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RSOsPR10 Expression in Response to Environmental Stresses is Regulated Antagonistically by Jasmonate/Ethylene and Salicylic Acid Signaling Pathways in Rice Roots

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Plant roots play important roles not only in the absorption of water and nutrients, but also in stress tolerance. Previously, we identified RSOsPR10 as a root-specific pathogenesis-related (PR) protein induced by drought and salt treatments in rice. Transcripts and proteins of RSOsPR10 were strongly induced by jasmonate (JA) and the ethylene (ET) precursor 1-aminocyclopropane-1-carboxylic acid (ACC), while salicylic acid (SA) almost completely suppressed these inductions. Immunohistochemical analyses showed that RSOsPR10 strongly accumulated in cortex cells surrounding the vascular system of roots, and this accumulation was also suppressed when SA was applied simultaneously with stress or hormone treatments. In the JA-deficient mutant *hebiba*, *RSOsPR10* expression was up-regulated by NaCl, wounding, drought and exogenous application of JA. This suggested the involvement of a signal transduction pathway that integrates JA and ET signals in plant defense responses. Expression of *OsERF1*, a transcription factor in the JA/ET pathway, was induced earlier than that of *RSOsPR10* after salt, JA and ACC treatments. Simultaneous SA treatment strongly inhibited the induction of *RSOsPR10* expression and, to a lesser extent, induction of *OsERF1* expression. These results suggest that JA/ET and SA pathways function in the stress-responsive induction of RSOsPR10, and that OsERF1 may be one of the transcriptional factors in the JA/ET pathway.

Keywords: ERF1 • Jasmonic acid • *Oryza sativa* • Pathogenesis-related protein 10 (PR10) • Salicylic acid • Stress response.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; ET, ethylene; GUS, β-glucuronidase; JA, jasmonic acid; LUC, luciferase; OE, overexpressing; OsERF1, *Oryza sativa* ethylene responsive factor 1; PR, pathogenesis-related; RSOsPR10, root-specific *Oryza sativa* PR10; qRT-PCR, quantitative real-time PCR; RT–PCR, reverse transcription–PCR; SA, salicylic acid

Introduction

Plants are constantly exposed to a variety of biotic and abiotic stresses. To survive these challenges, plants have developed elaborate mechanisms to perceive external signals and to manifest adaptive responses with appropriate physiological changes. At the molecular level, the perception of environmental stimuli and the subsequent activation of defense responses require a complex interplay of signaling cascades. Pathogenesis-related (PR) proteins have been well defined as plant proteins that are induced not only during pathogen infection but also in response to abiotic stresses, including wounding, drought and high salinity. The major families of PR proteins have been grouped into 17 different classes, primarily on the basis of

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their amino acid sequence identity, small molecular masses and acidic pIs (van Loon et al. 2006). Several PR proteins have anti-microbial enzymatic activity, such as chitinase (PR3, PR4, PR8 and PR11), β-1,3-glucanase (PR2) and RNase (PR10). However, the biological and/or biochemical functions of other PR proteins remain unknown. ABA, ethylene (ET), jasmonic acid (JA) and salicylic acid (SA) have been reported to be major signaling molecules in plant stress responses, and their signaling cascades have been widely investigated (Zeevaart and Creelman 1988, Reymond and Farmer 1998, Turner et al. 2002). In general, SA is an important signal for general defense responses and especially for attack by biotrophic pathogens in so-called systemic acquired resistance (SAR), the JA/ET signaling pathway is involved in responses to wounding and abiotic stresses such as drought and high salinity and also in the defense signaling against necrotrophic pathogens, and ABA has a crucial role in responses to a wide range of abiotic stresses, including drought, salt and cold. In the JA/ET pathway, the transcription factor ethylene responsive factor 1 (ERF1) integrates JA and ET signals (Lorenzo et al. 2003, McGrath et al. 2005, Hu et al. 2008). These signaling pathways work together or antagonistically in different stress responses, but the detailed mechanisms remain obscure. In Arabidopsis, JA/ET signaling induces the defensin PDF1.2 (PR12) and SA signaling up-regulates PR1; these two processes show an antagonistic relationship (Koornneef et al. 2008). Recently, one key component, NPR1, was reported to have a role in fine-tuning the antagonistic regulation of SA and JA/ET signaling to control gene expression (Leon-Reyes et al. 2009, Spoel et al. 2009). Currently, it is proposed that NPR1 mediates the fine, highly complex systems that are induced by various environmental stresses.

Since a major pollen allergen (Bet v1) from white birch was first identified as a PR10 class of proteins, there have been many studies on PR10 proteins in diverse plant species (Hashimoto et al. 2004, Liu and Ekramoddoullah, 2006, Hwang et al. 2008, Lebel et al. 2010, Xie et al. 2010, see also references cited therein). Those studies revealed that these proteins are responsive not only to biotic attacks but also to various environmental stresses, such as drought, high salinity, low and high temperatures, wounding and UV exposure. PR10 proteins, therefore, have been postulated to have important roles in plant defense responses against biotic and abiotic stresses. For instance, some PR10 proteins have been suggested to have anti-microbial RNase activity (Park et al. 2004, Liu et al. 2006, Kim et al. 2008). Some PR10 proteins are possibly involved in saline and desiccation tolerance (Pnueli et al. 2002, Srivastava et al. 2004, Jain et al. 2006). Recent genetic, structural and bioinformatic studies have revealed a variety of functions of PR10 proteins, including cytokinin-, flavonoid- and steroid-binding activities (Fernandes et al. 2008, Zubini et al. 2009), membrane permeability (Mogensen et al. 2007) and norcoclaurine synthase activity (Lee and Facchini, 2010). Furthermore, a Bet v 1-superfamily has been defined on the basis of structural similarities of the protein (Radauer et al. 2008), and it includes a member of the PR10 family and the recently reported PYR/PYL/RCAR family of ABA receptors (Ma et al. 2009). This superfamily can be divided into numerous subfamilies with low sequence similarity, but structural analyses of the proteins belonging to this superfamily have shown a common structural feature of a fold that acts as a versatile scaffold for the binding of bulky hydrophobic ligands, thus achieving a variety of functions.

PBZ1, a rice PR10 protein, was first characterized as a probenazole-inducible protein in rice (Midoh and Iwata 1996). Later, three highly homologous *OsPR10* genes were reported in rice: *OsPR10a* (encoding PBZ1), *OsPR10b* and *OsPR10c* (McGee et al. 2001). The biotic and abiotic stress-inducible nature of the *OsPR10a* gene has been investigated. Expression of *OsPR10a* is induced by some plant hormones and defense-related signaling molecules, including JA, ABA, SA and kinetin (Moons et al. 1997, Lee et al. 2001, McGee et al. 2001, Hwang et al. 2008). However, there are no reports on the signaling molecules and signaling pathways involved in *OsPR10a* induction in rice. Moreover, the physiological activities and functions of OsPR10 in the plant defense response are still unknown.

In the course of our rice proteome analyses, another gene homologous to *OsPR10a* was identified in the root (Hashimoto et al. 2004, Patent No. US 7,605,303 B2). This root-specific gene, *RSOsPP10*, encodes a novel root-specific protein. *RSOsPR10* mRNA was up-regulated by NaCl, drought and by exogenous applications of JA, ET and probenazole, but not by SA and ABA. Subsequent studies revealed that the induction was at the transcriptional level, and overexpression of RSOsPR10 in rice and bent-grass plants resulted in greater drought tolerance (T. Terakawa et al. in preparation, Patent Publication No. US 2009/0100552).

Here, we show the involvement of the JA/ET signaling pathway in *RSOsPR10* induction during responses to high salinity and wounding, and the antagonistic inhibition by exogenous SA treatment at the transcriptional level. Our results also indicated the possible involvement of the transcription factor OsERF1 in the induction of *RSOsPR10*, and, to some extent, in the suppression by SA. Immunohistochemical analyses showed that the RSOsPR10 protein accumulated mainly in the root cortex cells after stress treatments, suggesting that this protein plays a role in protecting the inner vascular system in the root during environmental stresses. We discuss the regulation of RSOsPR10 expression in relation to plant defense responses in rice.

Results

Accumulation of RSOsPR10 protein in rice roots after abiotic stresses and application of plant hormones

We analyzed the expression and accumulation of RSOsPR10 protein in rice seedlings after abiotic stresses (100 mM NaCl, drought and wounding) and treatments with plant hormones

[JA and the ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC)] using a specific antibody raised against recombinant RSOsPR10 (**Fig. 1A**). The expression of RSOsPR10 protein was induced approximately 12 h after the treatments began. The protein accumulated until 72 h, and was found only in the root parts. Previously, we showed that SA did not induce *RSOsPR10* expression (Hashimoto et al. 2004). In the present study, we investigated the effects of SA on RSOsPR10 induction by these treatments (**Fig. 1B**). Strong inhibitory effects were observed by simultaneous application of SA with NaCl, wounding, JA and ACC treatments. These results supported the idea that there is an antagonistic effect of the SA signal transduction pathway on the JA/ET pathway in the induction of RSOsPR10. This antagonistic relationship has also been reported for other plants in their responses to various external stresses (Koornneef et al. 2008, Leon-Reyes et al. 2009, Spoel et al. 2009). We investigated the dose-dependent effects of NaCl, JA, ACC and SA on RSOsPR10 induction. Concentrations of 30–50 mM NaCl, 5–10 μM JA and 10 μM ACC were sufficient for protein accumulation, and 100 μM SA was sufficient for the complete inhibition of the NaCl induction (**Fig. 1C**).

Tissue-specific expression of RSOsPR10 in rice roots

We used immunohistochemical analyses to examine the accumulation of RSOsPR10 protein in the root after NaCl, drought and JA treatments. The RSOsPR10 protein was localized specifically in root cortex cells, which surround the vascular system (**Fig. 2A**). No signals were present when non-immune serum was used, and when no stress was applied. Simultaneous application of SA diminished the signal (**Fig. 2B**), as was found in the expression analyses (**Fig. 1B**). Within the cortex cells, RSOsPR10 was localized in the cytosol, as indicated by the presence of the signal in the shrunken cytosolic compartment after plasmolysis (**Fig. 2C**). After 24 h of NaCl treatment, we estimated the amount of protein in the cortex cells from the band intensities of RSOsPR10 after SDS–PAGE and Western blot analyses. RSOsPR10 accounted for as much as 1% of cytosolic total soluble proteins, as determined by calculating the amount of protein relative to that of recombinant RSOsPR10 produced in *Escherichia coli*.

Expression of *RSOsPR10* mRNA in rice roots after abiotic stresses and treatments with plant hormones

We used reverse transcription–PCR (RT–PCR) to analyze expression levels of *RSOsPR10* mRNA in rice seedlings after the stress and plant hormone treatments (**Fig. 3A**). Expression of *RSOsPR10* was induced around 12 h after salt treatment, whereas its induction was earlier after drought, wounding, JA and ACC treatments. These results, together with the expression pattern of RSOsPR10 protein shown in **Fig. 1**, suggest that the root-specific expression of RSOsPR10 was regulated at the transcriptional level. We also investigated the effects of SA on induction of *RSOsPR10* mRNA (**Fig. 3B**). Strong inhibitory effects were observed by simultaneous application of SA with NaCl, JA and ACC. These results support the idea that there is an antagonistic effect of SA on the JA and ET (or JA/ET) signal transduction pathway that regulates induction of *RSOsPR10* at the transcriptional level. This antagonistic relationship between the SA and JA/ET signaling pathways has been reported in various plants and in response to various external

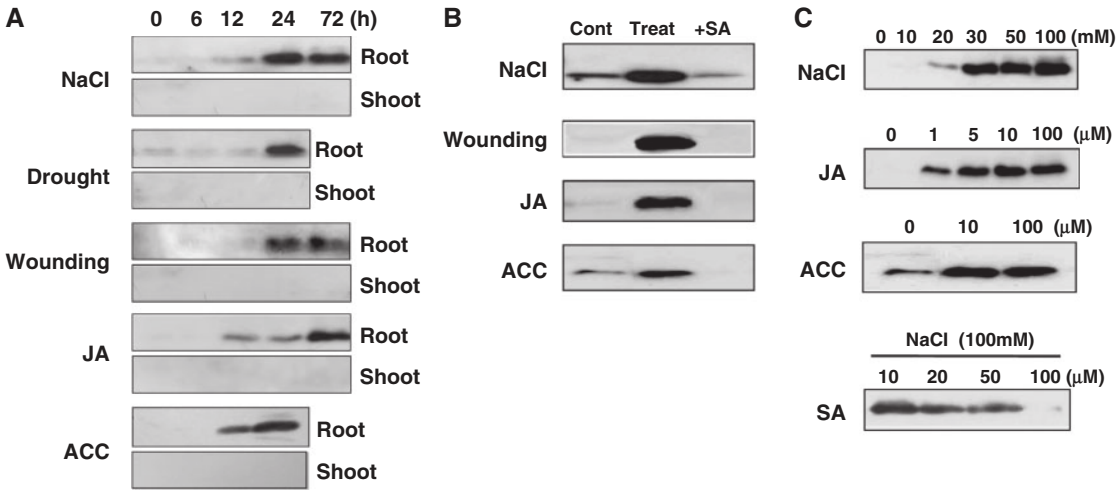

Fig. 1 Regulation of RSOsPR10 protein expression after stress and application of plant hormones. Two-week-old rice seedlings were treated with 100 mM NaCl, drought (air-dried), wounding (chopping), 100 μM JA, 100 μM ACC or 100 μM SA (A and B), or concentrations as indicated (C). Total protein (20 μg) was loaded on each lane. (A) Western blot analysis of RSOsPR10 expression. RSOsPR10 accumulated in roots, but not shoots, after wounding, NaCl, drought, JA and ACC treatments for the indicated times. (B) Induction of RSOsPR10 protein was completely suppressed by simultaneous application of SA with the indicated treatments for 24 h. (C) Dose-dependent effects of NaCl, JA, ACC and SA on expression of RSOsPR10. Treatments at the indicated concentrations were applied for 12 h.

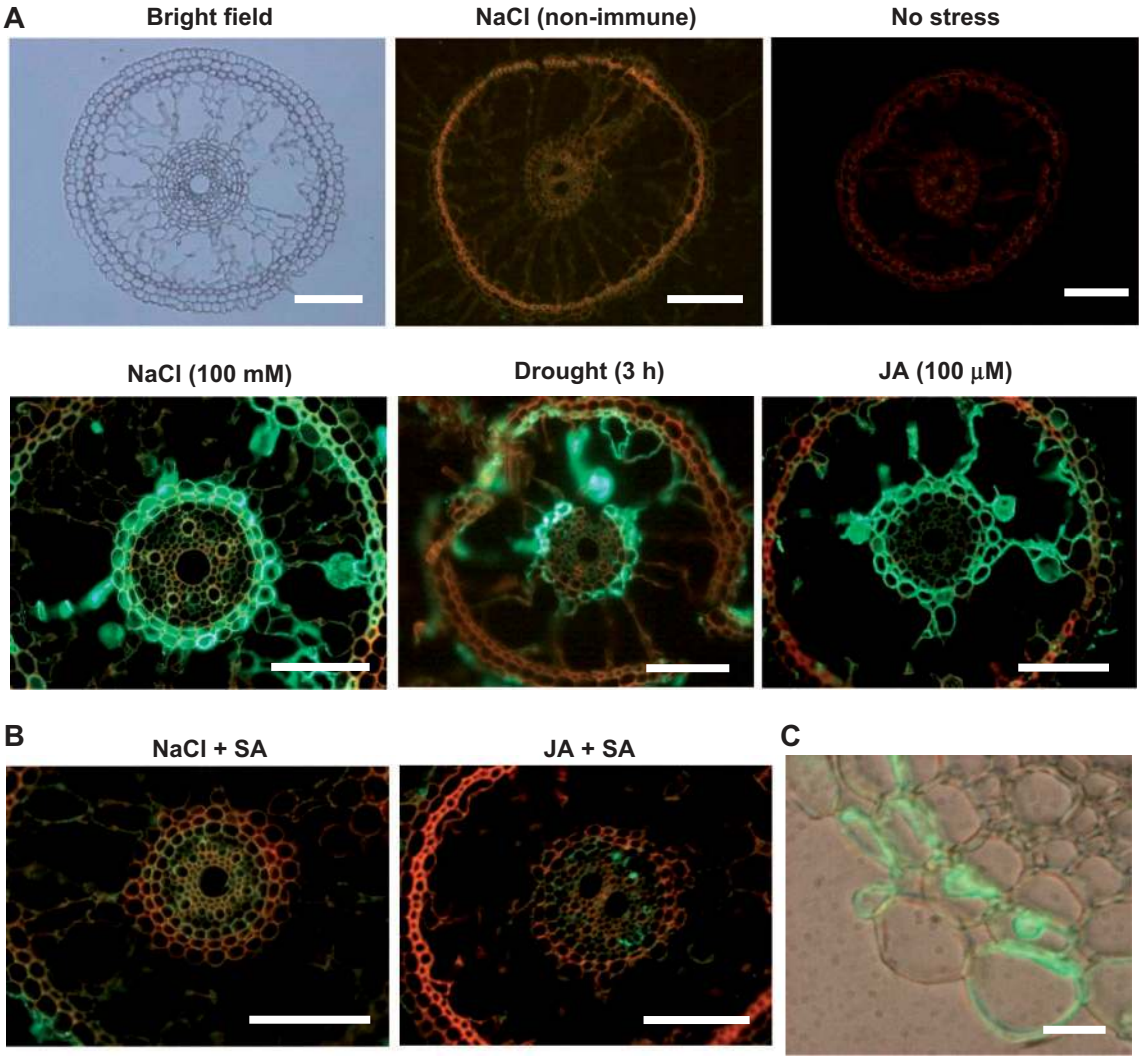

Fig. 2 Immunohistochemical detection of RSOsPR10 in rice root. (A) Green fluorescent signals in cortex cells after NaCl, drought and JA treatments. No signals were detected in non-stressed plants and non-immune serum control. Signal was concentrated around the inner and outer cell layers of the cortex. Bars = 10 μm. (B) Signals induced by NaCl and JA were almost completely inhibited by simultaneous application of SA (100 μM). Bars = 10 μm. (C) Signal was detected in the intracellular space after plasmolysis. Bar = 2 μm.

stresses (Koornneef et al. 2008, Leon-Reyes et al. 2009, Spoel et al. 2009). We investigated the dose-dependent effects of NaCl, JA, ACC and SA on expression of *RSOsPR10* mRNA. The results showed similar trends to those observed for protein expression (as shown in **Fig. 1C**); 50 mM NaCl, 10 μM JA and 20 μM ACC were sufficient to induce *RSOsPR10* mRNA, while 100 μM SA completely inhibited NaCl-induced accumulation of *RSOsPR10* mRNA (**Fig. 3C**).

Expression of *RSOsPR10* in seedlings of the JA-deficient mutant *hebiba*

RSOsPR10 was induced by exogenous application of JA; therefore, JA is possibly an endogenous signaling substance that is involved in expression of *RSOsPR10* in vivo. To examine further the role of JA, we determined the effects of endogenous JA on *RSOsPR10* expression in the JA-deficient mutant *hebiba* (*Oryza sativa* cv. Nihonmasari background). The mutant is deficient in the activity of a key enzyme in JA biosynthesis, resulting in almost no JA production in the seedlings (Riemann et al. 2003). NaCl and JA induced *RSOsPR10* expression in the mutant, similar to that in the wild type (**Fig. 4A**). Wounding and drought treatments also induced *RSOsPR10* expression in *hebiba* seedlings (**Fig. 4B, C**). The results clearly showed that JA biosynthesis is not always necessary for induction of RSOsPR10, although exogenous JA is a strong inducer, indicating the presence of JA-independent and JA-dependent pathways for *RSOsPR10* induction.

Fluctuation of JA and SA contents after NaCl and drought treatments

We measured the changes in endogenous JA and SA contents in rice roots after NaCl and drought treatments. NaCl caused

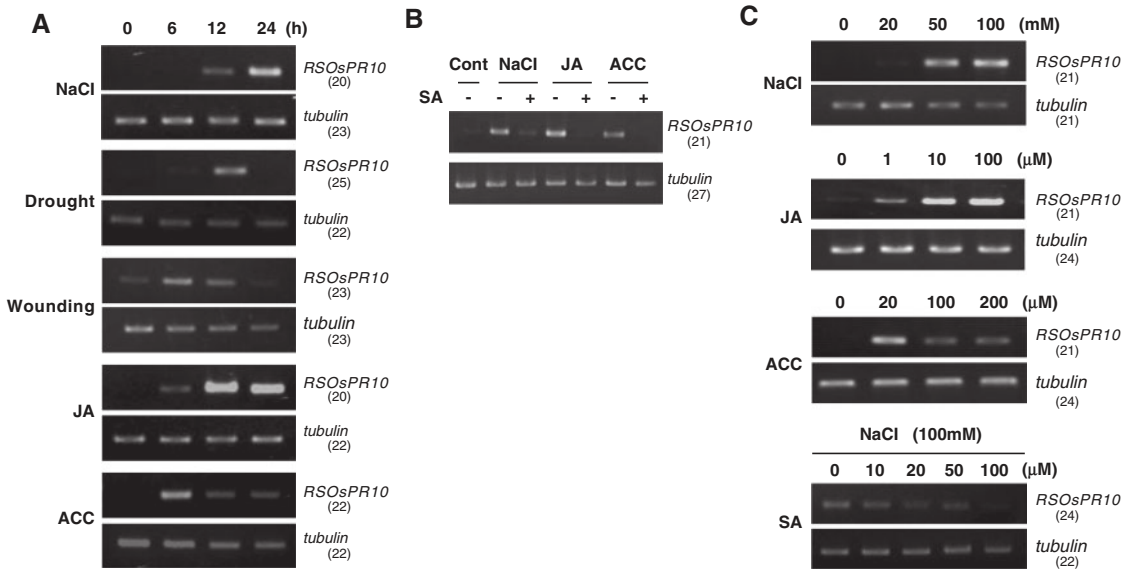

Fig. 3 Regulation of *RSOsPR10* mRNA expression after stress and signaling molecule treatments. Two-week-old rice seedlings were treated with 100 mM NaCl, drought (air-dried), wounding (chopping), 100 μM JA, 100 μM ACC and 100 μM SA for the indicated times (A and B). NaCl, JA, ACC and SA were used at the indicated concentrations (C). Cycle numbers for PCR are shown in parentheses. (A) RT–PCR analysis of *RSOsPR10* mRNA expression. Accumulation of *RSOsPR10* mRNA at the indicated times during abiotic stress (NaCl, drought and wounding) and exogenous JA and ACC treatments. (B) Induction of *RSOsPR10* mRNA was completely suppressed by simultaneous SA treatment. (C) Dose-dependent effects of NaCl, JA, ACC and SA on expression of *RSOsPR10* mRNA. All treatments were applied for 12 h.

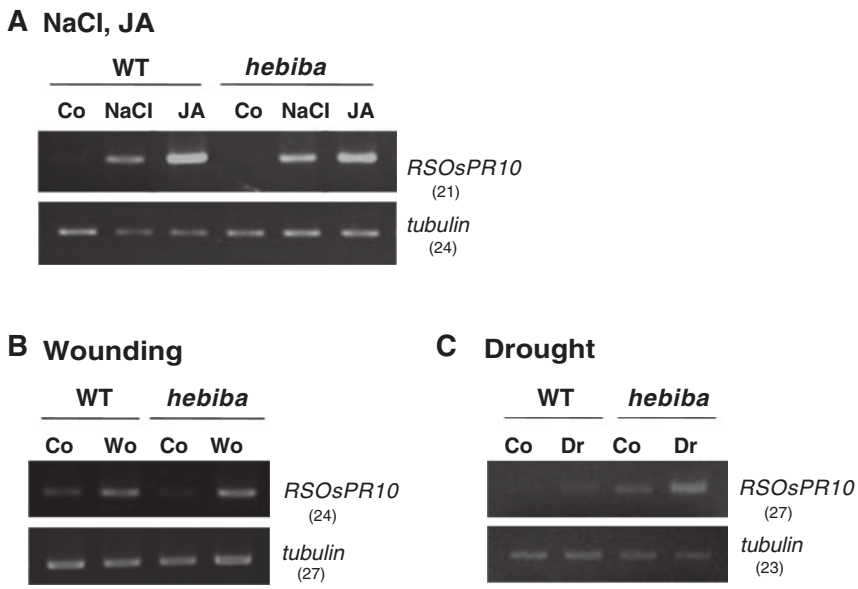

Fig. 4 Expression of *RSOsPR10* after NaCl and JA (A), wounding (B) and drought (C) treatments in roots of the wild-type (WT) and JA-deficient mutant *hebiba* (*O. sativa* cv. Nihonmasari). Two-week-old rice seedlings were treated with NaCl (100 mM), JA (100 μM), wounding (Wo) or drought (Dr; air-dried) for 12 h; Co, non-treated control. Details are the same as in **Fig. 3**.

slight fluctuations in both JA and SA contents during a 24 h treatment (**Fig. 5**). The contents were approximately 10 ng g^{-1} FW of JA and 100 ng g^{-1} FW of SA (corresponding to concentrations of approximately 0.05 and 0.7 μM, respectively), if JA and SA are evenly distributed in the root tissue. In contrast, drought treatment for 1 h induced about 100- and 10-fold increases in JA (~1,500 ng g^{-1} FW) and SA (~1,000 ng g^{-1} FW), respectively, corresponding to concentrations of about 8 μM JA and 7 μM SA. The concentration of exogenously applied JA required to induce *RSOsPR10* was 10 μM, and 100 μM SA was

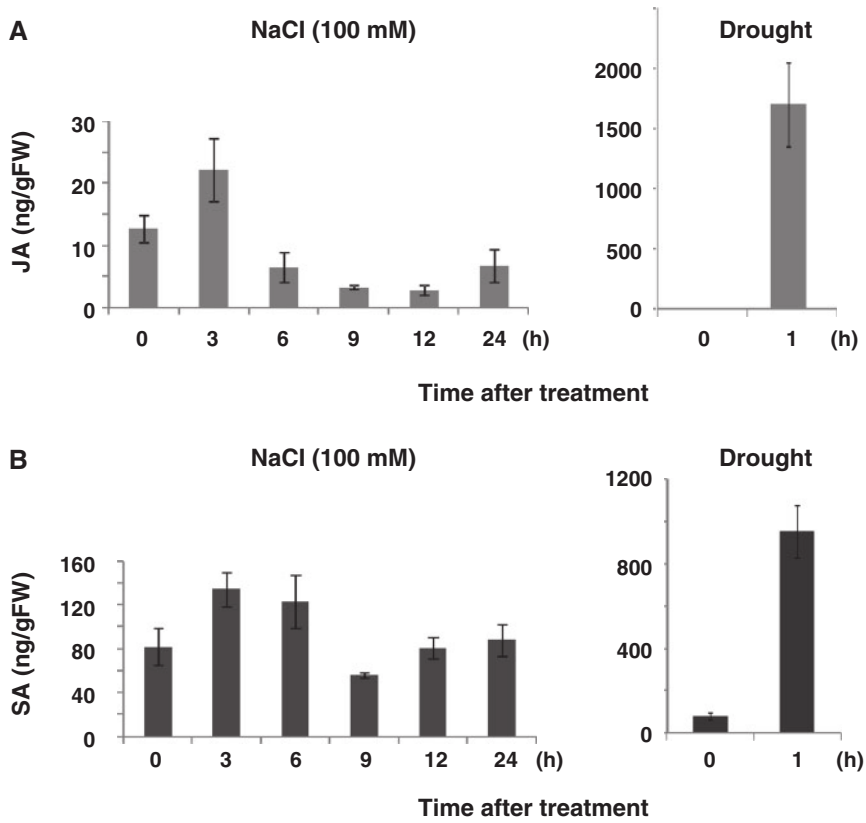

Fig. 5 JA (A) and SA (B) levels in rice roots after NaCl and drought treatments. Two-week-old rice seedlings were treated with NaCl (100 mM) and drought (air-dried), and JA and SA levels in the roots were determined at the indicated times (h) by LC-ESI-MS/MS. Values are means ± SE ($n = 5$).

required to inhibit the NaCl-induced increase in *RSOsPR10* (see **Figs. 1C**, **3C**). Therefore, the observed JA and SA contents after NaCl treatment were insufficient to induce or inhibit RSOsPR10 expression. After drought treatment, the concentration of JA (approximately 8 μM) could induce *RSOsPR10* accumulation, but that of SA (approximately 7 μM) would be insufficient to inhibit induction. However, as shown in the JA-deficient mutant (see **Fig. 4C**), drought treatment induced *RSOsPR10*, suggesting that a JA-independent signaling pathway induces RSOsPR10 expression in the response to drought stress. One possibility is that the ET pathway is responsible for the JA-independent induction of RSOsPR10. These results and those from the JA-deficient mutant indicate the presence of an ET pathway for the induction of RSOsPR10. In addition, exogenous JA induced RSOsPR10, even at concentrations as low as 1 μM (see **Figs. 1C**, **3C**). Together with these results, the finding that RSOsPR10 production was induced by both JA and ACC strongly suggests that a JA/ET signaling pathway operates for RSOsPR10 expression.

Expression of *OsERF1* transcription factor after NaCl, wounding, JA and ACC treatments

Since all of the above results indicated that a JA/ET signaling pathway operates for *RSOsPR10* expression, we further investigated the role of *ERF*, a transcription factor that acts downstream of JA and ET in stress responses of many plant species. ERF regulates the transcription of the genes by binding to the target DNA sequence (GCC-box) (Lorenzo 2003). Because two GCC-boxes are located at 2.8 and 3.3 kb upstream of the *RSOsPR10* gene, there is a possibility that the transcription factor ERF regulates *RSOsPR10* gene expression. First, we determined whether ACC induced the expression of 23 genes in the AP2/ERF family, all of which are known to be abundantly expressed in rice roots [RiceXpro database (http://ricexpro.dna.affrc.go.jp/), SALAD (http://salad.dna.affrc.go.jp/salad/)]. Of these 23 genes, the expression of only one, *OsERF1*, was affected by ACC (data not shown). Therefore, we analyzed the expression pattern of the *OsERF1* gene in more detail. *OsERF1* mRNA was transiently increased slightly earlier than that of *RSOsPR10* after NaCl and JA treatments, but the time course change of mRNA levels of *OsERF1* after wounding and ACC treatments appears similar to that of *RSOsPR10* (**Fig. 6A, B, D, E**). Even in the JA-deficient mutant *hebiba*, the expression of both *RSOsPR10* and *OsERF1* genes was up-regulated (**Fig. 6F**). These results suggest that OsERF1 might be involved via the JA/ET signaling cascade in inducing *RSOsPR10* expression.

Drought treatment induced *RSOsPR10*, but did not affect *OsERF1* expression (**Fig. 6C**). Together with the fact that ABA did not affect RSOsPR10 expression at either the transcriptional

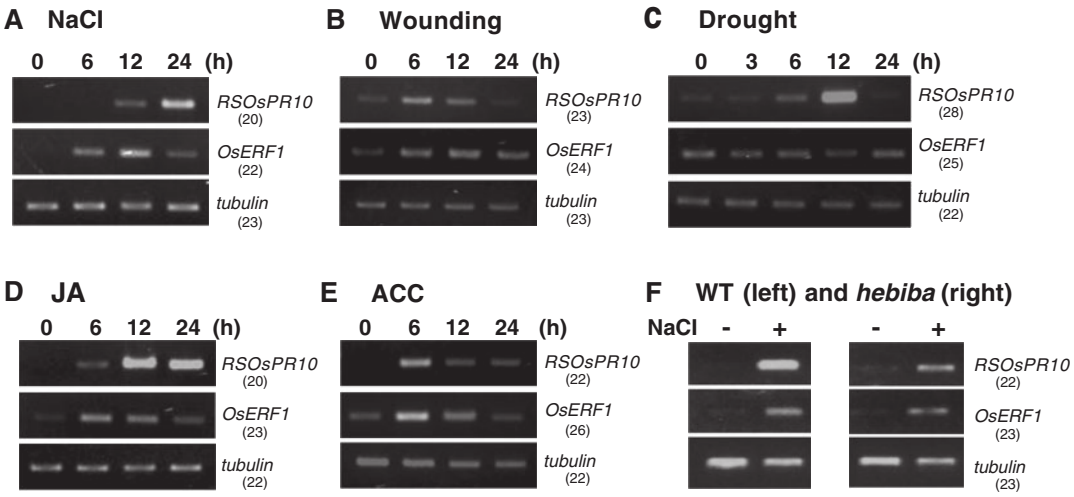

Fig. 6 Expression of *RSOsPR10* and *OsERF1* in roots of 2-week-old wild-type rice seedlings (Nipponbare) after NaCl (A), wounding (B), drought (C), JA (D) and ACC (E) treatments for the indicated periods, and after NaCl treatment in wild-type (WT, Nihonmasari) and *hebiba* seedlings (F). Details are the same as in **Fig. 3**.

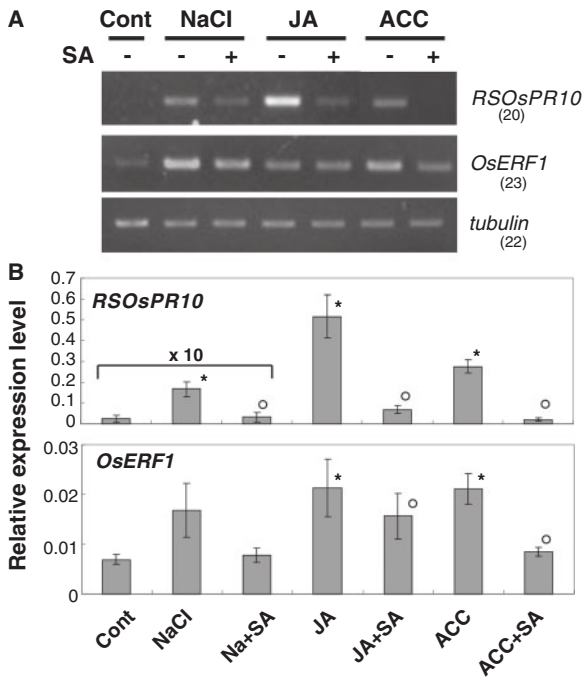

Fig. 7 Effects of SA on expression of *RSOsPR10* and *OsERF1* after NaCl, JA and ACC treatments. (A) RT–PCR analysis. Two-week-old rice seedlings were treated with NaCl (100 mM) or JA (100 μM) for 12 h and ACC (100 μM) for 6 h with or without SA (100 μM). Details are the same as in **Fig. 3**. (B) qRT-PCR analysis. All treatments were applied for 12 h. The relative ratio of gene expression was normalized against that of *ubiquitin*. Relative expression levels of RSOsPR10 after NaCl or NaCl+SA application and non-treated control are shown in a scale of ×10. Experiments were performed in biological triplicate and technical duplicate. Values represent the mean ± SE ($n = 6$). Asterisks indicate a significant difference ($P < 0.05$) between control and each treatment, and circles indicate a significant difference ($P < 0.05$) between the absence and presence of SA within the same treatments.

or the translational levels (Hashimoto et al. 2004), and therefore the endogenous signal substance(s) and transduction pathways of *RSOsPR10* induction after drought stress are still obscure.

Effect of SA on expression of *OsERF1* after NaCl, JA and ACC treatments

To investigate the possible mechanism of the antagonistic effect of SA on transcription of *RSOsPR10*, we used RT–PCR analyses to examine *OsERF1* expression after simultaneous SA treatment with NaCl, JA and ACC treatments (**Fig. 7A**). SA strongly inhibited *RSOsPR10* induction, with concomitant but weaker repression of *OsERF1*, at least after NaCl and ACC treatments. We analyzed the relationship between *RSOsPR10* and *OsERF1* expression patterns using quantitative real-time PCR (qRT-PCR). *OsERF1* suppression by SA + JA was detected with a significant difference ($P < 0.05$) (**Fig. 7B**), indicating that the increase of *OsERF1* expression by JA treatment was also repressed by SA treatment.

Discussion

Signaling pathways for induction of RSOsPR10 in response to abiotic stresses, such as salt, wounding and drought

The results of the present study suggest the possible involvement of a JA/ET pathway in which the transcription factor OsERF1 may have some function(s) in inducing *RSOsPR10* in response to salt and wounding stresses. There have been several studies on the possible involvement of a JA pathway in plant defense responses in rice (Kim et al. 2009, Ye et al. 2009). In the JA biosynthesis rice mutant *hebiba*, *RSOsPR10* was clearly

induced by exogenous JA (see **Fig. 4**), indicating the presence of a set of factor(s) regulating RSOsPR10 expression downstream of JA signaling. Interestingly, JA biosynthesis was strongly induced by drought stress but only marginally induced by salt stress (see **Fig. 5**). *OsERF1*, however, was induced by NaCl, wounding, JA and ET treatments. The maximal expression of *OsERF1* was observed slightly earlier than that of *RSOsPR10*. The profile was quite reliable in explaining its role in *RSOsPR10* induction, and showed similarities to expression of other defense-response genes, such as PDF1.2 in Arabidopsis (Lorenzo et al. 2003, Koornneef et al. 2008). Thus, it appears that OsERF1 may at least partially function in the ET and JA pathways for *RSOsPR10* induction in rice roots. Hu et al. (2008) also reported that overexpression of OsERF1 in Arabidopsis up-regulated ET-responsive genes such as β-chitinase and PDF1.2 (PR12).

We have some preliminary results from promoter analysis of *RSOsPR10* gene expression with rice lines expressing green fluorescent protein (GFP) under the control of 0–0.6 kb and 0–1.9 kb promoter fragments of *RSOsPR10*. However, the reporter was expressed in non-stressed rice seedlings, and expression was not root specific (data not shown). This indicates that important promoter elements must be located further upstream from the coding sequence. From database searches, we identified two GCC-boxes around the −3 kb region in the upstream region of the *RSOsPR10* gene. Therefore, we are now examining 0–2, 0–3 and 0–4 kb promoter::GUS and luciferase (LUC) constructs; the GUS constructs for expression in transgenic rice plants and the LUC constructs for a transient expression system with cultured rice cells. The promoter analysis will provide some answers about the involvement of OsERF1 in *RSOsPR10* induction.

Antagonistic effect of SA on JA/ET signaling pathways in regulation of RSOsPR10 expression

The antagonistic regulation between JA/ET- and SA-dependent defense pathways has been widely studied in Arabidopsis (van Wees et al. 2000, Miao and Zentgraf, 2007, Koornneef et al. 2008). The NPR1 protein integrates these pathways via its interactions with transcription factors such as TGAs and WRKYs (Leon-Reyes et al. 2009, Spoel et al. 2009, Zander et al. 2010). In rice, NPR1 and WRKYs share regulatory functions in defense responses (Lee et al. 2004, Chern et al. 2005, Shimono et al. 2007, Takatsuji et al. 2010). There were few reports about the antagonistic effects exhibited by SA and other phytohormones in rice. In the present study, we could not identify any SA-related signal transduction components that inhibited RSOsPR10 induction. Thus, SA induced some OsTGA(s) or OsWRKY(s) that are postulated to repress expression of *RSOsPR10* (**Fig. 8**). Analyses of multiple databases revealed that there are 13 TGAs and 97 WRKYs in rice (*O. sativa japonica*). Among these, 11 TGAs (RiceXpro database (http://ricexpro.dna.affrc.go.jp/)) and 56 WRKYs are expressed in the root (Rushton et al. 2010). Recently, SA signaling was examined in OsNPR1 overexpression and knock-down and OsWRKY45 knock-down rice lines (Chern et al. 2005, Shimono et al. 2007, Sugano et al. 2010). We intend to examine the expression of *RSOsPR10* in these mutants with or without SA treatment. As mentioned above, it is possible that OsERF1 functions to up-regulate *RSOsPR10* gene expression by binding to one or both of the GCC-boxes. In the present study, SA signaling only partially inhibited *OsERF1* expression (see **Fig. 7**). Therefore, stress-responsive expression of RSOsPR10 may be regulated by complex fine-tuning mechanisms, which involve concerted interactions with several transcription factors, including OsERF1, TGA(s) and WRKY(s).

Importance of RSOsPR10 in roots

As mentioned above, we have obtained results with rice and bent-grass overexpressing (OE) RSOsPR10 (T. Terakawa et al. in preparation, Patent Publication No. US 2009/0100552). Under growth chamber conditions, the OE rice exhibited increased drought tolerance. Our preliminary results indicated a possible function for RSOsPR10 in promoting root growth and root mass, which could lead to increased tolerance to soil desiccation and low nutrient conditions. OsRMC (*O. sativa* root mender curling) is involved in JA-mediated root development in rice, and RSOsPR10 expression was induced in *OsRMC* knock-down rice plants (Jiang et al. 2007). Methyl JA enhancement of adventitious rooting was also observed in tobacco cells (Fattorini et al. 2009). In the present study, stress and JA treatments induced accumulation of RSOsPR10 specifically in root cortex tissues (see **Fig. 2**). This indicates positive functions of JA-mediated signaling and RSOsPR10 in root growth in the response to environmental stresses. Further accumulation of data from OE rice and/or other plants (e.g. maize or soybean) in field conditions with and without environmental stresses will provide more information about the roles of RSOsPR10 and its homologs in plants.

Materials and Methods

Plant materials, growth conditions and stress treatments

We used rice (*O. sativa* L, cv. Nipponbare) and the JA-deficient rice mutant *hebiba* (*O. sativa* L, cv. Nihonmasari background) (Riemann et al. 2003). Nipponbare seeds were grown in water under controlled environment conditions (27°C, 12 h light/12 h dark cycle) as described previously (Hashimoto et al. 2004). *hebiba* plants were male sterile, such that they have to be maintained through the heterozygotes. Homozygotes were selected mostly according to Riemann et al. (2003). After germination at 27°C in the dark, candidates were transferred onto a floating polyethylene board with holes approximately 5 mm in diameter under a green safety light. They were cultured under 4 μmol m^{-2} s^{-1} red light at 25°C for 3.5 d. The homozygous

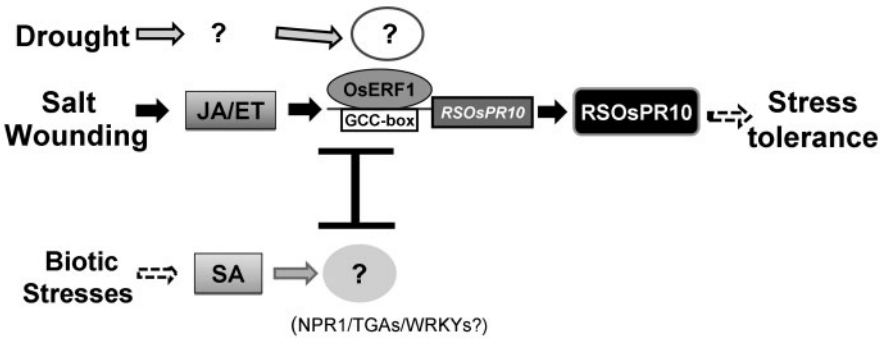

Fig. 8 Proposed model of the signaling pathway for RSOsPR10 expression in response to abiotic stresses in rice. The present work indicates that JA/ET signaling and the OsERF1 transcription factor are involved in up-regulation of *RSOsPR10* expression by salt and wounding stresses. The signaling pathway for drought-induced *RSOsPR10* expression remains to be studied. Antagonistic suppression of RSOsPR10 expression by SA may be regulated in part by OsERF1, and via some other unknown pathway(s), probably regulated by NPR1/TGAs/WRKYs.

mutant seedlings were selected by comparing the length of coleoptiles with that of the wild type.

Wild-type and *hebiba* seedlings (2 weeks old) were treated with NaCl, JA, ACC and SA by dipping the roots into water containing 100 mM NaCl (WAKO), 100 μM (+/−)-JA (Sigma), 100 μM ACC (Research Organics, Inc.), 100 μM SA (WAKO) or a combination of these chemicals. For the drought treatment, seedlings were air-dried on paper towels under controlled environment conditions (27°C, 55–65% relative humidity). For the wounding treatment, roots of seedling were cut into approximately 3–5 mm segments, and these segments were floated on fresh water. After treatments for appropriate time intervals, root samples and, if appropriate, shoot samples (sheaths and leaves) were collected and frozen in liquid nitrogen. All samples were stored at −80°C until use.

Protein extraction and Western blot analysis

Rice roots [from three seedlings, approximately 0.15 g (for some experiments, about 0.09 g of shoots)] were excised and immediately frozen in liquid nitrogen. The samples were ground by a cell disruptor (Micro-Smash, TOMY; www.digital-biology.co.jp) with two stainless steel beads (diameter: 5 mm) and crude proteins were extracted in 500 μl of extraction buffer [100 mM potassium phosphate buffer, pH 7.4, containing complete protease inhibitor cocktail (Roche Diagnostics; www.roche.com)] at 4°C in a bead cell disruptor. After centrifugation the proteins were concentrated using Amicon Ultra 0.5 ml centrifugal filters (molecular weight cut-off: 10,000) (Millipore; www.millipore.com). The amounts of extracted proteins were measured using a Pierce 660 nm protein assay (Thermo; www.piercenet.com). Protein samples (20 μg) were separated by SDS–PAGE [15% (w/v) polyacrylamide]. Electroblotting onto polyvinylidene fluoride (PVDF) membranes and visualization of immunoreacted peptide bands was performed as described elsewhere (Nishimura et al. 2009) using a 2,000-fold dilution of anti-RSOsPR10 antibody and a 5,000-fold dilution of peroxidase-conjugated secondary antibody (Vector Laboratories; www.vectorlabs.com), and a chemiluminescence system (ECL Plus kit; GE Healthcare; www.gelifesciences.com) followed by X-ray film exposure.

To generate recombinant RSOsPR10 with a His_6-tag (His_6-RSOsPR10), the full-length RSOsPR10 ORF was cloned into the *Nde*I and *Bam*HI sites of the pET16b vector (Novagen). The His_6-RSOsPR10 protein was expressed in *Escherichia coli* (BL21 DE3) and His_6-RSOsPR10 was purified using an Ni-NTA purification system (Invitrogen) according to the manufacturer's instructions. The purified antigen was used to immunize rabbits for the production of polyclonal antibodies (Qiagen, Japan). Specificity and affinity of the antigen for RSOsPR10 were assessed using crude root proteins extracted before and after stress (NaCl) treatment. Purified recombinant His_6-RSOsPR10 served as the positive control.

Immunohistochemical analysis of RSOsPR10

Sample preparation, cryosectioning and indirect immunofluorescence staining were performed as described by Endo et al. (2008) with minor modifications, as follows: phosphate-buffered saline (PBS) containing 3% bovine serum albumin (BSA) was used for blocking and 2% skim milk was used for immunoreactions. Primary anti-RSOsPR10 rabbit antibody and secondary anti-rabbit Alexa488 chicken antibody were used at 500- and 200-fold dilutions, respectively. The immunofluorescent images were acquired using an epifluorescent microscope (BX51, Olympus) equipped with a digital camera system (DP50, Olympus).

RNA extraction, RT–PCR and qRT-PCR

Total RNA was extracted from rice roots using the RNeasy Plant Mini Kit (Qiagen), and contaminating DNA was removed using RQ1 RNase-Free DNase (Promega). Total RNA (1 μg) was subjected to cDNA synthesis using oligo(dT) primers and M-MLV reverse transcriptase (Promega) according to the manufacturer's protocol. *RSOsPR10-*, *OsERF1-* and *α-tubulin*-specific primer

combinations (shown in **Supplementary Table S1A**) were used to amplify the transcripts. PCR was performed with the HotStarTaq Master Mix Kit (Qiagen) as follows: 94°C for 1 min, 55°C for 1 min and 72°C for 2 min. Numbers of cycles are indicated in each figure.

For qRT-PCR analysis, total RNA extraction, DNA elimination and cDNA synthesis were performed as described above. qRT-PCR was performed with a LightCycler 480 (Roche Diagnostics) using SYBR Green I Master mix. The primers used are listed in **Supplementary Table S1B**. The relative expression level of mRNA was determined using *ubiquitin* as the reference gene. All qRT-PCR experiments were performed in biological triplicate and technical duplicate.

Determination of JA and SA contents

Rice roots (approximately 40 mg FW) were frozen in liquid nitrogen. After grinding the tissue sample with metal beads, 2 ml of 80% acetonitrile containing 1% acetic acid was added and the mixture was gently shaken for h. Then, 40 ng of d_2-JA (Kanto chemicals) and 400 ng of d_6-SA (Sigma-Aldrich) were added as internal standards. After centrifugation, the supernatant was collected and the residue was re-extracted with 2 ml of 80% acetonitrile containing 1% acetic acid for 10 min. The supernatants were combined and subjected to cation exchange purification, and endogenous JA and SA were quantified by LC-ESI-MS/MS (liquid chromatography-electrospray ionization-tandem mass specrometry) as described previously (Yoshimoto et al. 2009).

Supplementary data

Supplementary data are available at PCP online.

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