RICE SALT SENSITIVE3 binding to bHLH and JAZ factors mediates control of cell wall plasticity in the root apex

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Plasticity of root growth in response to environmental cues and stresses is a fundamental characteristic of plants, in accordance with their sessile lifestyle. This is linked to the balance between plasticity and rigidity of cells in the root apex, and thus is coordinated with the control of cell wall properties. However, mechanisms underlying such harmonization are not well understood, in particular under stressful conditions. We have recently demonstrated that RICE SALT SENSITIVE3 (RSS3), a nuclear factor that mediates restrictive expression of jasmonate-induced genes, plays an important role in root elongation under saline conditions. In this study, we report that loss-of-function of RSS3 results in changes in cell wall properties such as lignin deposition and sensitivity to a cellulose synthase inhibitor, concomitant with altered expression of genes involved in cell wall metabolism. Based on these and previous phenotypic observations of the *rss3* mutant, we propose that RSS3 plays a role in the coordinated control of root elongation and cell wall plasticity in the root apex.

Growth and development of plant roots is sustained by continuous cell division at the root apex and subsequent cell differentiation. The direction and extent of root elongation is flexible and responsive to various environmental factors such as gravity, water and nutrient availability, and stressful conditions. To ensure the flexibility of root growth, it is important for the plant to maintain a balance between rigidity and plasticity of root cells, which may be correlated with cell wall properties. Rigidity or plasticity of cell wall may affect not only cell division and cell elongation but also the organization of differentiated cells. Cell walls are synthesized or reconstituted, depending on the process that cells undergo, whereas physical and chemical structures of cell walls are altered by environmental conditions.[1,2] For example, in tobacco cells, the cell wall exhibits weakened tensile strength under high-salinity conditions, which is associated with a decrease in cellulose content, compositional changes in cell wall-associated proteins, and reorganization of pectin.[1] However, it is largely unknown how the coordinated control between growth of root cells and cell wall properties is achieved.

RICE SALT SENSITIVE3 (RSS3) is a nuclear factor that interacts and forms a ternary complex with class-C bHLH transcription factors and JASMONATE ZIM DOMAIN (JAZ)

proteins.[3] A loss-of-function mutant of RSS3 (*rss3*) was originally identified in a screen of salt-sensitive mutants.[4] Under conditions without salinity stress, *rss3* shows a moderate reduction in root tip cell elongation but exhibits severe inhibition of root growth under salinity stressed conditions.[3] This is accompanied by aberrant cellular arrangement, formation of oblique cell plates, swelling of cells, and impaired root flexibility. Cell swelling and abnormal cytokinesis are also observed in cellulose-deficient mutants such as *radial swelling1* (*rsw1*) and *rsw2*.[5,6] In addition, reduced root cell elongation and aberrant cell expansion are observed in the *cobra* mutant deficient in cellulose microfibril organization[7] or roots grown in the presence of isoxaben, a cellulose synthase inhibitor.[8] These observations raise the possibility that the *rss3* mutant has a defect in the cell wall.

In the previous study, it was revealed by transcriptomic analysis of the root tip and subsequent enrichment analysis that many genes involved in cell wall metabolism are preferentially upregulated in *rss3*.[3] These include genes encoding biosynthetic enzymes for lignin, such as PAL, COMT, and CAD (**Fig. 1A**). Lignin is a phenylpropanoid polymer derived from monolignols and a major component of the secondary cell wall,[9] where lignin is deposited between the space of cellulose microfibrils. Since
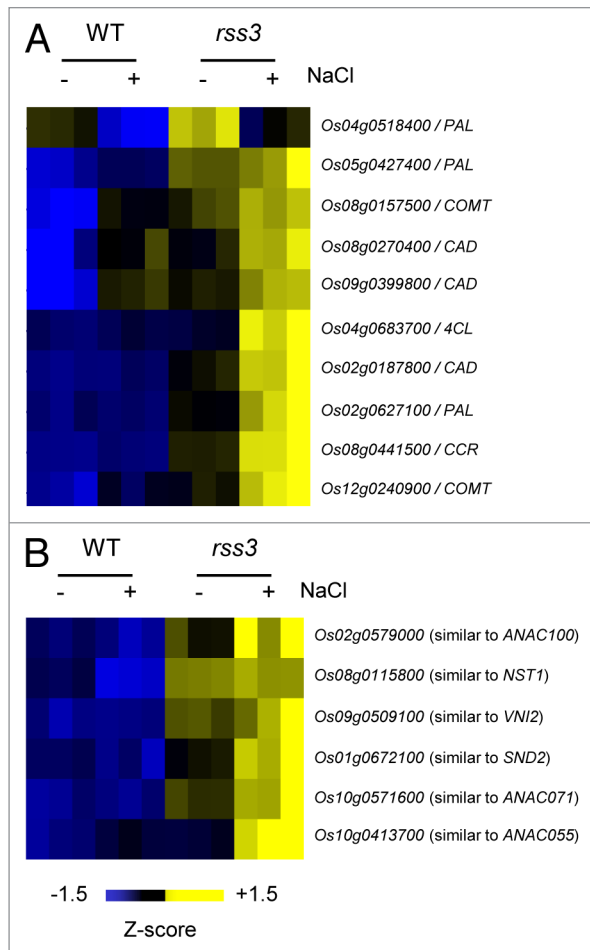
Figure 1. Altered expression of genes encoding lignin biosynthetic enzymes (**A**) and NAC family transcription factors (**B**) in the *rss3* root tip grown in the absence and presence of 100 mM NaCl.[3] Genes encoding lignin biosynthetic enzymes or NAC transcription factors that were upregulated in *rss3* were selected, based on the microarray data (GEO repository: GSE41442),[3] and levels of gene expression were visualized with TIGR Multi-experiment Viewer (MeV; http://www.tm4.org/mev/).

Figure 2. UV microspectrophotometry of cell walls in the root meristematic zone. (**A**) (first 4) Phenylpropanoid-derived compounds deposited in cell walls were observed in transverse sections (3 μm thickness) prepared from the meristematic zone of root tips (300 μm distal from the root cap tip) of 3-d-old wild-type and *rss3* grown in the absence (-) and presence (+) of 100 mM NaCl. (**A**) (second 4) Schematic drawing of the position of cell walls in the sections shown in the first 4 panels. (**B**) Quantification of UV absorbance in the cell wall observed in (**A**). Root samples were fixed in 3% glutaraldehyde, treated with 50% hypochlorous acid for deproteinization, and stained using 1% osmium tetroxide. After dehydration in a graded ethanol series, the root tips were embedded in epoxy resin and sliced with a diamond knife. Sections were mounted on quartz microscope slides, covered with a quartz coverslip, and observed at a wavelength of 278 nm under a microspectrophotometer (Zeiss MPM800), with a bandwidth of 20 nm and objective lens magnification of × 40. The levels of UV absorption in the cell wall and cytosol (for the background) were quantified using ImageJ software (http://rsbweb.nih.gov/ij/). Measurements were taken from at least 4 different positions for each sample and averaged for calculation. Data of at least 3 biological replicates were used for each condition (Mean ± SD n ≥ 3). Asterisk indicates *P* value < 0.05 (the Student t-test).

RSS3 expresses and functions in the meristematic zone (MZ) and in the region that borders the MZ within the elongation zone (EZ) of the root tip,[3] we monitored lignin contents in the cell wall areas in the MZ, by measuring the UV absorbance (278 nm) of ultra-thin sections under a UV microscope.[10] As shown in **Figure 2**, UV absorption in the cell wall areas was detected only at low levels in the wild type (WT), but at considerable levels in *rss3*. This indicates that lignin deposition into the cell walls in the MZ is restricted in WT, whereas it is ectopically induced in *rss3*. Because lignin confers rigidity to cell walls, lignin deposition may be a cause of the reduced cell elongation in *rss3* under conditions without salinity. However, this would not account for the impaired cell elongation in the *rss3* root under saline conditions, where the UV absorbance remained unchanged between WT and *rss3* (**Fig. 2**). Lignin deposition appeared to be perturbed by yet unknown cellular responses caused by salt
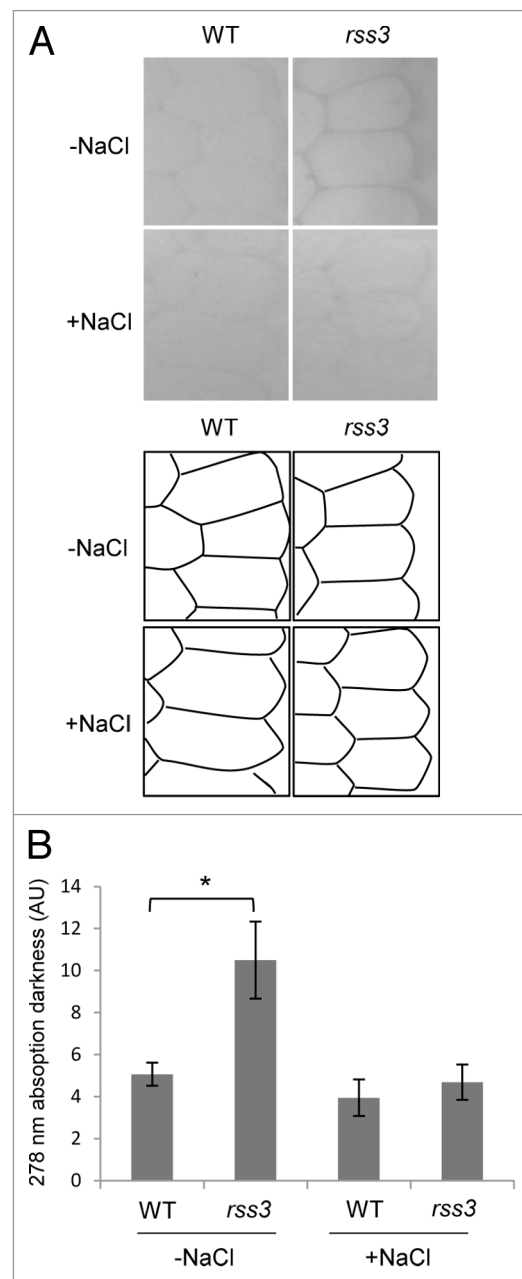
stress in *rss3*, in spite of activation of lignin synthetic gene expression. Expression of nearly half of salt stress-responsive genes is modified in *rss3*.[3] The largely altered salt stress responses are rather likely to be responsible for drastically impaired cell elongation in *rss3* under the salinity conditions.

It is known that lignin and cellulose biosynthesis is coordinately controlled. For example, chemical treatment or mutations that impair cellulose biosynthesis cause increased lignin deposition.[11-15] Oppositely, downregulation of lignin biosynthetic pathways results in increased cellulose synthesis.[16] Therefore, it is plausible that the increased lignin content in *rss3* may reflect the overall alteration of cell wall properties. To verify this possibility, we examined the effect of isoxaben, a cellulose synthase inhibitor, on root growth of WT and *rss3*. As shown in **Figure 3A**, root growth is impaired by isoxaben in WT and *rss3* in dose-dependent manners. Noticeably, a low concentration of isoxaben (0.01 μM) inhibited root growth of *rss3* but not that of WT (**Fig. 3A**), indicating that *rss3* is hypersensitive to the inhibitor. The inhibition by 0.01 μM isoxaben in *rss3* was accompanied by reduced sizes in both MZ and EZ (**Fig. 3B**). The size reduction was concomitant with a significant decrease in cell number of the MZ but not of the EZ (**Fig. 3B**). This suggests that cell division is affected in the mutant by inhibiting cellulose synthase. In contrast, the size reduction in the EZ probably reflects that inhibition of cellulose synthase leads to a decrease in cell elongation in the *rss3* root tip. The hypersensitivity of *rss3* to the cellulose synthase inhibitor implies that drastic changes in characteristics of cell walls are induced when cellulose synthesis is inhibited in *rss3*, where some unknown changes in cell walls have already occurred.

There have been cumulating lines of evidence that interference with cell wall integrity causes increased jasmonate production and ectopic lignin deposition that is mediated by jasmonate-dependent and -independent mechanisms.[11-13] Therefore, the hypersensitivity to isoxaben may also be explained by that jasmonate signaling provoked by inhibition of cellulose synthase is enhanced in *rss3*. This seems consistent with our observation that jasmonate-responsive genes are excessively induced in *rss3*,[3] and the report that accumulation of monolignol is induced by jasmonate.[17] To the contrary, however, it was reported that cell wall damage-induced lignin deposition was increased by mutations in the jasmonate signaling pathway at least in the EZ of the root tip in *Arabidopsis*.[14,15] Future research is needed to determine which components other than lignin are affected in the cell walls in *rss3*, and how overall properties of cell walls are controlled in the MZ.

The regulation of genes encoding enzymes for cell wall metabolism by RSS3 and its binding proteins may be mediated by secondary wall master regulators. In *Arabidopsis*, MYB-type (MYB46, MYB58, and MYB63) and NAC-type (SND1, SND2, VND6, VND7, NST1, and NST2) transcription factors function as positive regulators of secondary cell wall synthesis.[18-24] Some rice NAC-type transcription factors have also been described to control secondary cell wall metabolism.[25] In *rss3*, several *NAC* genes that belong to the subfamily, in which *Arabidopsis NST1* and *SND2* are classified, were upregulated (**Fig. 1B**). Thus, these
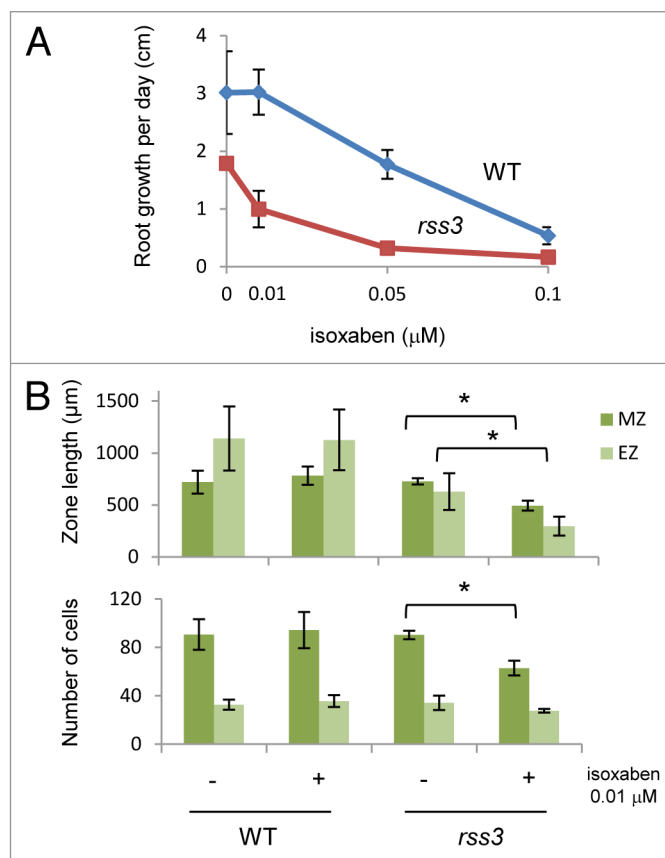

Figure 3. Sensitivity of the roots of wild-type and *rss3* to isoxaben. (**A**) Dose-dependent effects of isoxaben on root growth. Mean ± SD, n > 4. (**B**) Effects of isoxaben on the size and number of cells in the meristematic zone (MZ) and elongation zone (EZ) of the root tip. Mean ± SD, n = 3. Asterisks indicate the Student t-test *P* value < 0.05. Surface-sterilized seeds were germinated on an agar-based medium [agar 0.8%, 1 mM KH_2PO_4, 0.05% MES-KOH (pH 5.8)] containing 0.01 μM, 0.05 μM, and 0.1 μM isoxaben or mock solution (1×10^{-4}% DMSO in final) in a rectangular petri dish to treat rice seedlings with isoxaben (Wako, 092–05961). Seedlings were grown on the medium in plates tilted at a 75° angle.

factors may participate in the control of cell wall metabolisms in the MZ in the root apex. Interestingly, it was reported that lignin and cellulose synthetic genes are oppositely regulated in rice.[26] In contrast, however, we could not find preferential downregulation of cellulose synthetic genes in the *rss3* root tip in our microarray analysis.[3]

Mutants with altered cell wall composition exhibit defects in root elongation that are enhanced under stress conditions. For example, mutation of the chitinase-like protein AtCTL1 causes ectopic deposition of lignin, aberrant cell shape, and hypersensitivity to heat, salt, and dehydration stresses.[27-29] Furthermore, cellulose-deficient mutants *rsw1* and *rsw2* are sensitive to heat and salt stress.[6,30] Therefore, cell wall integrity is likely important for both root elongation and stress tolerance. Changes in the cell wall properties other than lignin deposition may also be a cause of hypersensitivity to salt stress in *rss3*.

In conclusion, we propose that RSS3, which binds to the JAZ and class-C bHLH factors, has a role to modulate cell

wall properties. Together with the observation that the RSS3 complex functions to sustain root growth by preventing an excessive jasmonic acid response at the root apex,[3] the regulation mediated by RSS3 might be important for coordinated control between root elongation and cell wall plasticity under particular environmental stress conditions.

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