Plasticity of root growth in response to environmental cues and stresses is a fundamental characteristic of land plants. However, the molecular basis underlying the regulation of root growth under stressful conditions is poorly understood. Here, we report that a rice nuclear factor, RICE SALT SENSITIVE3 (RSS3), regulates root cell elongation during adaptation to salinity. Loss of function of RSS3 only moderately inhibits cell elongation under normal conditions, but it provokes spontaneous root cell swelling, accompanied by severe root growth inhibition, under saline conditions. RSS3 is preferentially expressed in the root tip and forms a ternary complex with class-C basic helix-loop-helix (bHLH) transcription factors and JASMONATE ZIM-DOMAIN proteins, the latter of which are the key regulators of jasmonate (JA) signaling. The mutated protein arising from the rss3 allele fails to interact with bHLH factors, and the expression of a significant portion of JA-responsive genes is upregulated in rss3. These results, together with the known roles of JAs in root growth regulation, suggest that RSS3 modulates the expression of JA-responsive genes and plays a crucial role in a mechanism that sustains root cell elongation at appropriate rates under stressful conditions.

INTRODUCTION

In land plants, roots absorb water and soluble nutrients, in addition to anchoring the shoot system. The spatial distribution of roots, including primary and lateral roots, largely depends on environmental signals, such as water availability, gravity, and light, which are perceived by the root apex (Kiss et al., 2002, 2003; Kobayashi et al. 2007) and modulate regional cell elongation rates via mechanisms that involve auxins (Swarup et al., 2005; Kaneyasu et al., 2007).

Postembryonic root growth is sustained by continuous cell proliferation and subsequent cell differentiation. Cell division is maintained in the root apical meristem, or meristematic zone (MZ), which provides cells for elongation in the basal region, called the elongation zone (EZ). After elongation, cells undergo further differentiation or maturation in the maturation zone (MTZ), where root hairs and thickened secondary cell walls are formed. In contrast with root orientation, the rate of root growth is elaborately regulated in response to environmental changes at the level of both cell division and cell elongation. For example, when a plant grows under conditions of low phosphate availability, primary root growth stalls and the number and length of lateral roots increases (Svistoonoff et al., 2007). In the primary root, the phosphate deficiency is perceived in the apical region, resulting in the arrest of cell division and subsequent inhibition of cell elongation. This mode of regulation is mediated by LOW PHOSPHATE ROOT1 (LPR1), which encodes a multicopper oxidase (Svistoonoff et al., 2007). LPR1 has been proposed to modify a growth regulator in response to phosphate depletion in the apical region of the primary root. This in turn leads to the expression of genes, including those responsive to reactive oxygen species and to jasmonate (JA) and its derivatives (Chacón-López et al., 2011).

JAs are a class of plant hormone involved in defense-related responses caused by wounding and pathogens and in the regulation of developmental processes, including fruit ripening, pollen production, root growth, tendril coiling, and senescence (Creelman and Mullet, 1997; Wasternack, 2007). JAs play an important role in gene regulation by physically interacting with members of the R/B-like (or MYC-type) basic helix-loop-helix (bHLH) transcription factors, such as Arabidopsis homolog of myelocytomatosis oncogene MYC2. In the presence of...
l-le-conjugated JA, the F-box protein CORONATINE INSENSITIVE1 (COI1) binds to JAZs, resulting in their polyubiquitination and subsequent degradation by 26S proteasome (Chini et al., 2007; Thines et al., 2007). This releases the repression of R/B-like bHLH transcription factors from JAZs, leading to the transcriptional regulation of downstream target genes. In rice (Oryza sativa), overexpression of JAZ9, also called TIFY11a, using a stress-inducible promoter was reported to alleviate the growth inhibition resulting from salt and dehydration stress (Ye et al., 2009), although the downstream factors involved in the process were not identified.

In Arabidopsis, exogenously supplied JAs inhibit root growth, in terms of both cell division and cell elongation (Chen et al., 2011). This inhibition is completely suppressed in coi1 (Lorenzo et al., 2004; Chen et al., 2011). By contrast, loss-of-function mutations of MYC2 (jasmonate-insensitive1) exhibit weaker suppression of JA-induced root growth inhibition than coi1, suggesting that activation of MYC2 does not account for all of the JA-mediated inhibition of root growth (Lorenzo et al., 2004). Consistent with this, MYC2 was found to influence the expression of only a subset of JA-responsive genes (Dombrecbt et al., 2007). Moreover, myc2 combined with loss-of-function mutations of MYC3 and MYC4, the R/B-like bHLHs closely related to MYC2, further suppressed the JA-induced root growth inhibition, but the myc2 myc3 myc4 triple mutant was still relatively sensitive to JA than coi1 (Fernández-Calvo et al., 2011). Recently, MYC2 was suggested to reduce root meristem activity by repressing PLETHORA expression upon the activation of JA signaling (Chen et al., 2011). However, the transcriptional regulatory mechanisms that inhibit root cell elongation after JA perception remained unclear.

Salinity represents a widespread problem in agriculture. Despite extensive studies of the salt stress response and tolerance using a model dicot, Arabidopsis (Zhu, 2001; Yamaguchi and Blumwald, 2005; Takeda and Matsuoka, 2008; Qin et al., 2011), the mechanism underlying the control of root growth under salinity is still not well understood, especially in monocots. We previously showed that a rice protein RICE SALT SENSITIVE1 (RSS1), whose stability is controlled in a cell cycle phase-dependent manner, is required for the maintenance of meristem functions in the shoot and root under stressful conditions (Ogawa et al., 2011). In rss1 roots, cell proliferation activity is reduced upon salinity stress, resulting in a decrease in the number of cells in both the MZ and EZ. This finding illustrates the importance of the coordinated regulation of cell division and elongation in response to environmental changes.

In this study, we show that a rice nuclear factor, RSS3, has a regulatory role in root cell elongation. RSS3 interacts not only with JAZs, but also with non-R/B-like bHLH transcription factors and forms an RSS3-JAZ-bHLH ternary complex in the nucleus. Loss of function of RSS3 activates the expression of a subset of JA-induced genes in the root apex and restricts cell elongation but does not primarily affect cell division activity. Under high-salinity conditions, rss3 mutants exhibit severely inhibited root growth, concomitant with root cell swelling. These results reveal a molecular mechanism for the modulation of JA-responsive gene regulation, which is especially crucial for the control of root elongation in response to stressful conditions.

**RESULTS**

**The rss3 Mutant Has a Defect in Root Cell Elongation**

In the course of a genetic screen of rice to identify loci responsible for salt tolerance (Ogawa et al., 2011), we identified a recessive mutant designated rss3. When grown under high-salinity conditions, rss3 exhibited severely impaired root growth compared with the wild type (Figures 1A and 1B; see Supplemental Figure 1A online). Root growth in the absence of salinity stress was also inhibited in rss3, but only moderately. The length of cells in the MTZ of roots of rss3 plants grown under unstressed conditions was markedly shorter than those of the wild type (see Supplemental Figure 1B online). The inhibition of root growth in rss3 appeared to be primarily due to reduced cell elongation because the size of the EZ, but not of the MZ, was reduced in the mutant, and the number of cells in both the EZ and MZ was not affected in the mutant (Figures 1C and 1D). The exaggerated inhibition of root growth under salinity conditions also appeared to be mainly due to compromised cell elongation but was accompanied by aberrant cellular arrangement and the formation of oblique cell plates in the EZ, swelling of the cells in the EZ (see Supplemental Figures 2A and 2B online), and a waviness of the root surface at the MTZ (Figure 1E). Moreover, rss3 roots show impaired flexibility, in a salinity-dependent manner. When extracted from the medium, the wild-type roots exhibited a paintbrush-like shape, but rss3 roots did not, due to stiffness (Figure 1A). These rss3 phenotypes were observed both in seminal and adventitious crown roots, although rss3 showed a slight decrease in the number of crown roots, regardless of the salinity conditions (see Supplemental Figure 1C online). Collectively, these results suggest that RSS3 has an important role in root cell elongation and has additional functions in regulating root elongation under high-salinity conditions.

When seedlings were grown under continuous light, root coiling was frequently observed in the wild type under nonstressed conditions but not under salinity stress. By contrast, root coiling was not observed in rss3, even under nonstressed conditions (see Supplemental Figures 3A and 3B online). This is not due to a defect in the gravity-sensing machinery since root gravitropism is not impaired in rss3 (see Supplemental Figure 3C online). These results also suggest that the flexibility of rss3 roots is impaired.

**RSS3 Encodes a Nuclear Protein Homologous to the Regulatory Domain of the R/B-Like bHLH Transcription Factors**

The causative gene of the rss3 mutant was identified by map-based cloning as Os11g0446000 (Figure 2A). Among four genes located in the mapped region, we found a deletion in Os11g0446000 in rss3. Transformation with a genomic fragment of the wild-type allele of Os11g0446000 complemented the recessive mutant phenotype of rss3 (see Supplemental Figure 4A online). RSS3 encodes a protein consisting of 458 amino acids. RSS3 contains a region homologous to the postulated regulatory domain conserved among the R/B-like bHLH transcription factors (Li et al., 2006), which contains four conserved regions (I to IV) (Figures 2B and 2C). Region II is predicted to constitute the central sequence of the JAZ interacting domain (Cheng et al.,
RSS3 does not contain a bHLH domain or another known DNA binding motif. 

**rss3** contains a 47-bp deletion at the junction between the 6th intron and 7th exon (Figure 2A), which results in an altered splicing pattern (see Supplemental Figure 4B online) and the production of a mutated protein that lacks 15 amino acids in region IV and six amino acids in the subsequent C-terminal nonconserved region (Figure 2C).

In seedlings, **RSS3** was expressed predominantly in the roots, particularly in the root tip (see Supplemental Figure 5 online). A truncated form of **RSS3** transcript was expressed in the mutant roots, at levels that were apparently higher than those of the nondeleted **RSS3** transcript in the wild type (see Supplemental Figure 4C online). When enhanced green fluorescent protein (EGFP)-tagged **RSS3** was expressed under the control of the **RSS3** promoter, EGFP signals were detected at high levels in the MZ and at low levels in the EZ of the root tip (Figure 2D, left). The fusion protein was localized almost exclusively in the nuclei of the MZ cells (Figure 2D, top right). By contrast, nonfused EGFP expressed under the control of the constitutive *actin* gene promoter was localized in both nuclei and the cytoplasm of MZ cells. In the EZ region bordering the MZ, **RSS3**-EGFP was detected in both the nucleus and the cytoplasm (Figure 2D, bottom right). These data suggest that **RSS3** functions as a nuclear protein in the root tip and are consistent with the mutant phenotypes.

**RSS3 Interacts with the Transcription Factors bHLH089 and bHLH094**

We then used yeast two-hybrid (Y2H) screening to search for a protein that interacts with **RSS3**. Because the full-length **RSS3** caused a self-activation in the Y2H system, we used a version of **RSS3** that lacked the C-terminal 140 amino acids (**RSS3**N140) as bait and identified a non-MYC-type bHLH transcription factor (bHLH094) as an **RSS3** binding protein. The cDNA for bHLH094 expressed in the screening was truncated and encoded only the C-terminal part (amino acids 143 to 256) of this protein (Figure 3A). An analysis using further deletions of the truncated bHLH094 revealed that a region (amino acids 143 to 200) corresponding to the bHLH and subsequent C-terminal regions was necessary and sufficient for the interaction with **RSS3** (Figure 3B). We also examined the ability of a deletion series of the **RSS3** bait to interact with bHLH094 and identified a region of **RSS3** (amino acids 1 to 203) as being responsible for the interaction (Figure 3C). Importantly, the 21-amino acid sequence missing from **RSS3** in the **rss3** mutant was required for the interaction with the bHLH factor.
In vivo interaction of RSS3 and bHLH094 was examined by bimolecular fluorescence complementation (BiFC) and acceptor photobleaching Förster resonant energy transfer (apFRET) analyses (Figure 4). When expressed in onion (Allium cepa) epidermal cells, yellow fluorescent protein (YFP)–fused RSS3 localized to both the nucleus and cytoplasm, whereas cyan fluorescent protein (CFP)–fused bHLH094 (bHLH094-CFP) localized exclusively to the nucleus (Figure 4A). For BiFC assay, the N-terminal region of RSS3 fused to the N-terminal half of YFP (YFPN-RSS3N203) was coexpressed with the full-length bHLH094 fused to the C-terminal half of YFP (bHLH094-YFPC). The complemented YFP signals were predominantly detected in the nucleus (Figure 4B), indicating the in vivo interaction of the two proteins. In the apFRET assay, bHLH094-CFP was expressed together with YFP-RSS3N203 or YFP-RSS3full-length in onion epidermal cells. We measured the strength of the CFP signal before and after photobleaching of the YFP and calculated the efficiency of FRET (see Methods for details). After photobleaching of YFP-RSS3N203 or YFP-RSS3full-length in the nucleus, an increase in bHLH094-CFP signal was observed (Figures 4C to 4E), reflecting the interaction of the YFP and CFP fusion proteins. By contrast, energy transfer was not detected when nonfused YFP was expressed as a control, instead of YFP fused to RSS3N203 or RSS3full-length (Figures 4C to 4E). These
results strongly suggest that RSS3 and bHLH094 can interact in plant cells.

We also examined which types of bHLH factors bind to RSS3. Among more than 150 bHLH factors in rice, bHLH094 belongs to the class-C group (Li et al., 2006). The distinguishing feature of class-C members is the conservation of bHLH sequences and the subsequent C-terminal regions (see Supplemental Figure 6 online). To examine whether RSS3 interacts with other rice class-C bHLH factors, we selected bHLH089, which is classified in the same minor clade that bHLH094 belongs to, and three representative bHLH transcription factors from other clades of

Figure 3. The Interaction between RSS3 and bHLH094 in Yeast.

(A) Schematic representation of the full-length rice bHLH094 protein and its derivative (Y2H prey) obtained by Y2H screening. The bHLH domain is represented in blue, and the region that is conserved specifically in the class-C bHLH subfamily is shown in orange. AD, activation domain of GAL4.

(B) Analysis of the domains of bHLH094 required for binding to the bait RSS3^N318 as determined by Y2H assay. Numbers indicate terminal amino acid residues of bHLH094 fused to AD.

(C) bHLH094 interacting domains of RSS3 analyzed by a Y2H assay. Numbers indicate the terminal amino acid residues of RSS3 fused to GAL4 DNA binding domain (DBD). rss3 indicates the truncated protein translated in rss3 with a 21–amino acid deletion. Plasmids carrying AD alone (B) or DBD alone (C) were used as controls. Yeast transformants were spotted onto control medium [(-L,W), lacking Leu and Trp] or selective medium [(-A,H,L,W), lacking Ade, His, Leu, and Trp], and the activation of ADE2 and His3 reporter genes was monitored.

Figure 4. The in Vivo Interaction between RSS3 and bHLH094.

(A) The subcellular localization of rice bHLH094-CFP and YFP-RSS3^N203 in onion epidermal cells. bHLH094 localized exclusively to the nucleus, whereas RSS3 localized to both the nucleus and cytoplasm. c, cytoplasm; n, nucleus.

(B) BIFC assay to verify interaction between RSS3 with bHLH094. YFP^N, RSS3^N203 and bHLH094-YFP^C or YFP^C were transiently expressed with mRFP1 in onion epidermal cells.

(C) Representative images of the apFRET assay using bHLH094-CFP and YFP-RSS3^N203 (left four) or nonfused YFP (right four). Signal intensities of the acceptor and donor in onion cells before and after acceptor photobleaching were analyzed. Pseudocolor images represent fluorescence intensities.

(D) Evaluation of the interaction between YFP-RSS3^N203 and the class-C bHLH proteins fused to CFP by an apFRET assay. FRET efficiency was shown with mean values ± SD; n = 3 to 8.

(E) apFRET analysis of full-length RSS3 fused to YFP and class-C bHLH proteins fused to CFP in the nucleus of onion cells. Mean ± SD; n = 3 to 5. The names of rice bHLHs are as reported (Li et al., 2006). The control indicates the combination of YFP and bHLH094-CFP. Asterisks indicate P value < 0.05 (Student’s t test). Bars = 200 µm in (A) and (B) and 20 µm in (C).
class-C (bHLH092, bHLH079, and bHLH096). The in vivo interaction between these bHLH factors and RSS3 was examined by the apFRET assay. By the combinatorial expression of the respective bHLH-CFPs with YFP-RSS3N203, FRET efficiency that reflects the physical interaction was positive with bHLH089 but negative with bHLH092, bHLH079, and bHLH096 (Figure 4D). Similarly, the interaction of RSS3full-length with bHLH094 and bHLH089, but not with bHLH092, was demonstrated by the apFRET assay (Figure 4E). These results indicate that RSS3 can specifically interact with bHLH094 and bHLH089. Interestingly, both full-length bHLH094 and bHLH089 lack the N-terminal regulatory domains that are conserved among R/B-like bHLH factors and RSS3 (Li et al., 2006). The genes encoding bHLH094 and bHLH089 were expressed in the root tip, although high levels of expression were also observed in the basal tissues of the shoot (see Supplemental Figure 5 online). Taken together, these results suggest that RSS3 acts as a regulatory module for bHLH094 and bHLH089 and thus is directly involved in transcriptional regulation in the root tip.

RSS3 Interacts with JAZ Proteins

As described above, RSS3, but not bHLH094 or bHLH089, contains the regulatory domain conserved among R/B-like bHLH transcription factors. Considering that region II of the regulatory domain is a part of the JAZ interacting domain, we speculated that RSS3 is capable of binding to JAZs. To test this possibility, the binding abilities of 12 of the 15 rice JAZs (Ye et al., 2009) to RSS3 were examined by the Y2H assay. This analysis showed that JAZ8, JAZ9, and JAZ11 interact specifically with RSS3 (Figure 5A). Binding of RSS3 to JAZ9 and JAZ11, but not to JAZ8, was further confirmed in vivo by the apFRET assay (Figures 5B and 5C).

JAZ9 and JAZ11 were preferentially expressed in the apical and distal regions of the root tip, respectively (see Supplemental Figure 5 online). A comparison of Arabidopsis and rice JAZ proteins revealed that Os-JAZ9 and Os-JAZ11 belong to a rice-specific clade (Ye et al., 2009; see Supplemental Figure 7 online). Both Os-JAZ9 and Os-JAZ11 contain a canonical ZIM domain and a Jas domain, which are highly conserved among JAZ proteins (Figure 5D; see Supplemental Figure 7 online). The Jas domain contains a JAZ degron, which contains a critical role for JA-Ile-dependent binding to COI1 (Chini et al., 2007; Melotto et al., 2008; Sheard et al., 2010). In several Arabidopsis JAZ proteins, the Jas domain and its surrounding region is involved in the interaction with MYC-type bHLH transcription factors (Chini et al., 2007; Shyu et al., 2012). Several Os-JAZs were demonstrated to interact with COI1 (Seo et al., 2011), suggesting that the
Rice JAZ homologs are able to function with COI1 in JA perception. Os-JAZ9 and Os-JAZ11 also contain the consensus sequence of the JAZ degron, including the two basic amino acid residues (6th and 7th relative to the first amino acid of the JAZ degron) and the strictly conserved Phe residue that are required for the interaction with COI1 and JA-Ile (Melotto et al., 2008; Sheard et al., 2010) (see Supplemental Figure 7B online). In addition, most of the amino acids that are involved in hydrogen bond formation in the complex that forms between JA-Ile, COI1, and the JAZ peptide are conserved in Os-JAZ9 and Os-JAZ11 and the two rice COI1 proteins (see Supplemental Figure 8 online). Moreover, both JAZ9 and JAZ11 was shown to bind to the rice COI1 homologs at least by Y2H assay in the presence of coronatine, a structural analog of JA-Ile (Lee et al., 2013). Therefore, Os-JAZ9 and Os-JAZ11 may also function through their binding to JA-Ile.

RSS3 Forms a Ternary Complex with Rice bHLH094 and JAZ9

Based on the finding that RSS3 has two different binding partners, we examined whether RSS3 is capable of forming a ternary complex with the bHLH factor and JAZ by yeast three-hybrid assay (Figure 6A). Positive interaction was observed when RSS3N318 was coexpressed with bHLH094 and JAZ9. Moreover, when the 21–amino acid deletion mimicking the rss3 mutation was introduced into RSS3N318 (RSS3N318_D21), the interaction was disrupted. In vivo ternary complex formation was further examined using a combination of the BiFC and apFRET assays (BiFC-apFRET) (Figures 6B and 6C). YFPN-RSS3N318 and Os-bHLH094-YFPc were coexpressed in onion epidermal cells, together with ECFP-fused Os-JAZ9 (ECFP-OsJAZ9). After photobleaching, the YFP fluorophore reconstituted from YFPN-RSS3N318 and OsbHLH094-YFPc, an increase in ECFP-OsJAZ9 signal was observed in the nucleus (Figures 6B and 6C). By contrast, no signal increase was detected when YFPN-RSS3N318 and OsbHLH094-YFPc were coexpressed with nonfused ECFP. These results suggest that RSS3, Os-JAZ9, and Os-bHLH094 form a ternary complex in the nucleus.

RSS3 and JAZ together Repress bHLH094-Mediated Transcriptional Activation

bHLH factors are known to bind to specific DNA sequences with a minimal consensus motif, called the E-box (CANNTG, where N corresponds to any nucleotide) (Atchley and Fitch, 1997). The strength of the interaction varies with the identity of N and differs between different bHLH transcription factors. For example, Arabidopsis MYC2 has high binding affinities for CACGTG (also called the G-box) and CATATTG, but not for CAATTG and CAGCTG sequences (Godoy et al., 2011). We examined whether Os-bHLH094 acts on the same DNA sequences as Arabidopsis MYC2. We constructed LUC reporter genes carrying a triplicate repeat of the respective E-box sequences fused to the upstream region of a cauliflower mosaic virus (CaMV) 35S minimal promoter and cotransformed rice cultured cell protoplasts with these constructs and bHLH094. As shown in Figure 7A, bHLH094 enhanced the expression of reporters carrying (CACGTG)3, (CACGTG)3, and (CACGTG)3, as analyzed by BiFC-apFRET. Representative images (B) and FRET efficiencies (C) of apFRET in onion cells using YFPN-RSS3N318, bHLH094-YFPc, and ECFP-JAZ9 or nonfused ECFP are shown. Pseudocolor images represent fluorescence intensities. Bar = 10 μm. Mean ± SD; n = 3 (ECFP) and 6 (ECFP-JAZ9). Asterisks indicate P value < 0.05 (Student’s t test).
Expression of Salt-Responsive Genes Is Largely Altered in rss3

To gain further insight into the function of RSS3, we performed a transcriptomic analysis of the root tip, using a rice 44K microarray. First, we analyzed the relationship between the effects of the mutant genotype (rss3 mutation) and those of salinity (100 mM NaCl treatment) using two-way analysis of variance (ANOVA). As illustrated in the Venn diagram (Figure 8A), nearly half (49.5%; 3366/6884) of the genes affected by salinity stress were also affected by rss3 (bold numbers). These 3366 genes also accounted for half (48.9%) of the 6884 genes affected by rss3. For most of these 3366 genes, the altered expression in rss3 was observed under either nonstress or salinity stress conditions, and the salt-responsiveness of these genes seemed not to be changed between the wild type and rss3 (Figure 8B). This is reflected by the small proportion of the interactively affected genes (genotype × salinity), the salt-responsiveness of which was modified in rss3 (183/3366; Figure 8A). The proportion of the 183 genes interdependently affected by salinity and rss3 was also small among the total number of affected genes (10372; Figure 8A). Similar tendencies were observed when the differentially expressed genes were further filtered with more stringent fold-change criteria (≥2 or <0.5) (Figure 8A, numbers in gray, parentheses). These results suggest that RSS3 has global effects on the salt stress response pathways but does not constitute stress response signaling mechanisms.

A Substantial Proportion of JA-Responsive Genes Are Activated in rss3

Prompted by the finding that RSS3 forms complexes with JAZ and bHLH proteins, we explored whether the expression of JA-responsive genes was affected by rss3 (Figure 8C; see Supplemental Data Sets 1A and 1B online). For this analysis, genes that were altered in expression by rss3 were further selected with a cutoff of 1.5-fold change and with significance evaluated by rank product (P < 0.01, percentage of false positive [pfp] < 0.05). JA-responsive genes were selected in the same manner using the data deposited by Miyamoto et al. (2012) in the Gene Expression Omnibus of the National Center for Biotechnology Information (GSE32633). Notably, 20% of 112 JA-inducible genes were upregulated in rss3 under nonstressed conditions (Figure 8C). Similarly, 28% of the JA-inducible genes were upregulated in rss3 under salinity stress. These correspond to approximately an eightfold (P = 4.5e⁻¹⁴, hypergeometric distribution) and 11-fold (P = 2.1e⁻²⁰, hypergeometric distribution) enrichment, as the percentage of genes upregulated in rss3 out of the total number of genes examined in the array was 2.5 and 2.6% under nonstressed and salinity conditions, respectively. Only a small portion of JA-induced genes was downregulated in rss3 in either nonstressed or salinity conditions (5 and 2%, respectively). The expression of genes that are repressed by JA was less significantly affected by rss3. These results suggest that RSS3 has a role in repressing a portion but not all of the JA-inducible genes. Among the JAreponsive genes, the expression of which was altered in rss3, we found no significant biases to certain gene categories with respect to cellular metabolism and/or molecular function (see Supplemental Data Set 1C online).

To further evaluate the role of RSS3 in the regulation of JA-inducible genes, we examined the expression pattern of several JA-responsive genes. Wild-type and rss3 seedlings were grown in a hypotonic culture medium and transferred into the medium in the presence or absence of 100 μM JA. In the wild-type root tips, the expression of JAZ7, JAZ8, and SECOLOGANIN SYNTHASE (SLS) was induced by JA within 6 to 16 h (Figure 8D). The expression levels of these genes after JA treatment were further increased in rss3 (Figure 8D), indicating that RSS3 has a role in mediating negative regulation of JA-induced gene expression. Taken together, our results suggest that the JAZ-RSS3-bHLH ternary complex regulates the expression of JA-responsive genes in root tips. It is likely that the repressive function of this complex is due to the JAZ component, as is the case for the well-known JAZ-MYC2 complex in Arabidopsis (Chini et al., 2007; Thines et al., 2007).

It is noteworthy that, in the absence of exogenously supplied JA, the JA-induced genes are upregulated in rss3 in the conditions used for microarray analysis (Figure 8C; see Supplemental Figure 9A online) but not significantly in the hypotonic culture conditions (Figure 8D). This suggests that difference in physiological state in the root tip between the experimental setups affects the expression of JA-induced genes. We confirmed that, in the growth conditions in which root elongation was reduced in rss3 (Figure 1), the expression levels of the JA-induced genes in rss3 was considerably higher than those in the wild type grown in the absence of exogenous JA (see Supplemental Figure 9B online).

Low Levels of JA Inhibit Cell Elongation but Not Cell Division in the Root Tip

JAs inhibit cell division in the Arabidopsis root tip (Chen et al., 2011). In Arabidopsis cultured cells, cell cycle arrest at G2 has been reported for treatment with 50 μM JA (Pauwels et al., 2008). By contrast, rss3 resulted in reduced cell elongation but did not affect meristematic activity in the absence of exogenous JA (Figure 1). To verify the possibility that this reflects the
derepression of JA-responsive genes in rss3, we examined if treatment of the wild type with low concentrations of JA would phenocopy the reduced root cell elongation phenotype of rss3. Even though JA response is provoked in rss3 without JA treatment, it was less than those in wild type grown in the presence of 1 or 5 μM JA (see Supplemental Figure 9B online). We therefore expected that these concentrations of JA would inhibit root cell elongation specifically in EZ in the wild type. In fact, 5 μM JA caused a decrease in the size of the EZ but not of the MZ in the wild-type root tip, whereas both the MZ and EZ were reduced in size by 10 μM JA. In the MTZ, a reduction in cell size was observed even with 5 μM JA (see Supplemental Figures 10A and 10B online).

To explore these concentration-dependent differential effects of JAs on root tip cells, we monitored the expression levels of a S-phase marker gene, PCNA, and a M-phase cyclin, CycB2;1. The expression of CycB2;1 was repressed by 10 μM JA, but not by 5 μM, while the expression of PCNA was not significantly changed by JA at either concentration (see Supplemental Figure 10C online). These results further support the above idea that the effect of rss3 is mimicked by low concentrations of JA.

DISCUSSION

JAs are known to regulate root elongation in a wide variety of plant species (Corbineau et al., 1988; Creelman and Mullet, 1997; Ulloa et al., 2002; Uppalapati et al., 2005). In this article, we have shown that RSS3 is a novel regulatory factor of transcription, particularly in the regulation of JA-induced genes, that controls root cell elongation. RSS3 is expressed predominantly in the root tip, particularly in the MZ and in the EZ region bordering MZ. The function of RSS3 in transcriptional regulation was mainly evidenced by the interaction of RSS3 with bHLH094 and bHLH089 in the nucleus. We also demonstrated that RSS3 binds to JAZ9 and JAZ11 and thereby forms a ternary complex with bHLH094 and JAZ9. It is conceivable that bHLH089 and JAZ11 also form a similar complex with RSS3, based on their structural similarities to bHLH094 and JAZ9, respectively. RSS3 is likely to mediate the repression of bHLH factors by JAZ proteins, some of which are proposed to mediate active repression through NOVEL INTERACTOR OF JAZ (NINJA) and TOPLESS (TPL) (Pauwels et al., 2010).

We propose that RSS3 represses the expression of JA-induced genes because a portion of JA-responsive genes was upregulated in the root tip of rss3, and the levels of expression of...
Figure 8. The Expression of Salt- and JA-Responsive Genes Is Altered in rss3.

(A) Venn diagram summarizing the gene expression patterns examined using rice 44K microarrays. Differentially expressed genes based on three parameters, genotype (wild type versus rss3), salinity (with or without 100 mM NaCl), and interaction (genotype × interaction), were categorized by two-way ANOVA (P < 0.01) (bold). The differentially expressed genes were further filtered by fold change criteria (≥2 or ≤0.5) (gray, parentheses). The numbers of nonredundant genes in each category are indicated. The interaction between genotype and salinity, genes that were interdependently affected by differences in the genotype and growth conditions.

(B) Heat map views of 3366 nonredundant genes whose expression was altered in rss3 and by salinity in (A). WT, the wild type.

(C) Proportion of JA-responsive genes among upregulated (red) and downregulated (blue) genes in rss3 under nonstressed and high-salinity conditions. The up- and downregulated genes in rss3 were categorized as described in Methods. The JA-responsive genes were selected using data deposited by Miyamoto et al. (2012) into the Gene Expression Omnibus of the National Center for Biotechnology Information (GSE32633) with a cutoff of 1.5-fold upregulation and rank product (P < 0.01, pfp < 0.05). Numbers indicate the amount of nonredundant genes in each category. P values indicate the hypergeometric distribution significance, evaluating the enrichment of JA-induced genes.

(D) The expression of JAZ7 (top), JAZ8 (middle), and SLS (bottom) after treatment with 100 μM JA or mock treatment, quantified by real-time RT-PCR analysis. Root tips of 5-d-old seedlings grown in the water were used. Expression levels were normalized by the levels of eEF-1α. The expression levels relative to those in the wild type before treatment with JA are shown. Statistical differences were determined by one-way ANOVA followed by Tukey posthoc analysis, and different letters indicate significant differences (P value < 0.05). Mean ± SD; n = 4. SLS, secologanin synthase.
JA-responsive genes were significantly increased in rss3 after JA treatment. bHLH094 enhanced the transcriptional activation of promoters carrying specific E-box sequences, but this was suppressed by the combined expression of RSS3 and JAZ9. Based on these data, we propose that the bHLH factors and RSS3 combine with JAZs to negatively regulate JA-responsive genes (Figure 9). This is reminiscent of the function of Arabidopsis MYC2, a transcriptional activator of JA-induced genes (Dombrecht et al., 2007). The activity of At-MYC2 is blocked by the binding of JAZs. Upon JA-ile perception by COI1, the JAZ is degraded, which leads to the recovery of the activation function of At-MY2 and thereby results in JA-responsive gene induction (Chini et al., 2007; Thines et al., 2007). Similarly, the binding of JAZ9 to RSS3-bHLH094 might repress bHLH094-mediated transcriptional activation. In this scenario, defects in the binding of RSS3 to bHLH094 in rss3 should release the repression of bHLH094-mediated gene expression as illustrated in Figure 9.

However, in the rss3 mutant, the JA-induced genes were not fully activated and were still responsive to exogenous JA (Figure 9, bottom). Moreover, gene induction level by bHLH094 alone in the reporter assay was moderate. Therefore, it is suggested that, in conjunction with bHLH089 and bHLH094, other positive regulatory factors are involved in the activation of the JA-responsive genes (Figure 9, depicted as factor X). Probably, such factors are themselves activated by JA but regulated independently of RSS3 and its binding JAZs. The action of the factor X may be dependent on the downstream gene promoter. A possible candidate for such regulators is a MYB transcription factor. In the case of PAL, for example, the promoter region contains potential cis-regulatory sequences for bHLH094 (CAGCTG) and R2R3 MYB factors (CACCAACC) (Takeda et al., 1999; Sugimoto et al., 2000), respectively. A JA-induced MYB exists in rice (Os11g0684000) (Lee et al., 2001). Regulation of this MYB seems to be independent of RSS3 because it was not significantly activated in rss3 in our microarray data (GSE41442). It is also possible that the activity of MYB factor(s) in rice is regulated by interaction with several JAZ proteins, as recently reported in Arabidopsis (Qi et al., 2011). Another candidate for the factor X can be a different type of bHLH that is directly repressed by a JAZ factor independently of RSS3.

Importantly, in rss3, JA induces further activation of genes (Figure 8D). Therefore, RSS3 may have an additional role in repression of the bHLH094/089-mediated gene expression, through unknown mechanism that is affected by the interaction between RSS3 and the bHLH factors (Figure 9, bottom right). The elevated levels of gene expression in rss3 suggest that in the wild type, RSS3 restricts JA responses in the root tip to a certain extent. In general, activation of JA signaling in response to unfavorable conditions causes root growth inhibition (Chacón-López et al., 2011). Under weak stress conditions, however, there should be a control to sustain root growth by preventing excessive JA response. RSS3 may function in such regulation in the root tip. By contrast, JA-induced expression might be fully activated in tissues other than the root tip, where RSS3 is absent.

Accumulating evidence suggests that JAZ factors, together with the F-box protein COI1, play central roles in JA signaling (Pauwels and Goossens, 2011; Kazan and Manners, 2012; Wager and Browse, 2012). JAs are converted to JA-ile, which in turn binds to the COI1-JAZ complex in conjunction with ionositol pentakiphosphate, which functions as a cofactor (Chini et al., 2007; Sheard et al., 2010). In JAZ factors, the C-terminal Jas domain has a crucial role in JA-ile-dependent binding to COI1 and the subsequent degradation of JAZ by the 26S proteasome (Chini et al., 2007; Melotto et al., 2008; Sheard et al., 2010). The strength of association with COI1 differs among JAZs, which correlates with sequence variation in the Jas domain (Shyu et al., 2012). The differential association of JAZs with COI1 may enable plants to respond dynamically to a wide range of JA concentrations. Rice JAZ9 and JAZ11, which interact with RSS3 (Figure 5) and with COI1 in the presence of coronatine (Lee et al., 2013), also contain the JAZ degron, the core sequence of the Jas domain, but an incomplete PY consensus at the C terminus (see Supplemental Figure 7B online). The Jas domain of JAZ9 and JAZ11 likely binds to COI1 because the amino acids responsible for complex formation are well conserved in these JAZ proteins (see Supplemental Figure 8 online). The PY sequence has only a limited effect on the binding to COI1 in vitro.

**Figure 9.** Possible Functions of RSS3 and Its Binding Proteins in JA-Inducible Gene Expression.

RSS3 is proposed to have a role in mediating JAZ-dependent inactivation of genes. Some JAZ factors have been reported to mediate active repression through NINJA and TPL (Pauwels et al., 2010). It is unclear whether rice JAZ9 and JAZ11 are involved in active repression or passive repression. Top left: In the absence of JA (-JA), bHLH094 (or bHLH089)-mediated transcription is repressed by a combination of RSS3 and JAZ9 (or JAZ11) proteins that form a trimeric complex with the bHLH factor. Bottom left: In rss3, this repression was abolished because RSS3 with the 21-amino acid deletion (RSS3D21) cannot bind to the bHLH factor. bHLH094 is a weak activator by itself, but the bHLH-mediated transcription is readily activated depending on other positive regulators. Top right: In the presence of JA (+JA), JAZ is possibly degraded by 26S proteasome by the proposed COI1-dependent pathway (Chini et al., 2007; Thines et al., 2007), leading to derepression of the bHLH-mediated transcription. Since RSS3 alone cannot activate bHLH094, another positive regulation (depicted as factor X) is likely involved in the gene activation by JA. The action of factor X might be dependent on RSS3, as well as RSS3, which in turn participates in the downstream gene promoters. In the wild type (WT), the JA-responsive genes are active but incompletely ("restricted"). Bottom right: In rss3, JA induces full activation of genes. This might be achieved by release from repression through the JAZ, the positive regulation with the factor X, and additional unknown mechanism that is interfered by interaction of RSS3 with the bHLH factors. [See online article for color version of this figure.]
(Chini et al., 2007; Chung et al., 2010; Sheard et al., 2010). In some cases, however, the lack of a sequence including PY causes changes in the stability of JAZ and affects JA sensitivity in plants (Chung and Howe, 2009; Chung et al., 2010). Therefore, we cannot rule out the possibility that JAZ9 and JAZ11 have unique features that do not occur in canonical JAZ proteins. The complex that forms between RSS3 and JAZs remains to be characterized in terms of its affinity for COI1 and its stability in the presence of JAs.

Previous studies reported that the N terminus of R/B-like bHLHs functions as a JAZ interacting domain (JID) (Chini et al., 2007; Fernández-Calvo et al., 2011). Within the JID, the region II is highly conserved both in RSS3 and in R/B-like bHLHs (Figure 2C). Moreover, a single amino acid substitution (D94N) of ATR2 (Arabidopsis MYC3) in the region II [M/I/V-L(G)W(G)DG-F/Y] resulted in a dominant mutation causing activation of Trp-related (2C). Moreover, a single amino acid substitution (D94N) of ATR2 (Arabidopsis MYC3) in the region II [M/I/V-L(G)W(G)DG-F/Y] caused changes in the stability of JAZ and affects JA sensitivity in some cases, however, the lack of a sequence including PY that seems not to bind to JAZ factors (Fernández-Calvo et al., 2011). Interestingly, RSS3 has Met instead of Gly at this position, but nevertheless is able to bind to JAZ9 and JAZ11. Thus, variations within its amino acid sequence in the region II as well as their surrounding sequence may be responsible for the specificity of binding to respective JAZs.

The rice genome encodes two proteins homologous to RSS3, RSS3-LIKE1 and RSS3-LIKE2 (see Supplemental Figure 11A online). When RSS3-LIKE1 was constitutively expressed under a rice actin promoter in rss3, salt-dependent root growth inhibition and cell swelling were suppressed (see Supplemental Figures 11C and 11D online). Thus, RSS3 and its homologs possibly regulate overlapping target genes through members of the class-C bHLH factors, including bHLH089 and bHLH094. RSS3-LIKE1 and RSS3-LIKE2 are expressed not only in roots but also in other organs (see Supplemental Figure 11B online), as are bHLH089 and bHLH094. Therefore, similar regulatory mechanisms may also operate in tissues other than roots. Conceivably, the same is true for other plant species because genes homologous to RSS3 and bHLH089, such as Arabidopsis At1G53900 and At1G59640 (BPE; Szécsi et al., 2006), respectively, are found in the genomes of other plants. Interestingly, it has been reported that BPE also acts downstream of the JA signal and functions as a negative regulator of cell elongation in petals (Brioudes et al., 2009).

It remains to be clarified which factors downstream of the RSS3-JAZ-bHLH complex mediate the control of root growth. To classify the genes whose expression was altered in rss3, we applied our transcriptomic data for gene set enrichment analysis using MapMan (Thimm et al., 2004). Among the downregulated genes in rss3, highly enriched gene categories are related to abscisic acid–induced and late embryogenesis abundant genes (see Supplemental Figure 9C online). By contrast, genes upregulated in rss3 were classified into a wide variety of categories (see Supplemental Figure 9C online). Notably, many of them were related to cell wall metabolism (categories such as “cell wall modification,” “β-1,3 glucosidase,” “lignin biosynthesis,” “phenylpropanoids,” and “simple phenols”) (see Supplemental Figure 9C online). It is plausible that RSS3 regulates the expression of genes involved in cell wall metabolism, which are required for proper cell elongation and maturation. In fact, genes encoding enzymes involved in lignin biosynthesis and/or the metabolism of other cell wall components, such as PAL, COMT, Cellulase, and Peroxidase, are upregulated in rss3 (microarray data: GSE41442) (see Supplemental Data Set 1D online), which may result in changes in cell wall properties. This may in turn affect the elongation of rss3 root cells under nonstress conditions and cause further defects under salinity conditions. In tobacco (Nicotiana tabacum) cells, the physical and chemical structures of the primary cell wall are altered when the plants are cultured under high-salinity conditions; specifically, the wall exhibits weakened tensile strength, concomitant with a decrease in cellulose content, compositional changes of the cell wall–associated proteins, and reorganization of pectin (Iraki et al., 1989). It is reasonable to assume that RSS3 is involved in the regulation of genes involved in such processes. This may explain the impaired flexibility in rss3 roots. Experiments to examine how rss3 affects cell wall properties are underway.

We demonstrated that RSS3 globally modulates the expression of salinity stress–responsive genes. The link between this modulation and the RSS3-mediated regulation of JA-responsive genes remains unclear, although JAs are known to be involved in various stress responses (Wasternack, 2007; Takeuchi et al., 2011). Notably, Arabidopsis MYC2 was originally identified as a transcriptional activator of stress-responsive genes (Abe et al., 1997). The global effects of rss3 on the stress-responsive genes may be accounted for by assuming that the target cis-elements of Arabidopsis MYC2– or rice bHLH94-related Transcription factors prevail in primary stress-responsive genes, including the upstream transcription factors, and that they variously modulate the expression levels of the stress-responsive genes. Such bHLH–mediated controls would contribute to the adaptation of root growth in response to stress conditions.

In conclusion, we have shown that RSS3 forms a ternary complex with bHLH and JAZ factors. Moreover, our results indicate that RSS3, possibly through this complex, has an important role in stress-responsive gene regulation and the control of cell elongation, providing a novel molecular mechanism by which JA-responsive genes and plant growth are regulated. The RSS3-mediated regulation of cell elongation would allow the elaborate control of root growth in response to environmental stresses and also be linked to the regulation of cell division in the root tip. A further understanding of the mechanisms that sustain root growth under stressful conditions will enhance our understanding of how plants adapt to environmental changes.

METHODS

Plant Materials and Growth Conditions

The rss3 mutant was originally identified in a screen for salt-dependent phenotypes (Ogawa et al., 2011) in the rice (Oryza sativa) mutant population prepared by Miyao et al. (2003) (line ND2084). Wild-type and rss3
seeds (*O. sativa* cv Nipponbare) were surface sterilized and germinated on solid MS-based medium (Murashige and Skoog basal medium, 1% Suc, 0.25% gellan gum, and 0.05% MES-KOH, pH 5.8) in 13-cm-high capped bottles. Seedlings were grown at 25°C under a photoperiod of 14 h light (4000 lux, white light) and 10 h dark for 3 to 7 d. For the salinity stress test, 100 to 150 mM NaCl was applied to the medium. JA ([−]−JA; Wako) was dissolved in DMSO (Wako) to produce a 100 mM stock solution and stored at −20°C. For JA treatment of rice seedlings, JA or mock solution was applied to the solid MS-based medium, except that the short-term treatment with JA was performed in water.

**Microscopy Observation of Root Cells**

Root samples were treated with chloral hydrate solution (8 g chloral hydrate, 1 mL glycerol, and 2 mL water) or stained by the modified pseudo-Schiff propidium iodide technique (Truernit et al., 2008; Ogawa, hydrate, 1 mL glycerol, and 2 mL water) or stained by the modified pseudo-Schiff propidium iodide technique (Truernit et al., 2008; Ogawa, et al., 2011). The tissues were observed under a fluorescence microscope (Axio Imager.Mi; Carl Zeiss) or under a confocal laser scanning microscope (FV-1000D; Olympus).

**Positional Cloning of the RSS3 Gene**

Genetic mapping of rss3 was performed with F2 populations derived from crosses between rss3 and an indica cultivar, Kasalath. DNA extracted from 1501 F2 seedlings exhibiting the rss3 phenotype was used to type subspecies-specific polymorphisms for simple sequence repeat and cleaved amplified polymorphic sequence marker design (International Rice Genome Sequence Project, 2005; see Supplemental Table 1 online). Transcripts of four genes located in the mapped regions, i.e., Os11g0446000, Os11g0446500, Os11g0446700, and LOC_Os11g25970 (the first three genes were annotated in the Rice Annotation Project Database and the last in Michigan State University/The Institute for Genomic Research), were analyzed by RT-PCR. A truncation of Os11g0446000 mRNA was detected in rss3. The causative RSS3 gene was confirmed by complementation of rss3 using a genomic fragment (7.4 kb) of the wild-type allele of Os11g0446000. We cloned the RSS3 cDNA fragments by 5′- and 3′-rapid amplification of cDNA ends using GeneRacer (Invitrogen) and amplified the cDNA encoding the full-length RSS3 amino acid sequence.

**Sequence Alignment and Phylogenic Analysis**

Alignment of the amino acid sequences was performed using MAFFT software (http://mafft.cbrc.jp/alignment/software/) with the G-INS-i strategy and/or the MUSCLE algorithm from MEGA5.0 (http://www.megasoftware.net). To generate the graphical output, the sequences listed in Supplemental Data Set 2, TreeView (Page, 1996) and Genedoc (http://www.ncbi.nlm.nih.gov/gfx/genedoc/) were used.

**Plasmid Construction**

A 7.4-kb genomic fragment of RSS3 (4.0 kb of coding region, 2.5 kb of the upstream promoter region, and 0.9 kb of the downstream region) was amplified by PCR with the specific primers 5′-TCGAGGCTGCAG-AAGTGACCGCG-3′ and 5′-AGGCTTAAAGATTCGTCCGGAC-3′ and inserted into pOR-Blunt II-TOPO (Invitrogen). The fragment containing ProRSS3:RSS3 was excised with Xbal and Kpn1, inserted between XbaI and Kpn1 sites of the binary vector pCAMBIA1301 (CAMBIA), and then inserted for transformation of rss3 for the complementation assay. Furthermore, the plasmid was modified to construct ProRSS3:RSS3-EGFP. Briefly, the stop codon (TAG) of RSS3 was substituted into a codon (TCG) followed by an EcoRI-BamHI linker sequence. The EGFP gene fragment, derived from ProAct:EGFP (Ogawa et al., 2011), was then inserted between the EcoRI and BamHI sites. The resulting ProRSS3:RSS3-EGFP fusion gene, containing an amino acid junction sequence (Ser-Glu-Phe-Ala-Ala-Ala-Ser-Thr) between RSS3 and EGFP, was inserted between XbaI and Kpn1 sites of the binary vector pPZP2H-lac (Fuse et al., 2001). The binary plasmid constructs were used for Agrobacterium tumefaciens-mediated transformation. Introduction of the ProRSS3:RSS3-EGFP fusion gene into the rss3 mutant complemented the severely impaired root growth phenotype under salinity conditions.

For transient assays using rice protoplasts, cDNAs encoding RSS3, bHLH094, and JAZ9 were amplified by PCR with specific primer sets (see Supplemental Table 1 online) and subcloned into the pENTR/D-TOPO vector. These genes were then transferred using the LR recombination reaction (Gateway technology; Invitrogen) into a vector containing 35S: Sh:GW:stop (courtesy of Hirokazu Kato, Nagoya University), which consists of the CaMV 35S promoter, the first intron of maize (*Zea mays*) Sh1 with an internal deletion, the Gateway cassette, stop codons in three frames, and a nopaline synthase terminator. For constructing the reporter plasmid containing E-boxes: TATA: ILUC (E-box repeats fused to the CaMV 35S minimal promoter with firefly LUC), the GAL4 binding sequence (UAS of the UAS:TATA:ILUC plasmid (courtesy of Yutaka Sato, Nagoya University) was replaced with an artificial enhancer sequence that contains the triplicate of the E-box regulatory sequence (underlined), 5′-GATCCTCGAGCAACANTGAGAANACTGAGGATCC-3′.

For construction of YFP- RSS3 and Os-bHLH094-YFP driven by the CaMV 35S promoter, cDNAs for RSS3 and bHLH094 subcloned in the pENTR/D-TOPO vector were transferred to Gateway expression vectors (YFP/pUGW0 and cYFP/pUGW2) by LR recombination. The pDH51-mRFP1 plasmid (Zhong et al., 2008) was used as a control for the BiFC assay. For fluorescence observation and the apFRET assay, cDNAs encoding RSS3, class-C bHLHs, and JAZs were subcloned into the pENTR/D-TOPO vector and transferred to Gateway expression vectors (YFP/pUGW0, pDH51-GW-CFP, and ECFP/pUGW0) (Nakagawa et al., 2007; Zhong et al., 2008). Primer sets used for cDNA amplification are listed in Supplemental Table 1 online.

**Y2H and Three-Hybrid Assay**

Transformation of yeast (AH109) and the interaction assays were performed using Matchmaker Library Construction and Screening Kits (Clontech) according to the manufacturer’s instructions. Summary of screening of RSS3 binding proteins are described in Supplemental Table 2 online. Fragments of cDNA encoding RSS3, bHLH094, and JAZs were amplified by PCR with the primer sets listed in Supplemental Table 1 online. These cDNAs were derived from mRNA expressed in the root tip, except for JAZ12, which was from whole seedlings. Among the 15 JAZ genes reported by Ye et al. (2009), cDNAs for 12 JAZ genes other than JAZ5, JAZ13, and JAZ14 were amplified. Amplification of each JAZ was obtained by cloning into the NdeI and EcoRI site of pGBKTT7 or introduced by homologous recombination into pGAD77-Rec. For the yeast three-hybrid assay, RSS3 and bHLH094 were amplified by PCR and cloned into the NotI site or EcoRI and BamHI sites of pBRIIDGE (Clontech).

For screening of RSS3 interactors, rice cDNA libraries derived from root tissues of 7-d-old seedlings were amplified and cloned into the prey vector pGAD77-Rec in yeast. Using the N-terminal 318 amino acids of RSS3 as bait, a total of 72 × 10^6 clones were screened based on the activation of the HIS3 reporter gene. The 336 candidate clones were further selected by activation of the ADE2 and MEL1 reporter genes, resulting in 333 clones. Within the 40 high MEL1 expressing clones, nine candidate clones were selected by their reading frame and repeated reporter assay after retransformation of the respective plasmids (listed in Supplemental Table 2 online). For four candidate clones that carried transformation factors genes, the dependency of the bait plasmid was examined. As a result, only one clone was selected, which had a gene fragment for a DNA binding protein (Os07g0139300; bHLH094). By screening another cDNA library, derived from the root tips of rice
seedlings grown under salinity conditions (9.9 \times 10^3 clones), using the N-terminal 203 amino acids of RSS3 as bait, no positive clones were obtained after further selection.

Expression Analysis

Total RNA was extracted and purified using the RNeasy Mini Kit or RNeasy Micro Kit (Qiagen). Reverse transcription was performed using the QuantiTect Rev transcription kit (Qiagen). First-strand cDNA was amplified by PCR PrimeStar HS DNA polymerase (Takara) using the GeneAmp PCR System 9700 (Applied Biosystems) or real-time PCR with Power SYBR Green PCR master mix (Applied Biosystems) using Mx3000P (Agilent). The primer sets are listed in Supplemental Table 1 online. The quantified expression levels of the tested genes were normalized against those of UBQ, 25S rRNA, or \( eEF1-\alpha \) (Jain et al., 2006).

Transient Expression Assay Using Rice Protoplasts

Electroporation of rice cultured cell–derived protoplasts was performed as described previously (Hattori et al., 1995). Ten micrograms of each plasmid used for electroporation, with 5 \( \mu \)g of a reference plasmid encoding renilla \( LUC \) driven under a CaMV 35S promoter used for normalization. The activity of firefly and renilla luciferase was monitored by a luminometer (Lumat LB 9507; Berthold Technologies) using the PicaGene Dual Sea Pansy Luminescence Kit (Toyo-Ink). Each experiment was repeated at least three times using a single preparation of protoplasts.

BIFC, apFRET, and BIFC-apFRET Analysis

To evaluate the protein–protein interaction by BIFC, apFRET, or BIFC-apFRET analysis, transient assays were performed by particle bombardment using the PDS-1000/He biolistic particle delivery system (Bio-Rad). Plasmids encoding the protein of interest were adsorbed onto gold particles (1.6 \( \mu \)m in diameter) according to the manufacturer’s instructions (Bio-Rad). Gold particles were then bombarded into onion pieces at 7584 kPa under a vacuum of 94.82 kPa. The onion pieces were then incubated in a Petri dish for 12 h at 30°C in the dark. Epidermal cells were observed by fluorescence microscopy (Axio Imager.M1; Carl Zeiss).

The nuclei of onion epidermal cells expressing the proteins of interest were subjected to apFRET and BIFC-apFRET analyses using a confocal laser scanning microscope (LSM700; Carl Zeiss). ECFP or CFP was used as the FRET donor, and YFP or BiFC-derived YFP was used as the FRET acceptor. After bleaching the acceptor, FRET was monitored as an increase of donor fluorescence. Calculation of the FRET efficiency used the formula

\[
\mathrm{FRET \ efficiency} = \frac{\int \mathrm{donor} \times \int \mathrm{acceptor} - \int \mathrm{donor} \times \int \mathrm{bleached} \times \int \mathrm{bleached}}{100/\mathrm{donor} \times \mathrm{intensity} \times \mathrm{bleached}}
\]

Microarray and Statistical Analysis

For transcriptomic analysis, total RNA was extracted using the RNeasy Mini Kit (Qiagen) from root tips (1 mm long) excised from wild-type and rss3 seedlings that had grown for 3 d on the solid MS medium in the presence (+NaCl) or absence (−NaCl) of 100 mM NaCl. The excised root tips include the MZ and EZ, but not the MTZ, even in rss3 plants grown under salinity stress. Three biological replicates, each of which consisted of at least 15 individual root tips, were prepared. The quality and quantity of RNAs were evaluated using a NanoDrop ND-1000 UV-VIS spectrophotometer (NanoDrop) and Agilent 2100 bioanalyzer (Agilent Technology). The RNAs were labeled with a LOW RNA input linear amplification labeling kit (Agilent Technology). Four different aliquots (WT [−NaCl], WT [+NaCl], rss3 [−NaCl], and rss3 [+NaCl]) of Cy5-labeled cRNA (825 ng) and a control aliquot (WT [−NaCl]) of Cy3-labeled cRNA (825 ng) were prepared and used for hybridization using the Agilent-015241 rice gene expression 4X44K microarray (Agilent Technology). Scanned images were analyzed with Feature Extraction Software v10.5.1.1 (Agilent). Microarray data were normalized using the variance stabilization normalization method (Huber et al., 2002) packaged in R software (http://www.r-project.org/). After normalization, the relative log2 expression values of the genes in each sample were calculated by subtracting the Cy-3 value from the Cy-5 value. Differentially expressed genes were categorized using two-way ANOVA (P < 0.01) by genotype (the wild type versus rss3) and stress treatment (−NaCl versus +NaCl) with TIGR Multi-Experiment Viewer (http://www.tm4.org/mev/), where the P value was adjusted with multiple testing corrections (Benjamini and Hochberg, 1995). Alternately, differentially expressed genes were selected by a cutoff of 1.5-fold change and by rank product (P < 0.01, pfp < 0.05) (Bretting et al., 2004) of R, to examine the effect of rss3 on the JA-responsive genes. Relative log2 expressions were Z-scaled and visualized with R and Multi-Experiment Viewer, respectively. Functional categorization of genes was performed, based on the classification available in the Mapman program (http://mapman.gabipd.org/) (Thimm et al., 2004). The raw and processed microarray data are available at the Gene Expression Omnibus with repository number GSE41442.

For extraction of the JA-induced or JA-repressed genes, the microarray data by Miyamoto et al. (2012) were also normalized using the variance stabilization normalization method and filtered by a cutoff of 1.5-fold change and by rank product (P < 0.01, pfp < 0.05).

Accession Numbers

Sequence data from this article can be found in GenBank/EMBL databases under the accession numbers listed in Supplemental Table 3 online. The newly obtained cDNA sequence of Os11g0446000 encoding full-length RSS3 was deposited in GenBank under accession number AB753860.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. rss3 Displays Defects in Root Growth.

Supplemental Figure 2. rss3 Displays Aberrant Cellular Arrangement in the MZ.

Supplemental Figure 3. Responsiveness of rss3 to External Stimuli.

Supplemental Figure 4. Complementation Assay of the rss3 Mutant, Diagrammatic Representation of the rss3 Mutation, and Expression Levels of RSS3 in rss3.

Supplemental Figure 5. Expression Pattern of RSS3, bHLH089, bHLH094, JA29, and JAZ11 in Wild-Type Plants.

Supplemental Figure 6. Sequence Alignment of the RSS3 Interaction Domain of bHLH094, bHLH089, and Class-C bHLH Proteins in Rice.

Supplemental Figure 7. Comparison of the ZIM and Jas Domains of Rice and Arabidopsis JAZ Proteins.

Supplemental Figure 8. Comparison of the Amino Acid Residues of JAZ Proteins Required for the Interaction with COI1 and JA-Ile.

Supplemental Figure 9. Altered Expression of Genes in rss3.

Supplemental Figure 10. Dose-Dependent Effects of JA on Cell Division and Cell Elongation in the Root Tip of Rice.

Supplemental Figure 11. Characterization of RSS3 Homologs of Rice.

Supplemental Table 1. Oligonucleotides Used in This Study.

Supplemental Table 2. Summary of the Yeast Two-Hybrid Screen.

Supplemental Table 3. Accession Numbers of Genes and Proteins.

Supplemental Data Set 1A. Expression Analysis of JA-Induced Genes in rss3.
Supplemental Data Set 1B. Expression Analysis of JA-Repressed Genes in rss3.

Supplemental Data Set 1C. Functional Characterization of Genes Whose Expression Was Altered by JA and in rss3.

Supplemental Data Set 1D. Lists of the Categorized Genes Whose Expression Was Altered in rss3.

Supplemental Data Set 2. Sequence Alignments Used for Phylogenetic Analysis in This Study.

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AUTHOR CONTRIBUTIONS


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Tto1


RICE SALT SENSITIVE3 Forms a Ternary Complex with JAZ and Class-C bHLH Factors and Regulates Jasmonate-Induced Gene Expression and Root Cell Elongation

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