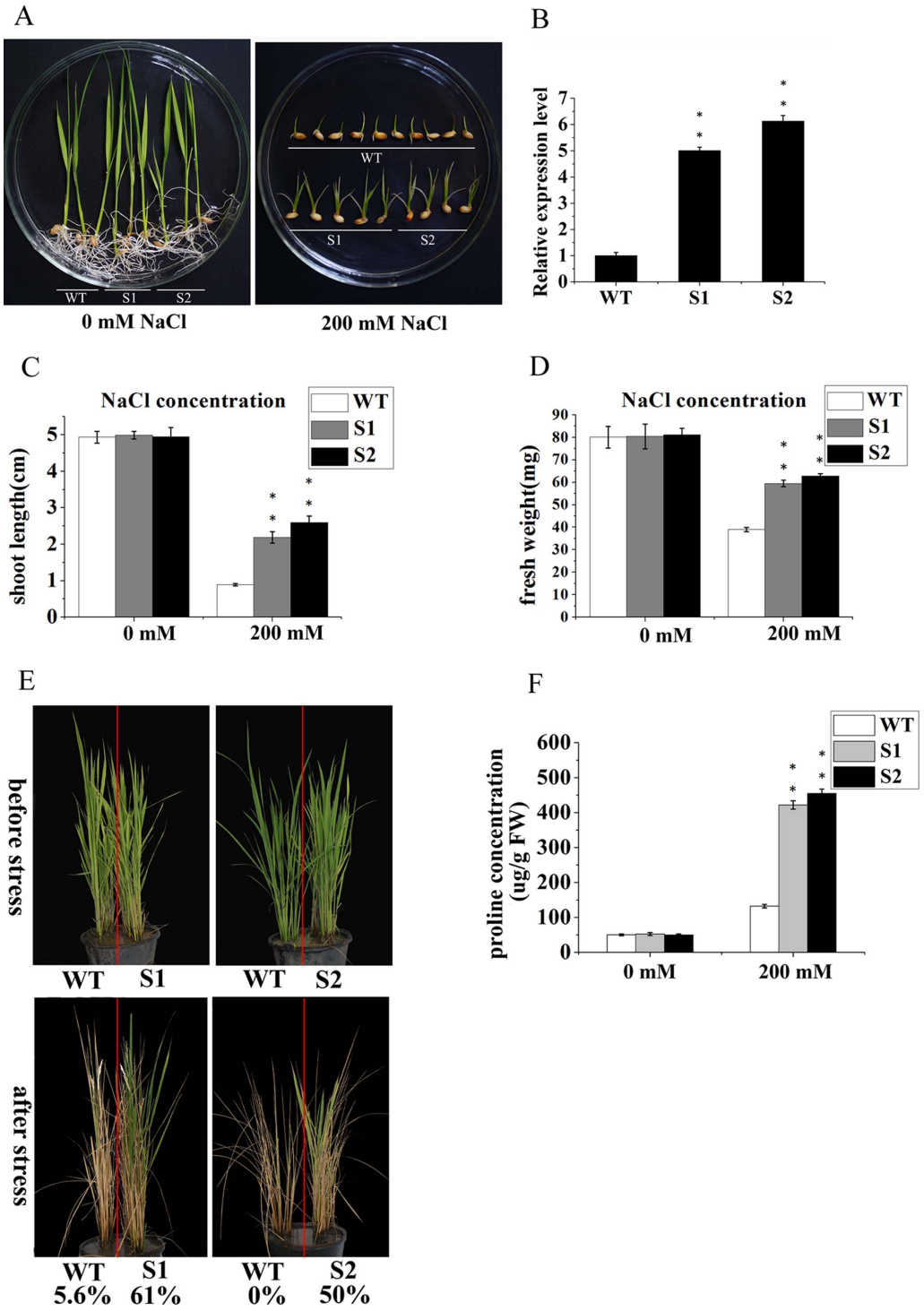

Fig. 4. Effect of *SIDP361* expression on salt tolerance. (A) Phenotypic comparisons among wild-type (WT) plants and transgenic lines *S1* and *S2* grown for 12 d in ½ MS media supplemented with either 0 mM or 200 mM NaCl. (B) Expression levels in WT control and transgenic lines *S*1 and *S*2. Data represent means ± SD from 3 independent experiments. **, values are significantly different from WT data at $P < 0.01$. (C, D) Lengths and fresh weights of shoots from WT and transgenic (*S1* and *S2*) rice seedlings after 12 d of growth in ½ MS media supplemented with 0 mM or 200 mM NaCl. All values are means ± SD. **, values are significantly different from WT data at $P < 0.01$. (E) Phenotypic comparisons and post-stress survival rates (%) for WT and transgenic rice seedlings, before induction of salt stress for 21 d (top panel) and following recovery for 7 d after severe salt stress (bottom panel). Survival values are means of 6 plants per plant type. (F) Levels of free proline in WT seedlings and *SIDP361*-overexpressing lines *S1* and *S2*) grown in ½ MS media supplemented with 0 mM or 200 mM NaCl. **, values are significantly different from WT data at $P < 0.01$, based on Student's *t*-tests.

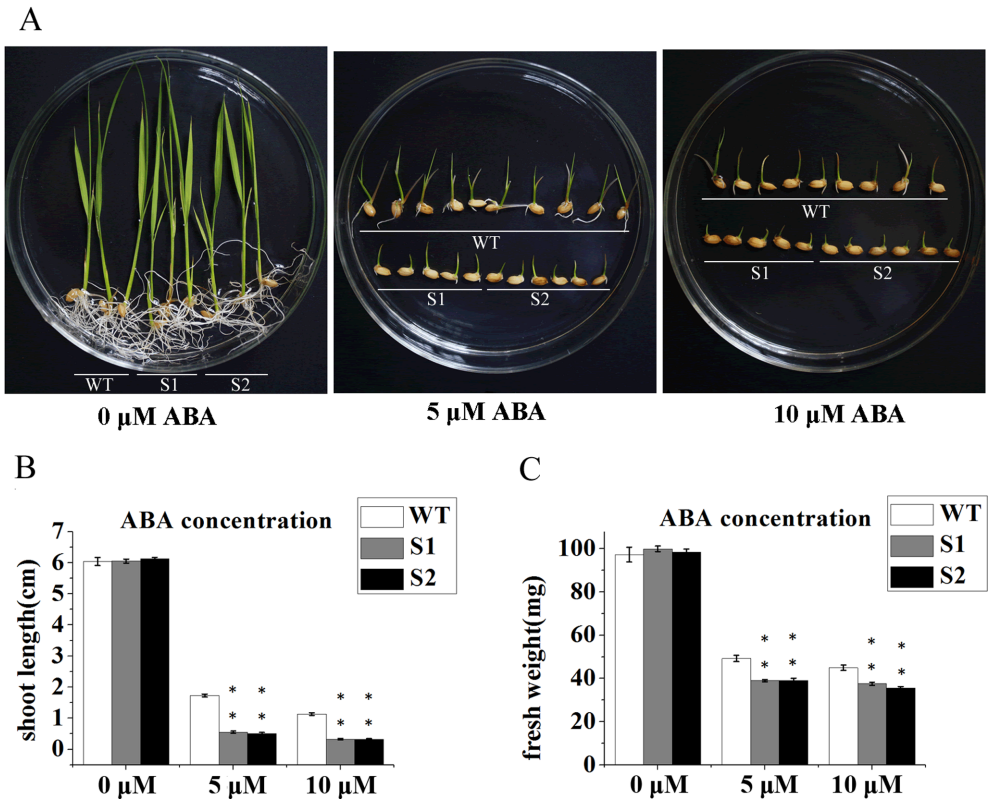

Fig. 5. Changes in morphology of WT and *SIDP361*-overexpressing rice seedlings in response to ABA. (A) Performance by WT (control) and transgenic lines *S1* and *S2* grown for 12 d in presence of 0, 5, or 10 μM ABA. (B, C) Lengths and fresh weights of shoots from WT (control) and transgenic rice lines *S1* and *S2* under different ABA concentrations. **, values are significantly different from WT data at $P < 0.01$, based on Student's *t*-tests.

Expression of Stress-related Genes in *SIDP361*-overexpressing Transgenic Rice

To explore the possible molecular mechanisms by which *SIDP361* confers salt tolerance, we determined the expression profiles for several well-known stress-responsive genes. These included *OsDREB1A* and *OsDREB2A*, encoding DREB-type transcription factors (TFs); *OsP5CS*, encoding a rate-limiting enzyme involved the biosynthesis of proline; *OsNAC5*, *OsSNAC1*, and *OsSNAC2*, encoding typical stress-related NAC-type TFs; *OsLEA3*, *OsLEA3-1*, *OsRab16a*, and *OsRab16b*, encoding late embryogenesis abundant (LEA) proteins; and *NCED3* and *DSM2*, encoding proteins involved in ABA biosynthesis. Under normal growing conditions, expression by many of these genes was significantly higher in the transgenic lines than in the WT rice (Fig. 6). The exceptions were *P5CS* and *DREB2A*, for which expression was not always significantly different among plant types. However, after plants were exposed to 200 mM NaCl for 3 h, all of these tested genes were substantially up-regulated in the transgenic lines when compared with the WT. Therefore, our findings suggested that the involvement of *SIDP361* in regulating these stress-related genes was the main explanation for why salt tolerance was improved in the overexpressing lines.

Discussion

DUF1644 is part of a large protein family that includes many members whose biological functions are still largely unclear. In this study, we reported the isolation and characterization of a novel rice DUF1644 protein, SIDP361. This protein is predominantly localized in the cytoplasm but can also be observed in the cytoplasmic foci. The latter occasionally resemble processing bodies and stress granules where mRNA decays and storage (Jan et al. 2013).

The 1.5-kbp promoter region of *SIDP361* contains ABRE, MYCRS, MBYRS, and DRE, which are respectively recognized as AREB/ABF and MYB, MYC, and DREB TFs. These *cis*-elements and their corresponding TFs are important for abiotic stress responses in plants (Yamaguchi-Shinozaki and Shinozaki 2006). Because ABRE, MYCRS, and MYBRS function as *cis*-acting elements for the expression of ABA-responsive genes, we believe that *SIDP361* is induced by ABA through these elements under abiotic stress conditions.

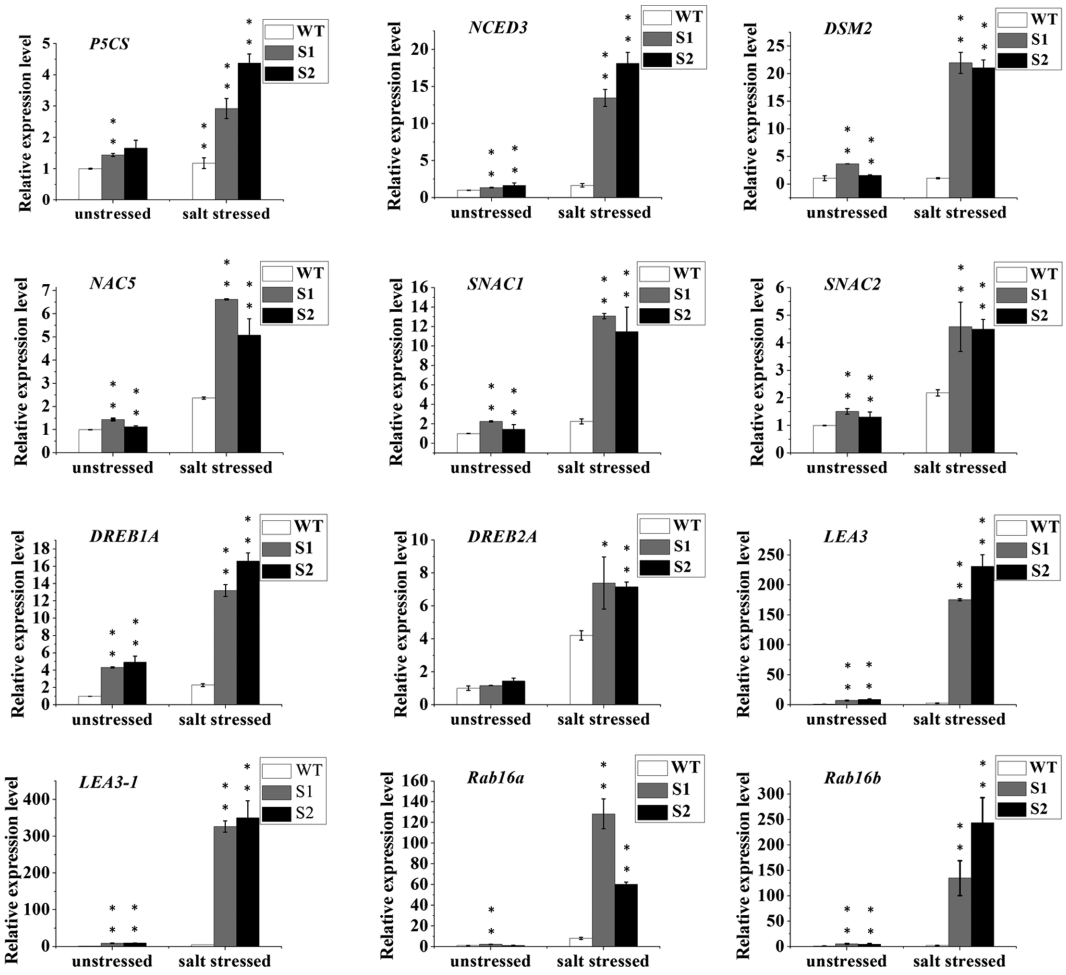

Fig. 6. Expression patterns for stress-responsive genes in WT (control) and *SIDP361*-overexpressing rice lines *S1* and *S2* under unstressed (0 mM NaCl) or salt-stressed (200 mM NaCl) conditions. Values are means and standard errors for 3 replicates. * and **, values are significantly different from WT data at $P < 0.05$ and $P < 0.01$, respectively, based on Student's *t*-tests.

The presence of numerous stress-responsive *cis*-acting elements in the *SIDP361* promoter and the strong induction of *SIDP361* by salt, drought, and ABA treatments lead us to conclude that this gene has a critical role in the ABA signaling pathway as well as in stress responses by rice plants.

Salinity conditions cause osmotic stress. Therefore, many plants accumulate compatible osmolytes to stabilize their membranes and protect their subcellular structures as a way to decrease osmotic potential in the cytoplasm (Bohnert and Shen 1998; Hare et al. 1998; McNeil et al. 1999; Diamant et al. 2001; Wang et al. 2003). Our results showed that the levels of proline were higher in *SIDP361*-overexpressing rice than in the WT after salt treatment. *P5CS* encodes a rate-limiting enzyme that leads to the biosynthesis of proline (Hong et al. 2000). Consistent with this, our Q-PCR data indicated that, under salt stress, the expression of *P5CS* was increased by 3.0- and 4.4-fold in transgenic lines when compared with the WT. This suggested that the elevated proline concentrations resulted partially from increased expression of *P5CS*.

The LEA proteins that accumulate in response to drought, salinity, or extreme temperatures are thought to function in protecting macromolecules so that the cellular structure can be maintained by adjusting osmotic pressures (Vierling and Kimpel 1992; Ingram and Bartels 1996). The expression of *lea* genes appears to be ABA-dependent (Mundy and Chua 1988; Skriver and Mundy 1990; Leung and Giraudat 1998). In our study, four LEA-encoding genes -- *OsRab16a* (*OsRab21*, *OsLEA29*), *OsRab16b* (*OsLEA26*), *OsLEA3*, and *OsLEA3-1* -- were significantly up-regulated in overexpressing plants regardless of the level of NaCl to which they were exposed. Osmotic adjustment is a major mechanism for developing tolerance to high salt. Thus, *SIDP361* may contribute to the accumulation of compatible osmolytes, e.g., free proline and LEA proteins, by activating the expression of genes related to osmolyte biosynthesis.

Plants accumulate more ABA under abiotic stress, which

then rapidly activates the ABA signaling pathway and regulates the expression of several ABA-responsive genes to ensure plant survival. In the ABA biosynthetic pathway, the oxidative cleavage of neoxanthin catalyzed by NCED is considered the rate-limiting step. For example, overexpression of *NCED3* in WT Arabidopsis plants results in increased ABA accumulations (Iuchi et al. 2001). We also found that overexpression of *SIDP361* promoted the expression of *NCED3* under salinity stress. Furthermore, the â-carotene hydroxylase gene *DSM2*, which significantly contributes to ABA synthesis (Du et al. 2010), was also significantly up-regulated in our overexpressing plants in the presence of high salinity. Our observations suggested that *SIDP361* functions as a positive regulator of ABA biosynthesis, which might partially explain why the transgenic plants had greater salt tolerance. Moreover, we showed here that the exogenous supply of ABA could induce the expression of *SIDP361* and that overexpression of this gene could increase the sensitivity of transgenic plants to exogenous ABA. All of these results strongly indicate that *SIDP361* regulates the response of transgenic rice seedlings to salt stress through an ABA-dependent signaling pathway.

The TFs that are induced early by abiotic stress play a crucial role in regulating plant responses because they can activate the expression of stress-inducible downstream target genes. Both *SNAC1* and *SNAC2* function as key positive regulators in the ABA signaling pathway, and their overexpression in rice leads to significantly improved tolerance to drought and salt stresses (Hu et al. 2006; Hu et al. 2008). In our study, expression of *SNAC1* and *SNAC2* was up-regulated under salinity by approximately 4.5- and 13.0-fold in the transgenic lines. *NAC5* is an important TF in the ABA-dependent signaling pathway and salt tolerance is improved in *OsNAC5*-overexpressing rice plants when compared with control plants (Sperotto et al. 2009, Song et al. 2011). Salt stress also caused *NAC5* to be up-regulated by 5.0-fold in our transgenics. Therefore, by activating those transcription genes, *SIDP361* may indirectly participate in regulating the expression of numerous downstream stress-related genes, thereby possibly contributing to a large extent to the enhanced salt tolerance by transgenic rice.

Both ABA-dependent and -independent regulatory systems are involved in controlling the expression of stress-regulated genes (Yamaguchi-Shinozaki and Shinozaki 2005). These include dehydration-responsive TFs DREB1A and DREB2A in ABA-independent pathways (Medina et al. 1999; Sakuma et al. 2006). Overexpression of *DREB1A* or *DREB2A* increases tolerance to abiotic stresses in Arabidopsis and rice (Dubouzet et al. 2003; Matsukura et al. 2010). We also found that, in the presence of high salt, our transgenic rice plants accumulated more mRNA of *DREB1A* and *DREB2A*, which suggested that *SIDP361* is an upstream regulator of those genes. However, because transcript levels of *DREB2A* were not affected in the transgenics under normal growing conditions, it appears that *SIDP361* does not directly activate the transcription of *DREB2A*. Overall, these data provide evidence that the stress-related genes induced by *SIDP361* operate in different pathways.

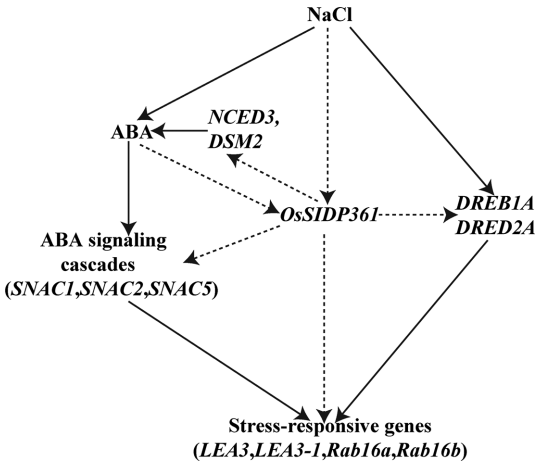

Fig. 7. Proposed model for regulatory network of *SIDP361* in stress signaling transduction pathway of rice. Expression may be induced in several ways: 1) ABA biosynthesis to activate ABA-relevant signaling cascades; 2) activation of ABA-dependent pathway through *SNAC1*, *SNAC2*, and *NAC5*; or 3) activation of ABA-independent pathway through *DREB1A* and *DREB2A* proteins. Solid lines represent confirmation from previously published data; dashed lines, conclusions drawn from current study. Arrows indicate direction of promotion.

The ABA-dependent and -independent stress signaling pathways converge at several points that are potential elements for cross talk (Knight and Knight 2001). ABRE, MYCRS, MYBRS, and DRE *cis*-elements are commonly present in the promoters of these genes, including the *AtRD29A* promoter. The *SIDP361* promoter also contains both ABRE and DRE *cis*-elements. To elaborate on the functioning of *SIDP361*, we developed the model shown in Fig. 7. First, salinity induces ABA accumulation, which triggers relevant signaling cascades (Ingram and Bartels 1996) and induces the expression of *SIDP361*. The overexpression of *SIDP361* then induces the expression of both *NCED3* and *DSM2*, suggesting that *SIDP361* has a positive feedback role in ABA biosynthesis in the presence of salinity stress. Second, *SIDP361* may be an important upstream factor of *NAC5*, *SNAC1*, and *SNAC2*, as it regulates the expression of these TFs. *OsNAC5* can also interact with *OsNAC5* and *SNAC1*. Moreover, *NAC5* can bind directly to the *LEA3* promoter and regulate the expression of *LEA3* in rice (Takasaki et al. 2010). Thus, the partial contribution of *SIDP361* to salt tolerance is achieved *via* the induction of *LEA3* transcription by up-regulating *NAC5* expression. Therefore, we propose that, in addition to

its role in the ABA biosynthesis pathway, *SIDP361* also positively modulates the ABA signaling cascades. Third, overexpression of *SIDP361* in rice is associated with increased accumulations of *DREB1A* and *DREB2A* transcripts, both of which participate in the ABA-independent signaling pathway. Altogether, we believe that our findings demonstrate that *SIDP361* is involved in ABA-dependent and -independent signaling pathways in response to salt stress.

Nevertheless, we also found that the transcript levels of stress-related target genes in transgenic plants were more significantly up-regulated under salt stress than under normal growing conditions. This implied, therefore, that *SIDP361* mediates the activation of those stress-responsive genes by requiring other factors that are induced under salt stress.

Our molecular characterization of *SIDP361* showed that its expression was induced by high salinity, drought, and ABA, but not by cold, heat, MeJA, or SA. The SIDP361 protein is mainly localized in the cytoplasm. *SIDP361*-overexpressing transgenic plants were hypersensitive to ABA and showed more tolerance to salt stress when compared with WT rice. Expression analysis also suggested that *SIDP361* is involved in regulating the biosynthesis of osmoprotectants, including free proline and LEA proteins. Finally, *SIDP361* expression elevated the transcription levels of many stress-related genes, including ABA-inducible genes and two ABA-independent genes, thereby indicating that *SIDP361* functions as an important positive factor in mediating between ABA-dependent and -independent signaling pathways during the plant response to salinity. All of our study results provide a solid argument that *SIDP361* would be a valuable tool for breeding crops with enhanced salt tolerance.

Table 1. Primer pairs used in real-time PCR

Gene	Primer sequence (5'-3')
Actin	F:5' TGTATGCCAGTGGTCGTACCA 3' R:5' CCAGCAAGGTCGAGACGAA 3'
SIDP361	F:5' CACCATGCCAAAGGACAGGAGC 3' R:5' TCAATGTGCAGGATCACCA 3'
SIDP361-GFP	F: 5' CACCATGCCAAAGGACAG 3' R: 5' TGCATGTGCAGGATCACC 3'
SNAC1	F:5' ATCCCTCACAACCCACAA 3' R:5' GTCCCTCTCCCTCCTCAT 3'
SNAC2	F:5' CAAGGGCGAGAAGACCAA 3' R:5' CAGCACCCAATCATCCAAC 3'
NAC5	F:5' AAGGGCGTCAAGACCAAC 3' R:5' AACACCCAATCATCCAACC 3'
DREB1A	F:5' GCCTCTTTTTTCTCTCTTTTC 3' R:5' AACTTGTTCCATCACATTACC 3'
DREB2A	F:5' GGAGGAATAGGAAGAAGGGA 3' R:5' GAGCGGGAACAAGAAAGAGA 3'
NCED3	F:5' GGTTTGTGGCGAATGTC 3' R:5' TCGTGGTGTGTTTCTG 3'
DSM2	F:5' TGGTGGCAGCGGTGATGT 3' R:5' ATGCGAGCGGGAGTTTGG 3'
P5CS	F:5' AGCCACAGATGGAGTTAGATG 3' R:5' GTCGGTGACAAGAAGTTGAGAT 3'
Rab16a	F:5' GCTCAAGCTCGGTACAACA 3' R:5' CCTCCCATTCCATCATCCT 3'
Rab16b	F:5' CATCTTACTGATAGCAACAACACT 3' R:5' GTCCATCCTCTCAAGCAAAT 3'
LEA3	F:5' GAATGATTTCCCTTTGGGTCTA 3' R:5' ACTCTGACGAAAACAACTGAAC 3'
LEA3-1	F:5' CGGCAGCGTCCTCCAACAG 3' R:5' GCCTCGTCTTCGGTCATCC 3'

F-forward primer; R-reverse primer

Materials and Methods

Plant Material and Stress Treatments

We used 'Taipei309' rice for all stress evaluations. Seeds were surface-sterilized with ethanol (70% v/v) for 5 min and then with diluted NaOCl (1:3 v/v) for another 5 min, followed by several rinses with sterile water. To measure *SIDP361* transcript levels in response to abiotic stress, we incubated those seeds in a half-strength Murashige and Skoog (MS) medium (Murashige and Skoog 1962) under controlled laboratory conditions (16-h photoperiod, 25°C/22°C day/night). After 14 d, the WT germinants were exposed to the following treatments: dehydration to simulate drought conditions (seedlings placed on dry filter paper), salinity (200 mM NaCl), oxidative stress (20 mM H_2O_2), cold (7°C), heat (45°C), ABA (100 μM), JA (100 μM), or SA (2 mM). Leaf tissues were harvested at 0, 1, 3, 6, 12, and 24 h during each treatment period, then immediately frozen in liquid nitrogen and stored at -80°C. Expression of *SIDP361* was monitored in various tissue types: stems, root, and leaves at the seedling stage; flowers at the flowering stage; and immature spikes at the heading stage. All sampling involved three technical replications.

To examine how the expression of stress-responsive genes differed between transgenic and WT plants, we used seedlings at the four-leaf stage and supplemented their growth media with 0 or 200 mM NaCl for 3 h. Their RNA was then extracted for Q-PCR assays.

Plasmid Construction and Plant Transformation

To generate the *SIDP361*-overexpression construct, we amplified full-length cDNAs of *SIDP361* by reverse transcription PCR (RT-PCR), using total RNA extracts and gene-specific primers (Table 1). Four nucleotides, CACC, were added in front of each gene-specific forward primer to facilitate the TOPO cloning of the PCR fragment into the TOPO-D entry vector. The RT-PCR product was subsequently cloned into a Gateway entry vector, pENTER/D-TOPO (Invitrogen, NY, USA). Afterward, the inserted fragments were introduced into the destination vector pH7WG2 from the pENTER/D-TOPO vector through a recombination reaction that utilized Gateway LR Clonase II Enzyme Mix (Invitrogen) according to the manufacturer's instructions. This step allowed *SIDP361* to be driven under the control of the cauliflower mosaic virus 35S (CaMV 35S) promoter (Karimi et al. 2002). The overexpression construct was then transferred into *Agrobacterium tumefaciens* strain EHA105 by the freeze-thaw method (Hofgen and

Willmitzer 1988). Finally, *Agrobacterium*-mediated transformation was performed with calli derived from mature embryos of WT 'Taipei309' rice to generate transgenic plants according to a published protocol (Nishimura et al. 2006).

RNA Isolation and Quantitative Real-time PCR

The total RNAs from all tissue types (see above) were extracted with TRIZOL reagent (Invitrogen), as recommended by the manufacturer. To minimize genomic DNA contamination, we treated the RNA with DNase I (Invitrogen) at room temperature for 15 min, then at 70°C for 10 min before converting it to cDNA, using a reverse transcriptase kit (M-MLV, Invitrogen) according to the manufacturer's instructions. All Q-PCRs were performed on optical 48-well plates with a StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) to monitor DNA synthesis. Each reaction contained 10 μL of SYBR Premix Ex Taq II (Takara), 2.0 ng of cDNA sample, and 200 nM of each gene-specific primer, in a final volume of 20 μL. The PCR thermal-cycle conditions included denaturing at 95°C for 3 min; then 40 cycles of 95°C for 30 s, 61°C for 30 s, and 72°C for 1 min. Relative expression levels were evaluated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001), using rice *Actin1* as the internal control. Three independent biological replicates were used for each experiment.

Analysis of *SIDP361* Promoter Sequence

For analyzing the *cis*-elements of the promoter sequence, a 1500-nucleotide upstream region from the putative ATG translation start codon of *SIDP361* (Accession Number: AK066561) was identified using the PLACE database (www.dna.affrc.go.jp/PLACE/index.html) (Higo et al. 1999).

Subcellular Localization of SIDP361-GFP Fusion Protein

To determine the subcellular localization of SIDP361 protein in 'Taipei309' rice, we used specific primers (Table 1) to amplify the full-length ORF of *SIDP361* without the stop codon. The amplified fragment was sub-cloned into the pENTER/D-TOPO vector. Verified inserts were then fused to the destination vector pMDC83 by recombination reactions that used LR clonase (Invitrogen) according to the manufacturer's instructions. This produced a SIDP361-Green Fluorescent Protein (SIDP361-GFP) fusion construct under the control of the CaMV 35S promoter (Karimi et al. 2002).

This fusion construct and the GFP control vector were transferred into rice calli by *Agrobacterium*-mediated transformation and GFP fluorescence was visualized under a confocal microscope (LSM 780, Carl Zeiss SAS, Jena, Germany). A GFP gene that lacked the target gene in the same pMDC83 plasmid was used as our control.

Evaluating Salt Tolerance in Transgenic Rice Plants

Surface-sterilized seeds from WT rice and our two transgenic lines *S1* and *S2* (at least 15 seeds per plant type) were placed on plates containing half-strength MS media supplemented with either 0 mM or 200 mM NaCl. After exposure for 12 d under long days (i.e., 16-h photoperiod) and 25°C/22°C (day/night), their phenotypic responses to salinity were assessed by photographing the seedlings and measuring shoot lengths and fresh weights. Triplicate observations were made. To evaluate seedling performance under salt stress, we placed WT and transgenic plants (*S1* and *S2*) in barrels containing nutrient soil and grew them in a greenhouse where they were irrigated with 500 mL of water containing 0 mM or 200 mM NaCl. After 21 d of growth under these stress conditions, the seedlings were returned to normal conditions for 7 d of recovery. Survival rates were calculated for each line.

Tests of ABA-sensitivity

Seeds of the WT and transgenic lines *S1* and *S2* were immersed in half-strength MS media supplemented with 0, 5, or 10 μM ABA, then treated for 12 d under a 16-h photoperiod (25°C/22°C, day/night) to assess the phenotypic responses of seedlings to ABA treatment. Shoot lengths and fresh weights were recorded and the seedlings were photographed. All data were analyzed for significant differences by Student's *t*-tests. The sensitivity tests were repeated twice, with each involving at least 15 seeds per plant type.

Measurements of Cellular Proline

At the four-leaf stage, WT and transgenic (*S1* and *S2*) rice plants were transferred to half-strength MS media containing 0 mM or 200 mM NaCl. After 2 d of treatment, leaf samples (approx. 0.5 g) were collected from each plant. Proline levels were determined by the sulfosalicylic acid method (Troll and Lindsley 1955). Free proline was measured using L-proline as the standard (Bates et al. 1973) and values were reported as micrograms per gram of fresh weight for the leaf samples.

Acknowledgments

This work is supported by the National Program of Transgenic Variety Development of China (Grant No.2014ZX08001-008 to Grant No.2014ZX08001-008) and the National Key Basic Researches Program of China (Grant No. 2012CB126312).

Author Contributions

ML designed research, performed research, and wrote the paper; LG performed research; CG analyzed data; LW contributed new analytical tools; LC provided English language support.

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