Supplementary Information

RSS1 regulates the cell cycle and maintains meristematic activity under stress conditions in rice

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Supplementary Discussion

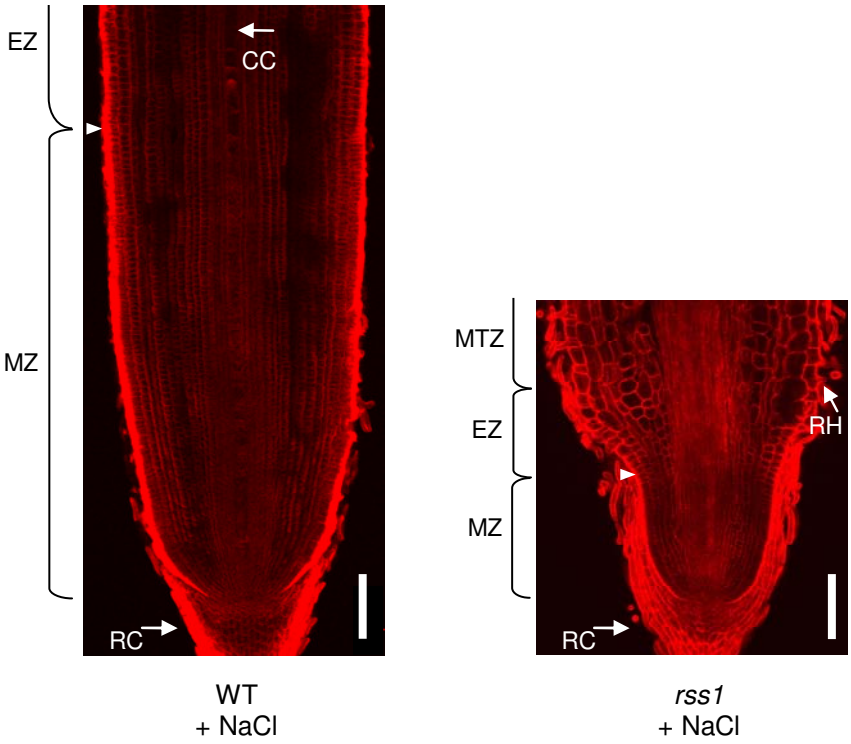
The loss of RSS1 in eudicots

It is believed that angiosperms diversified rapidly in the Mid-Cretaceous Period, referred to as Darwin's 'abominable mystery'[61]. Recent progress in palaeobotany and phylogenetics suggests that early angiosperm evolution can be traced to the relatively gradual radiation of Amborellales, Nymphaeales, and Austrobaileyales, followed by the more rapid (within 5 million years [Myr]) radiation of five lineages, including magnoliids, monocots, and eudicots, more than 120 Myr ago[22,23,62] (Supplementary Fig. S9).

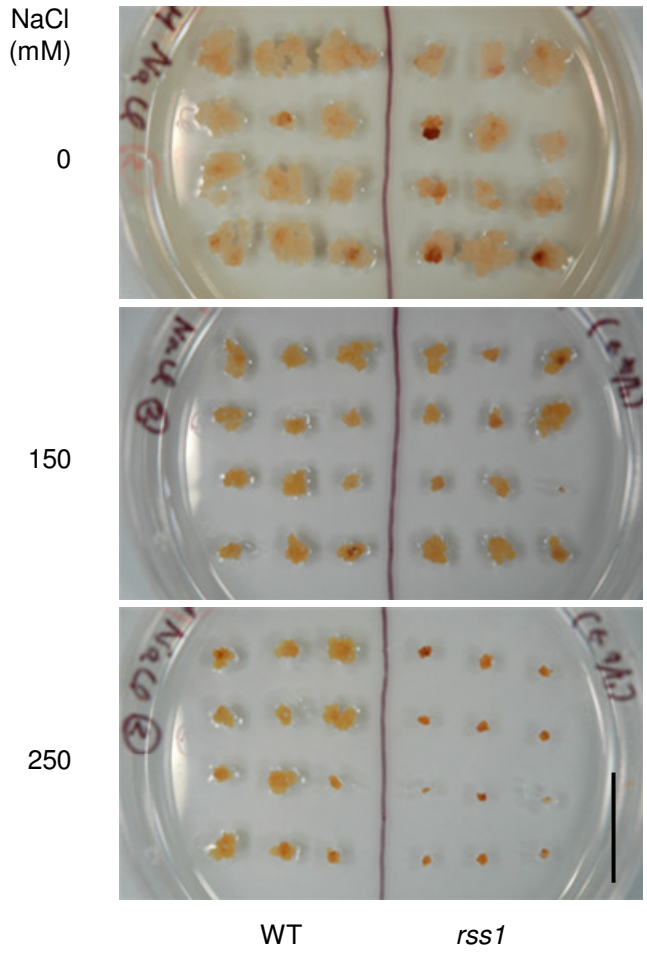
Among these lineages, RSS1 homologues have been found in Amborellales, Nymphaeales, magnoliids, and monocots. However, the RSS1-related sequences found in eudicots show only limited similarity and are confined to region I, which is not necessary for the salt-tolerance function of RSS1. Thus, the loss of RSS1 in eudicots probably occurred during or after the radiation of magnoliids, monocots, and eudicots. This timing nearly coincides with the structural changes in pollen that occurred during the same era. Eudicots have tricolpate pollen grains, whereas all the other angiosperms have monocolpate pollen grains.

The homologous region I sequences found in angiosperms are likely derived from the same source, considering the position of the intron between the shared DEN- and D-boxes (located 5-7 amino acids downstream of the DEN-box) (Supplementary Figs. S6 and S8). In eudicots, the subsequent C-terminal portion appears to consist of eudicot-specific sequences, but it is considerably diversified. The region I and eudicot-specific sequences are found in the core eudicots (asterids and rosids) and in lower eudicots, such as *Aquilegia* (Ranunculales)[63], suggesting that this arrangement arose early in the evolution of eudicots.

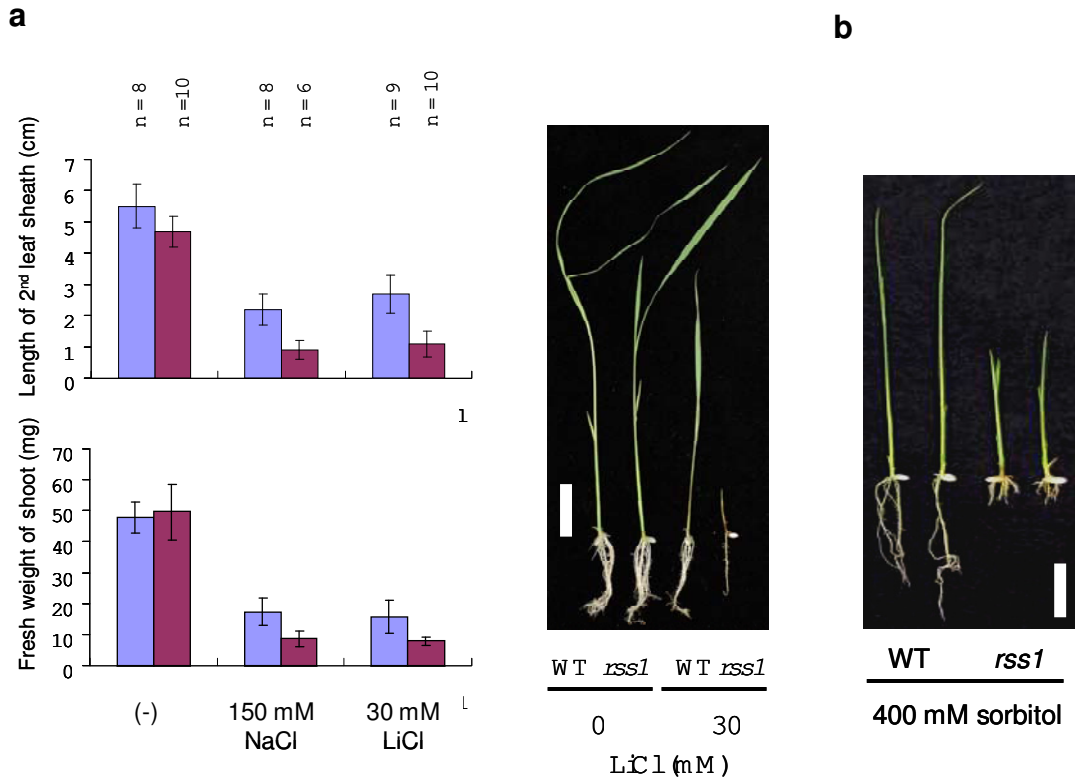
It is a mystery why RSS1 was lost in eudicots, despite its vital role in stress tolerance and the fact that "stress-relevant genes are ubiquitously present in the plant kingdom" [64]. It is noteworthy that most of the previous stress-relevant genes have been shown to be involved in inducible tolerance mechanisms in response to sudden environmental changes; in contrast, RSS1 contributes to a constitutive tolerance mechanism. Consistent with this, our transcriptomic analysis demonstrated that salt-inducible gene expression was not diminished in *rss1*. Based on studies of fossilised flora, angiosperms diversified and dominated freshwater environments rather than coastal, brackish water-related environments from the Aptian to the Albian[62,65,66]. Therefore, it is conceivable that ancestral angiosperms were not exposed continuously to high-salt conditions and could have diversified under relatively stable environmental conditions[65]. However, they still might have needed to acclimate to transient stresses, such as temporary changes in temperature or humidity. Under such circumstances, the tolerance system to continuous abiotic stress might have become less essential, allowing the loss of the prototypical RSS1 in the eudicot lineage. Meanwhile, other angiosperms retained the RSS1 system, possibly through selective pressures put forth after diversification.



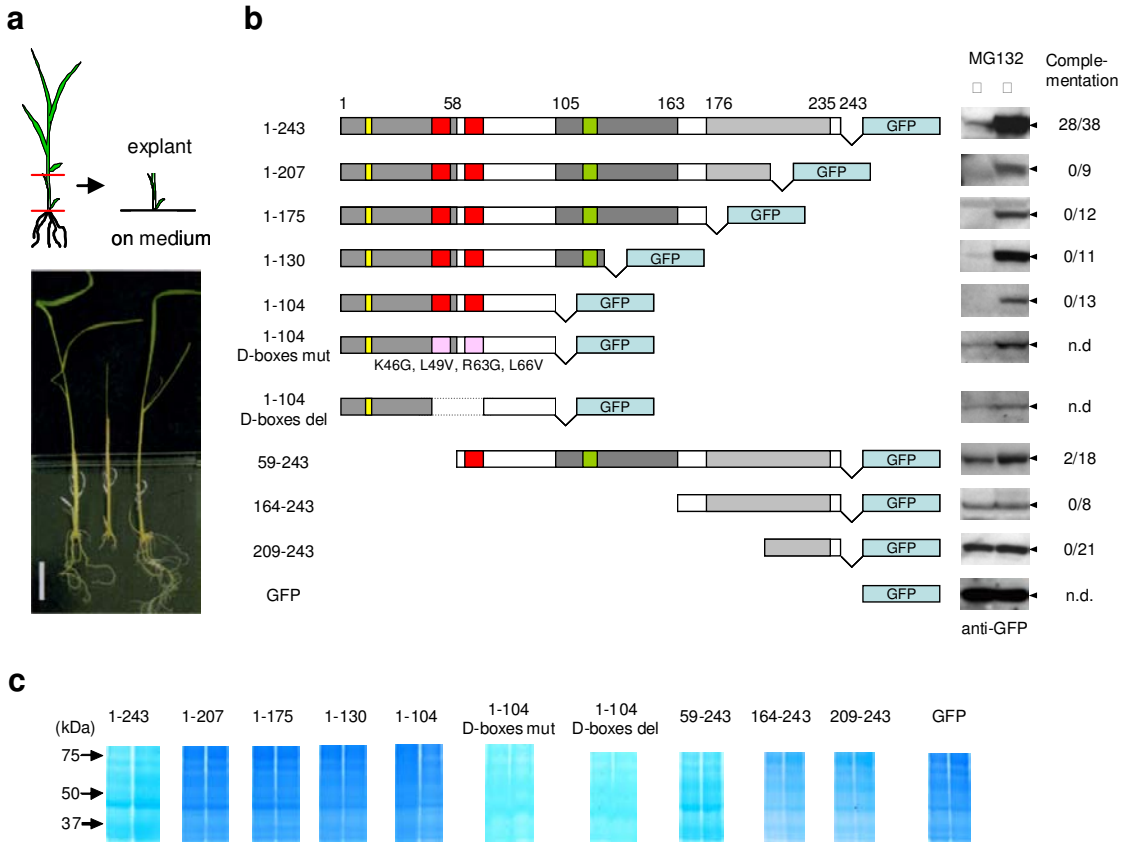
Supplementary Figure S1. ***rss1*** **causes salt-dependent diminution of the meristematic and elongation zones in roots.** Magnified view of the root tip of WT (left) and *rss1-2* (right) plants grown in the presence of 150 mM NaCl for 5 days. Root cells were stained with propidium iodide and observed under a confocal laser scanning microscope. Arrowheads indicate the upper end of the MZ flanked by the EZ. MTZ, maturation zone; RH, root hair; RC, root cap; CC, central cylinder. White bars, 100 μm.



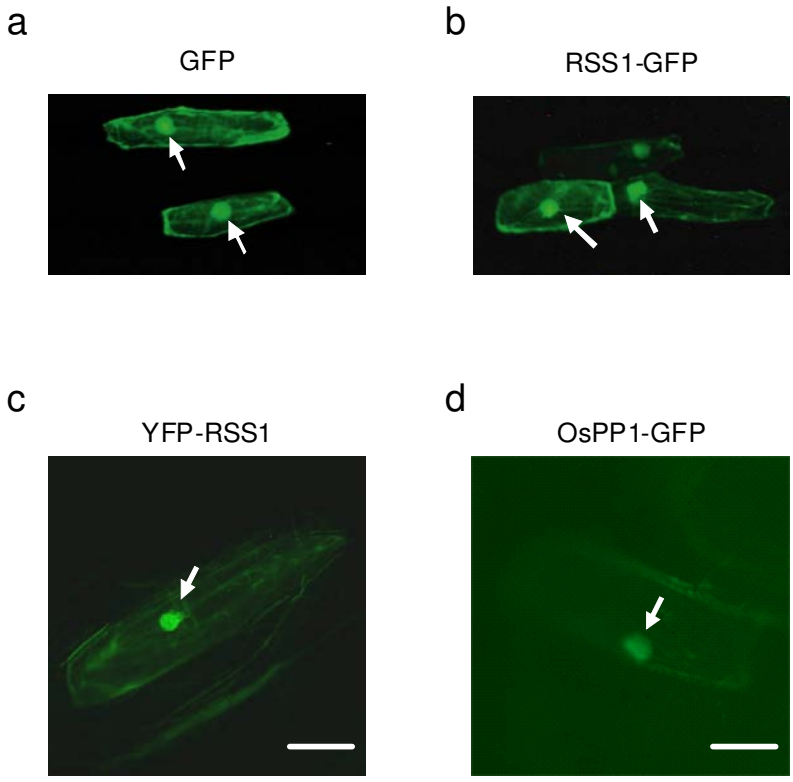
Supplementary Figure S2 ***rss1*** **calli are hypersensitive to salt.** Callus lines derived from WT or *rss1-1* seeds were grown in MS medium containing 2 mg/L 2,4-dichlorophenoxy acetic acid (2,4-D). Fresh callus pieces were then transferred to fresh medium containing the indicated concentrations of NaCl and cultured at 25°C for 3 weeks. WT (left) and *rss1-1* (right) calli incubated on the same plates are shown. Bar, 2 cm.



Supplementary Figure S3. Hypersensitivity of *rss1* to ionic and osmotic stresses. (a) *rss1* is hypersensitive to LiCl. (left) Growth inhibition of WT (blue) and *rss1-1* (magenta) seedlings grown in MS medium in the presence or absence of 150 mM NaCl or 30 mM LiCl for 11 days. The average length of the second leaf sheath (top) and fresh weight of the shoot (bottom) are shown with the s.d. The number of individuals that were considered is indicated (*n*). (right) Representative WT and *rss1-1* seedlings grown in the presence or absence of 30 mM LiCl for 11 days. (b) *rss1* is hypersensitive to osmotic stress. WT and *rss1-1* seedlings grown in MS medium in the presence of 400 mM sorbitol for 17 days. Bars, 2 cm.



Supplementary Figure S4. Mutational analysis of RSS1 by complementation and stability assays. (a) Complementation assay of *rss1* using the *RSS1* cDNA driven by the *actin* promoter (*pAct::RSS1*). (top image) Explants excised from transgenic lines were transferred onto MS medium containing 150 mM NaCl and examined for salt tolerance. (bottom) Growth of the representative explants under saline conditions. Left, WT transformed with the vector control. middle, *rss1* transformed with the vector control. right, *rss1* transformed with *pAct::RSS1*. Six out of eight *rss1* explants carrying *pAct::RSS1* exhibited a salt-tolerance phenotype, but all of the 15 *rss1* explants carrying the control vector exhibited the salt-sensitive phenotype. Bar, 2cm. (b) Rice calli expressing the wild-type and mutated *RSS1* fused to *GFP* driven by the *RSS1* promoter were cultured in the absence (-) or presence of 50 μM MG132 (+). Proteins extracted from the calli were analysed by immunoblotting using anti-GFP antibodies. For the complementation assay, *rss1* explants carrying the indicated transgenes were tested for salt tolerance, as in (a). The rate of the number of complemented lines per the number of tested lines is shown. n.d., not determined. The amino acid positions of RSS1 are shown on the top. D-boxes with amino acid substitutions are indicated with pink rectangles. (c) Protein staining as the loading control for the immunoblot analysis in (b).

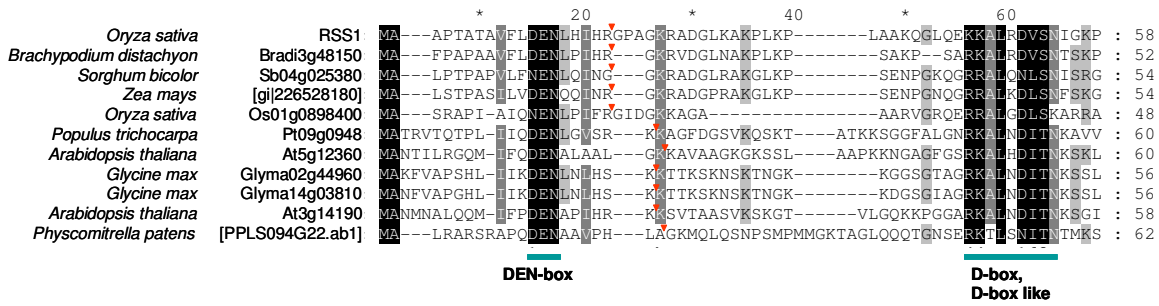


Supplementary Figure S5. Subcellular localisation of RSS1 and OsPP1. Plasmids carrying *GFP* (a), *RSS1-GFP* (b), *YFP-RSS1* (c), and *OsPP1-GFP* (d) driven by the CaMV 35S promoter were introduced into onion epidermal cells by the particle bombardment method. GFP or YFP fluorescence was observed under a fluorescence microscope and is visualised in green. Arrows indicate nuclei. Bars, 0.1 mm.

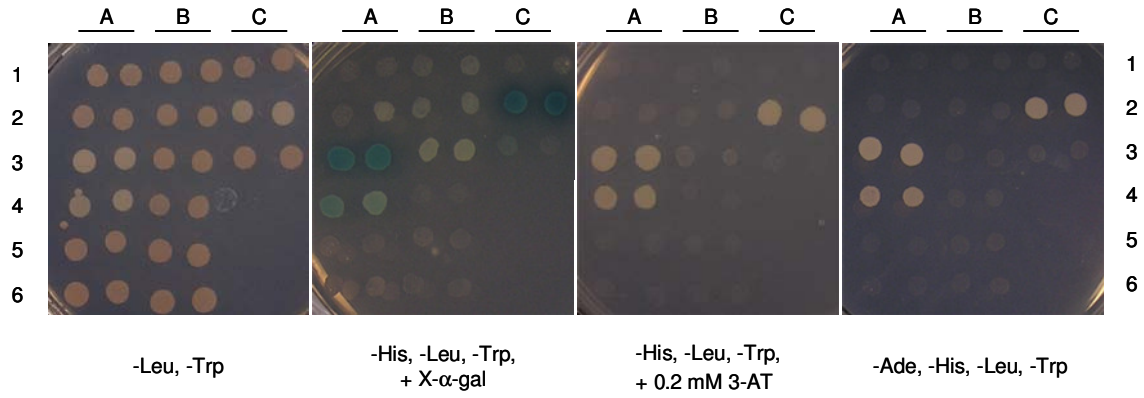
a

											ref.
Sea urchin Cyclin B	R	A	A	L	G	N	I	S	N		19
Patella Cyclin A	R	S	A	L	G	T	I	T	N		67
Starfish Cyclin B	R	G	A	L	E	N	I	S	N		67
Frog Cyclin A1	R	T	V	L	G	V	I	G	D		68
Yeast Cdc13	R	H	A	L	D	D	V	S	N		69
Mouse Cyclin B1	R	T	A	L	G	D	I	G	N		70
Human Cyclin A	R	A	A	L	A	V	L	K	S	G N	71
A. thaliana CycB1;1	R	Q	V	L	G	D	I	G	N		72
A. thaliana CycA2;3	R	A	V	L	G	E	I	T	N		73
Tobacco CycB1;1	R	R	A	L	G	D	I	G	N		74
Tobacco CycA3;1	R	V	V	L	G	E	L	T	N		74
Consensus	R	x	A V	L	x	x	I L V	x	N		
RSS1 (46-54)	K	K	A	L	R	D	V	S	N		
RSS1 (63-71)	R	K	P	L	Q	D	V	S	N		

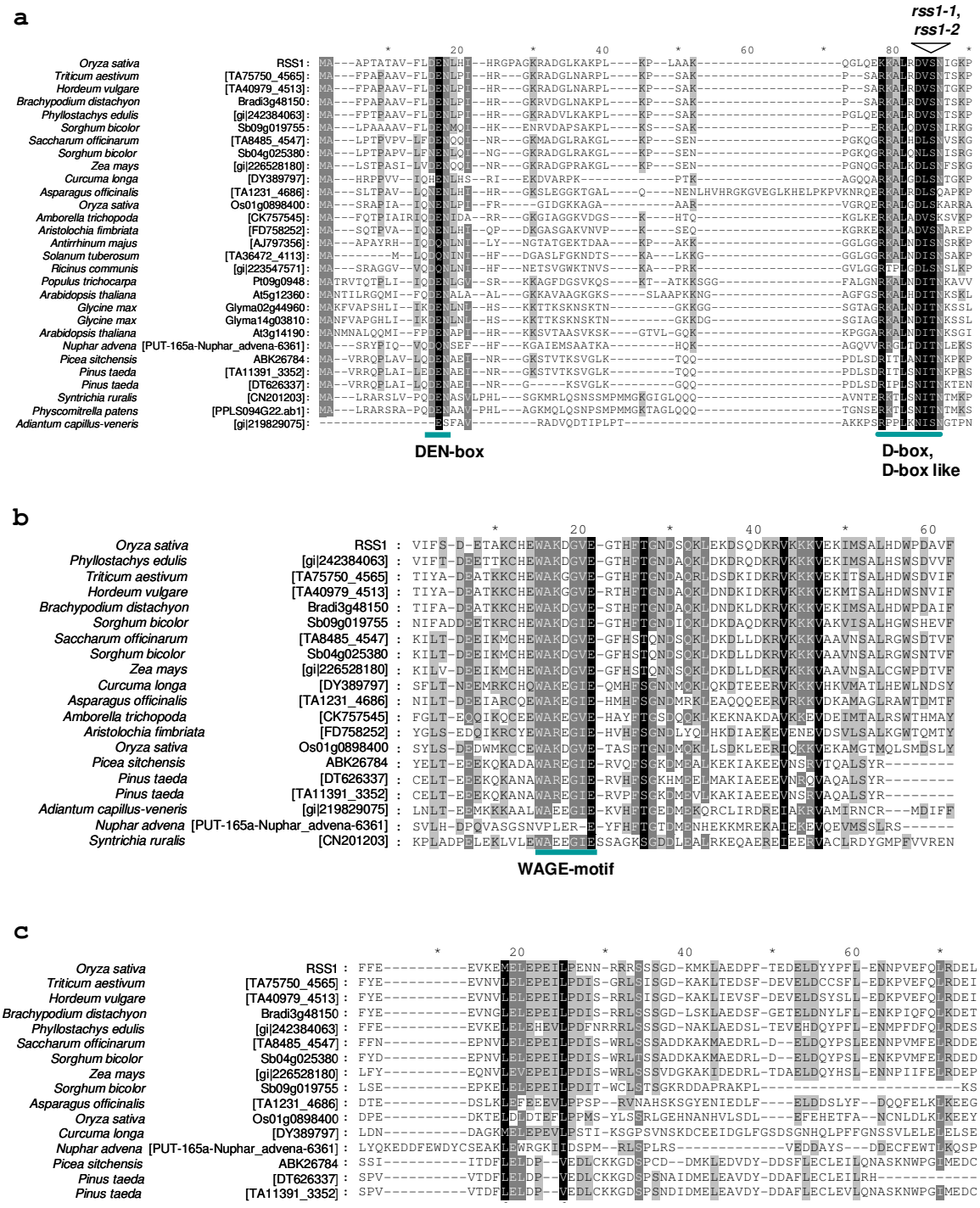
b



Supplementary Figure S6. Conserved sequence motifs in the N-terminal region of RSS1. (a) Primary sequences of the conserved D-box among cyclins A and B in animals and plants[19,67-74]. (b) N-terminal sequence of RSS1 and RSS1-related proteins. Proteins indicated with parenthetical names were deduced from expressed genes registered in GenBank or the DOE Joint Genome Institute (http://genome.jgi-psf.org/); all the other proteins are registered in the Plant GDB (http://www.plantgdb.org/). The DEN-box and D-box/D-box-like sequences are indicated. Highly conserved amino acids are shaded with three colour grades. The position of the intron between the D- and DEN-boxes is indicated by a red arrowhead. The lineages that the indicated plant species belong to are as follows: monocots (*Oryza sativa*, *Brachypodium distachyon*, *Sorghum bicolour*, and *Zea mays*); eudicots (*Populus trichocarpa*, *Arabidopsis thaliana*, and *Glycine max*); and mosses (*Physcomitrella patens* and *Syntrichia ruralis*). Note that the introns between the DEN- and D-boxes are located at nearly the same position, suggesting that the sequences homologous to region I are derived from the same source.

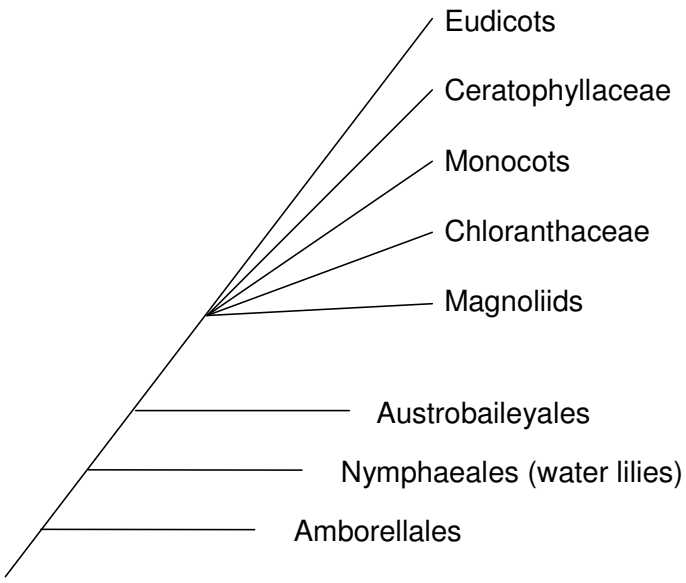


Supplementary Figure S7 An interaction of RSS1 with the subunit of APC/C by yeast two-hybrid system. The N-terminal regions of the RSS1 protein (a.a. 1-207 or 1-104) with or without deletion of the D- or DEN box were fused to the GAL4 DNA binding domain (Bait). The rice homologues of Cdh1 (OsCdh1;1 [Os01g0972900], OsCdh1;2 [Os03g0123300]) or Cdc20 (OsCdc20;1 [Os04g0599800]) were fused to the GAL4 activation domain (Prey). The following combination of the bait and prey proteins were co-expressed in the yeast cells carrying the *ADE2*, *HIS3* and *MEL1* reporter genes. Position 1A, RSS1[1-207] and OsCdh1;1. 2A, RSS1[1-207] and OsCdh1;2. 3A, RSS1[1-207] and OsCdc20;1. 4A, RSS1[1-104] and OsCdc20;1. 5A, RSS1[1-104]ΔD-box and OsCdc20;1. 6A, RSS1[1-104]ΔD-boxΔDEN-box and OsCdc20;1. 1B, bait vector and OsCdh1;1. 2B, bait vector and OsCdh1;2. 3B, bait vector, and OsCdc20;1. 4B, RSS1[1-104] and prey vector. 5B, RSS1[1-104]ΔD-box and prey vector. 6B, RSS1[1-104]ΔD-boxΔDEN-box and prey vector. 1C, RSS1[1-207] and prey vector. 2C, p53 and SV40 large T (positive control). 3C, Lamin C and SV40 large T (negative control). Two lines each of the yeast cells that expressed the respective bait and prey proteins were grown on the appropriate medium (which did not contain the indicated amino acids), supplemented with or without 0.2 mM of 3-Amino-1,2,4-triazole (3-AT) or 20 mg/L of X-α-gal. Activation of the *MEL1* reporter gene, encoding an α-galactosidase that hydrolyzes X-α-gal, results in blue colonies. 3-AT, a competitive inhibitor of the HIS3-gene product, was used to retard the growth of yeast transformants with a leaky HIS3 activity. The bait and prey plasmids carried the *TRP1* and *LEU2* marker genes, respectively. All the examined transformants were selected on the medium lacking Leu and Trp.

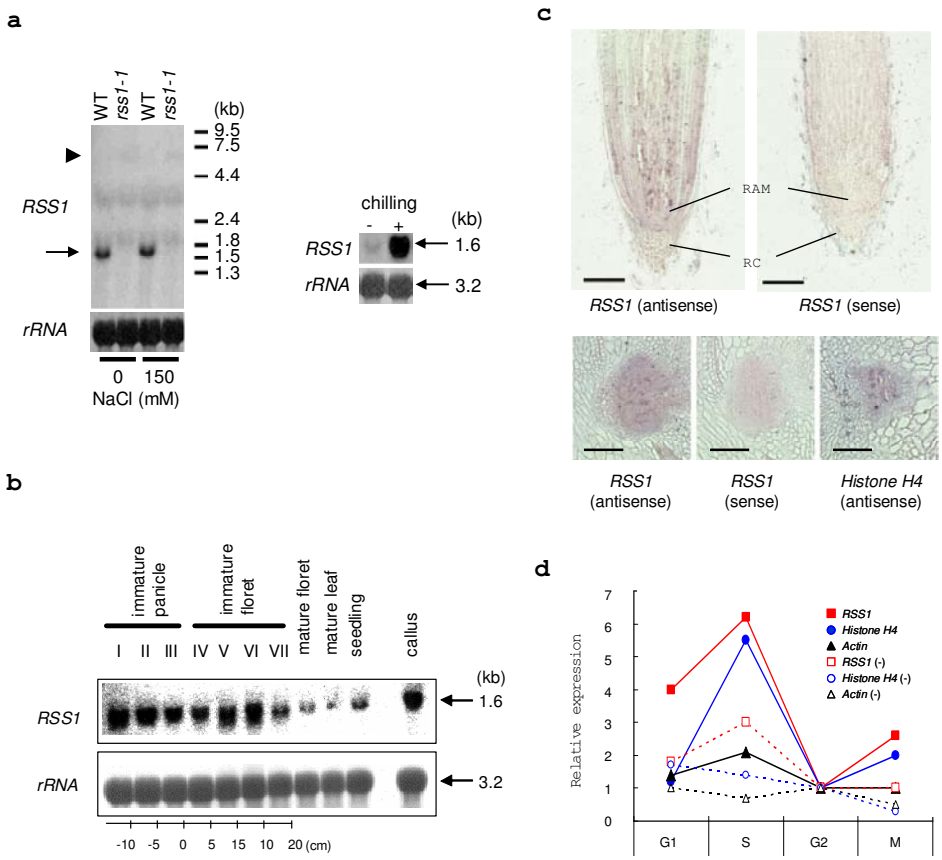


Supplementary Figure S8. Comparison of the primary sequences among RSS1 and its relatives. (a) region I. (b) region II. (c) region III. Proteins indicated with parenthetical names were deduced from expressed genes registered in GenBank, TIGR Plant Transcript Assemblies (http://plantta.jcvi.org/), or the DOE Joint Genome Institute (http://genome.jgi-psf.org/); all the other proteins are registered in the Plant GDB

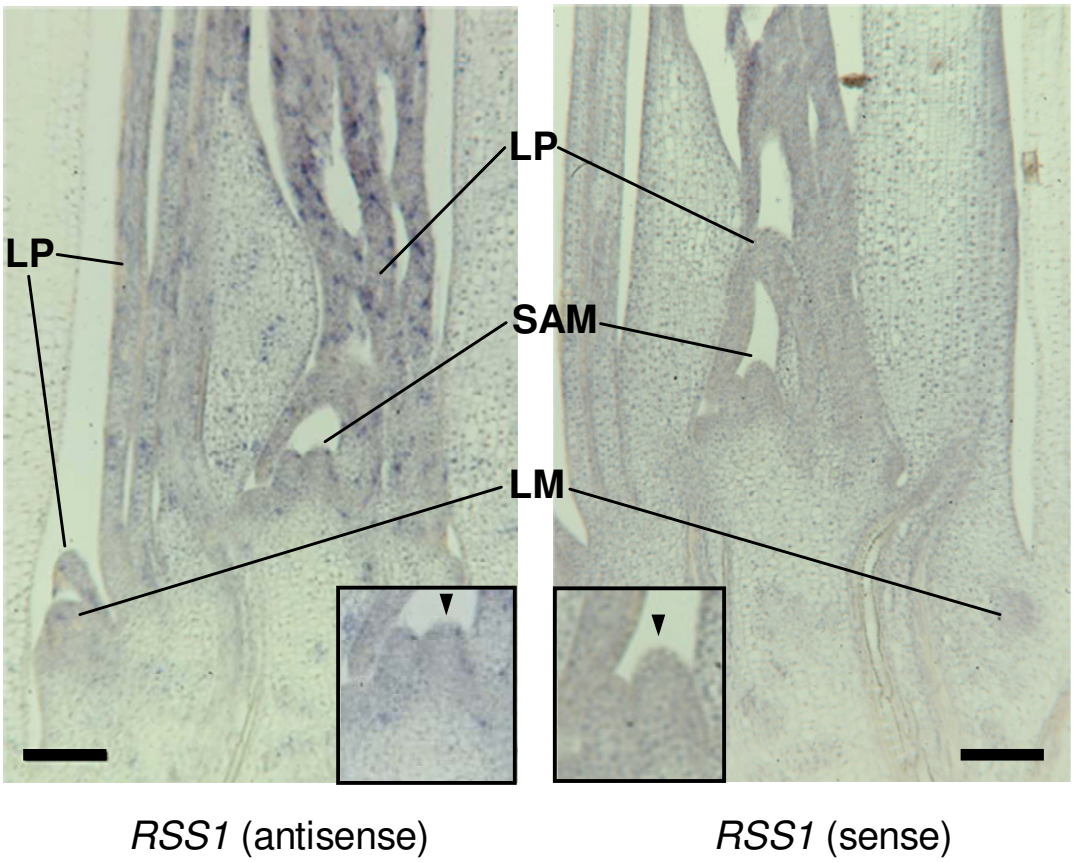
(http://www.plantgdb.org/). The sequences of the DEN-box, D-box/D-box-like, and WAGE motifs are indicated. The position of *Tos17* in *rss1-1* and *rss1-2* is indicated by a triangle. Highly conserved amino acids are shaded with three colour grades. The lineages that the indicated plant species belong to are as follows: monocots (*Oryza sativa*, *Triticum aestivum*, *Hordeum vulgare*, *Brachypodium distachyon*, *Phyllostachys edulis*, *Sorghum bicolor*, *Saccharum officinarum*, *Zea mays*, *Curcuma longa*, and *Asparagus officinalis*); Amborellales (*Amborella trichopoda*); magnoliids (*Aristolochia fimbriata*); eudicots (*Antirrhinum majus*, *Solanum tuberosum*, *Ricinus communis*, *Populus trichocarpa*, *Arabidopsis thaliana*, and *Glycine max*); Nymphaeales (*Nuphar advena*); gymnosperms (*Picea sitchensis*, *Pinus taeda*, and *Pinus taeda*); mosses (*Physcomitrella patens* and *Syntrichia ruralis*); and ferns (*Adiantum capillus-veneris*).



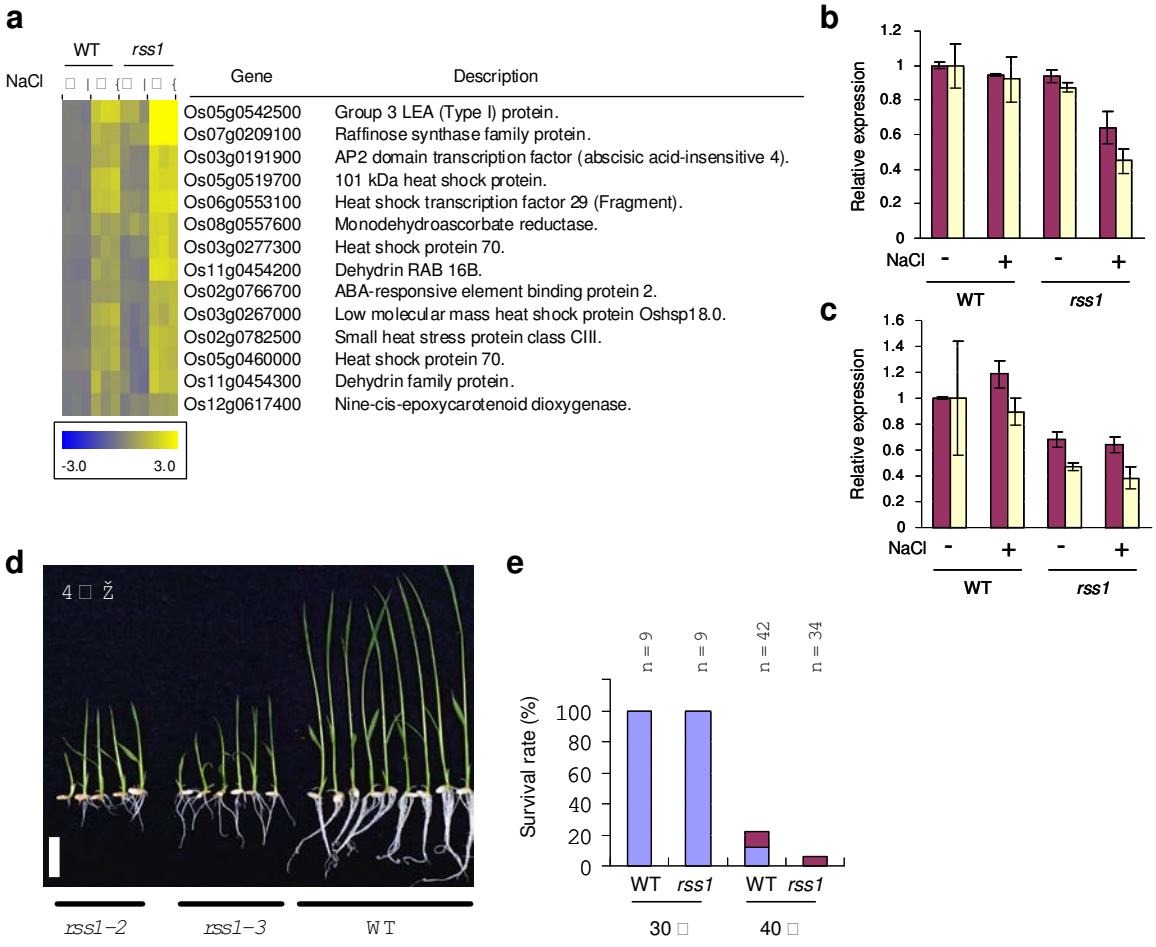
Supplementary Figure S9. Schematic illustration of the early evolution of angiosperms.



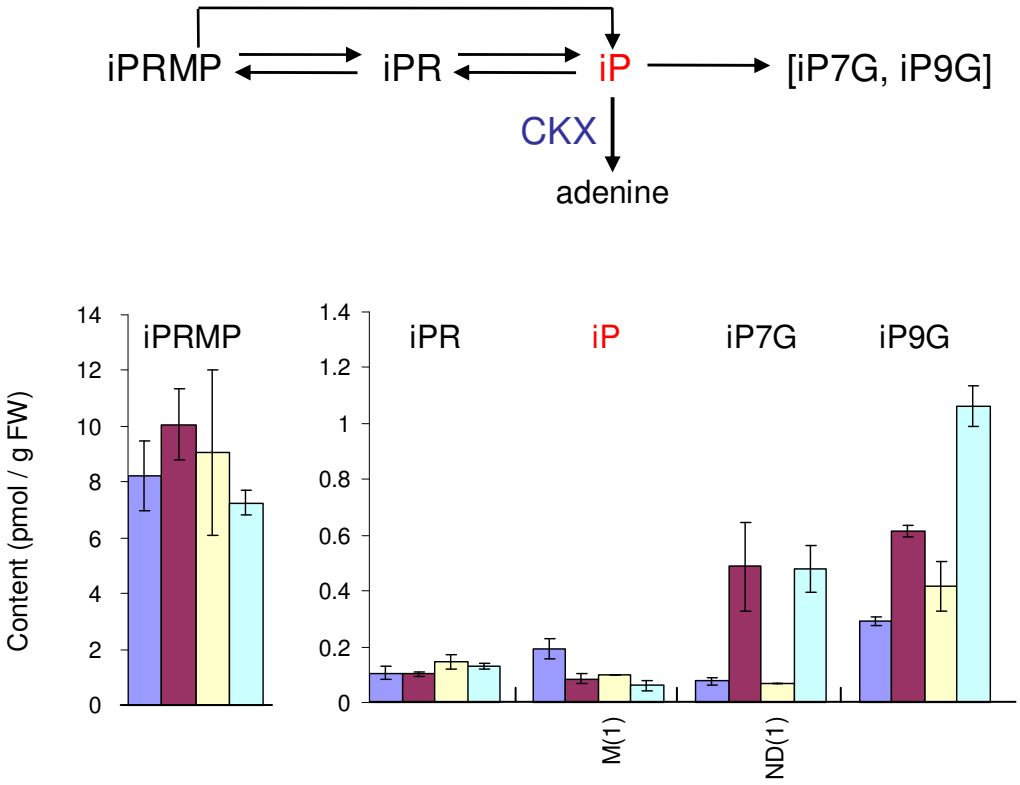
Supplementary Figure S10. Expression pattern of *RSS1*. (a) (left) Detection of the *RSS1* mRNA in WT and *rss1-1* seedlings grown on MS medium in the presence or absence of 150 mM NaCl for 2 weeks. The arrow indicates the position of the *RSS1* mRNA detected in WT. The arrowhead indicates the position of faint signals that correspond to *RSS1* mRNA carrying the *Tos17* insertion in *rss1-1*. (right) *RSS1* expression is up-regulated by cold treatment. One-week-old seedlings were treated with (+) or without (-) cold treatment at 4°C for one week. *rRNA*, loading control. (b) Expression of *RSS1* during reproductive growth. The mRNA levels of *RSS1* in the tissues indicated were analysed by RNA blotting. Panicles or florets at different developmental stages (I-VII; immature to mature) were classified according to the distances between the two auricles just below the flag leaf and next leaf, as indicated at the bottom (up to +20 cm). *rRNA*, loading control. (c) *In situ* localisation of *RSS1* in the root apices (top) and in primordia of the adventitious crown root (bottom). The expression pattern of *histone H4* in the root primordia is also shown. RAM, root apical meristem. RC, root cap. Bars, 100 μm. (d) Expression levels of *RSS1*, *histone H4*, and *actin* genes at the indicated cell cycle phases were analysed by northern blotting, and the quantified signal intensity is shown in the graph. The dashed lines indicate the RNA levels without synchronisation (-).



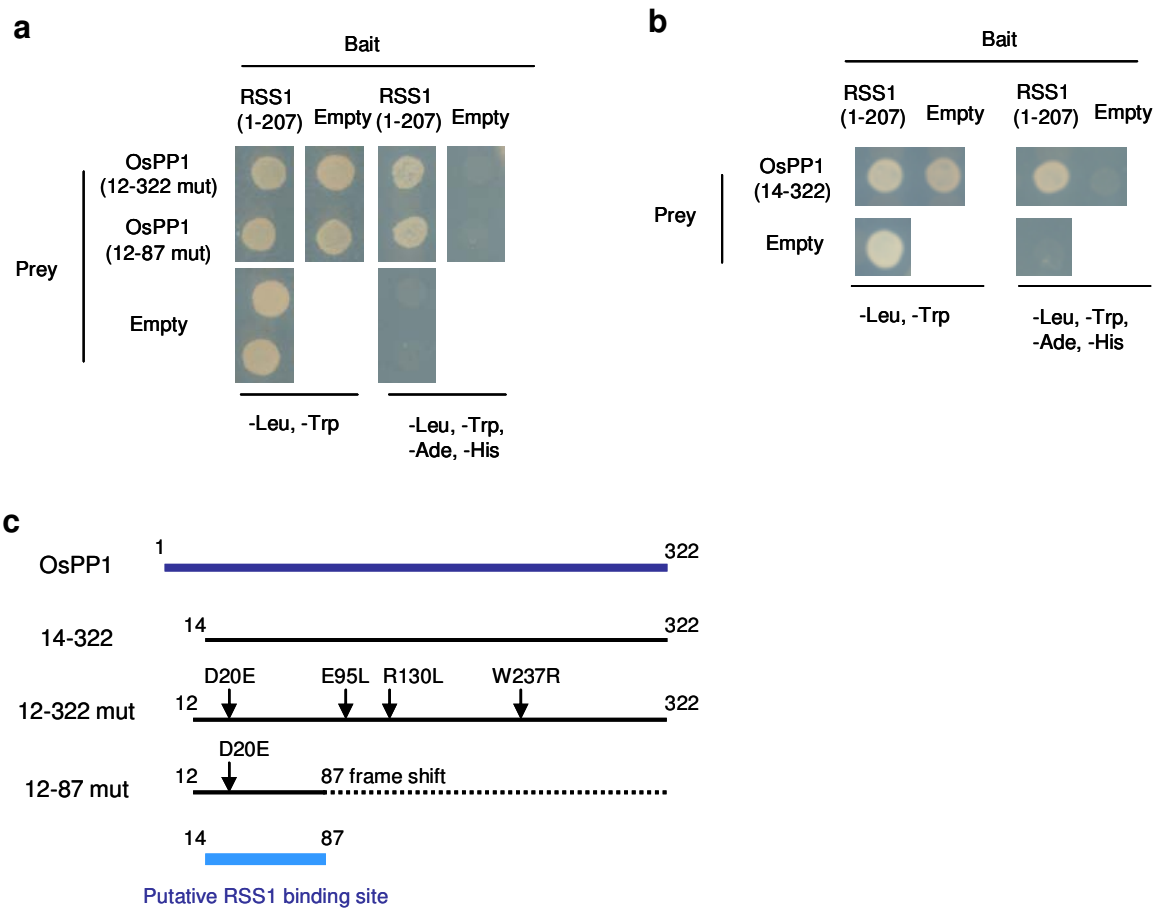
Supplementary Figure S11. Magnified views of the *in situ* localisation of *RSS1* mRNA. The longitudinal sections of the shoot apical meristem (SAM) region were hybridised with anti-sense (left) and sense (right) probes, specific to *RSS1*. Note that *RSS1* mRNA was detected in a patchy pattern, in the SAM and lateral meristem (LM), in addition to the leaf primordia (LP). Bars, 100 μm. (insets) Magnified views of the *in situ* localisation of *RSS1* mRNA in the SAM. The arrowheads indicate the position of the SAM.



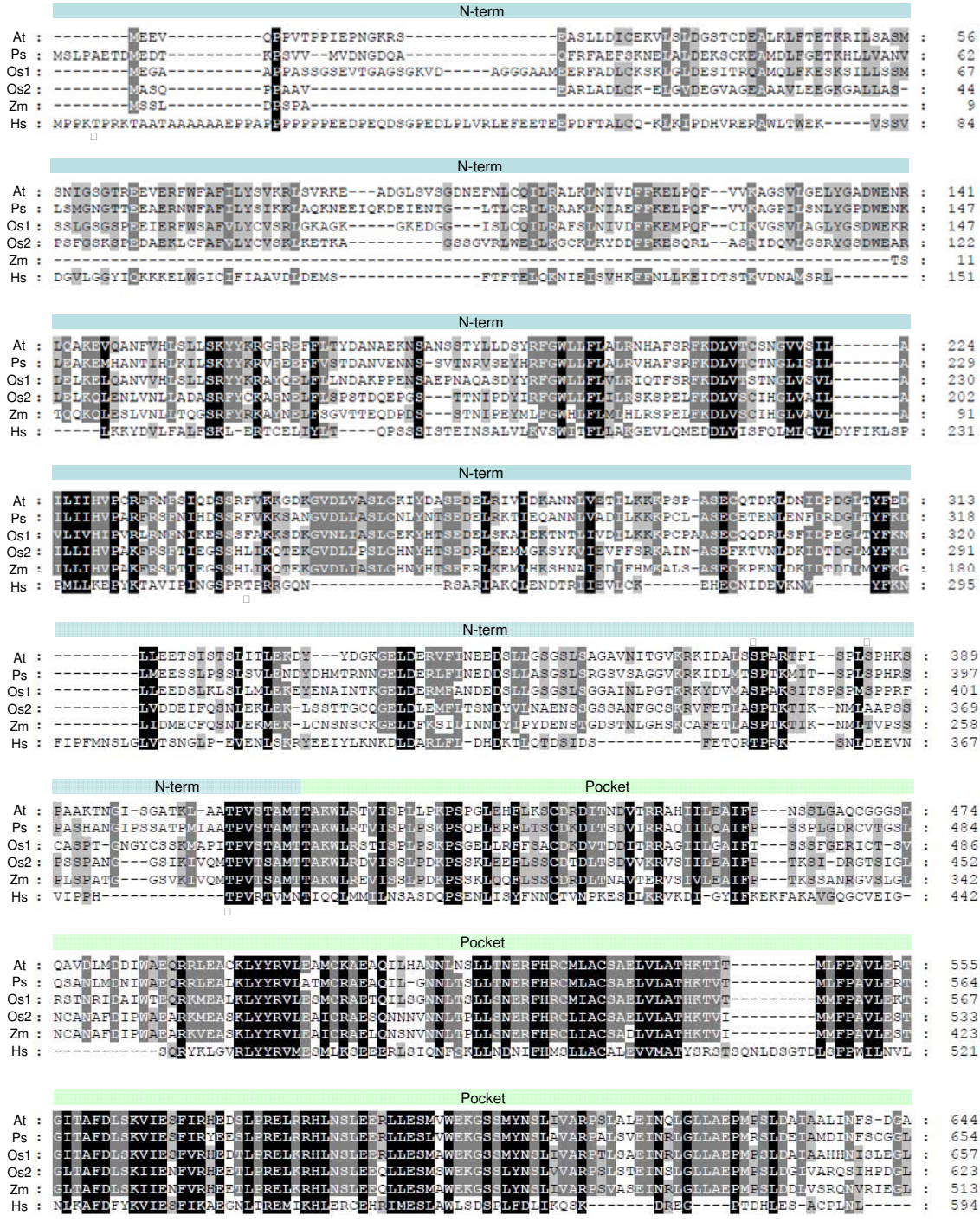
Supplementary Figure S12. ***rss1*** **affects abiotic stress responses.** (a) Heat map view of the expression pattern of genes possibly involved in stress tolerance under the category 'salt-inducible (long-term).' The expression levels are displayed as $\log_2$-transformed values. (b and c) Expression patterns of *OsNHX1* (b) and *OsDREB1A* (c) in the shoot basal tissues of seedlings grown under normal or salt-stressed conditions. Comparative analysis of WT and *rss1-2* plants by microarray (magenta) and of WT and *rss1-1* plants by quantitative RT-PCR with normalisation to *ubiquitin E2* expression (yellow). The data represent the mean ± s.d., $n = 3$. (d) *rss1* is hypersensitive to cold stress. Three-day-old WT and *rss1* seedlings were incubated at 4°C for 2 weeks, followed by incubation at 25°C for 4 days. Bars, 2 cm. (e) *rss1* is hypersensitive to heat stress. One-week-old WT and *rss1-1* seedlings were incubated in a chamber at 30°C or 40°C under continuous light (5,000 lux) for 1 week, followed by incubation at 25°C for 2 weeks. The number of surviving but seriously damaged plants (magenta) and of healthy plants (blue) were scored, respectively.



Supplementary Figure S13. Levels of cytokinins and related metabolites in the basal shoot tissues. (top) Schematic illustration of cytokinin biosynthesis and metabolism, adapted from previous reports[33,34]. (bottom) One-centimetre-long shoot basal tissues from one-week-old seedlings of WT (blue and magenta) and *rss1-2* (yellow and light blue) grown in the absence (blue and yellow) or presence (magenta and light blue) were examined. Active cytokinins (iP) are indicated in red. iP, N^6-(Δ^2-isopentenyl)-adenine; iPR, iP riboside; iPRMP, iPR 5-monophosphate; iP7G, iP-*N*-7-glucoside; IP9G, iP-*N*-9-glucoside. The data represent the mean ± s.d., n = 3. M(1), the average of two independent samples due to a single mistake. ND(1), the average of two independent samples due to one value being below the detection limit.



Supplementary Figure S14. A putative RSS1 binding region of OsPP1. (a, b) Interactions of RSS1 with OsPP1 carrying the indicated mutations by the yeast two-hybrid assay. The GAL4 DNA-binding domain fused to the truncated RSS1 (a.a. 1-207) and the GAL4 activation domain fused to OsPP1 were co-expressed in yeast cells carrying the reporter genes, *ADE2* and *HIS3*. (a). Interactions of RSS1 with OsPP1 clones carrying point mutations. (b) An interaction of RSS1 with OsPP1 carrying the N-terminal deletion. (c) A scheme of the mutated OsPP1 used in (a) and (b). The truncated *OsPP1* cDNA clones carrying point mutations ([12-322 mut] and [12-87 mut]) were identified by the interaction assay of the cDNA fragments, amplified by PCR from the positive clone of the initial screening. The OsPP1 cDNA fragment (14-322) was designed for the evaluation of the effects of the point mutations. The positions of the five point mutations and a putative RSS1 binding region are shown.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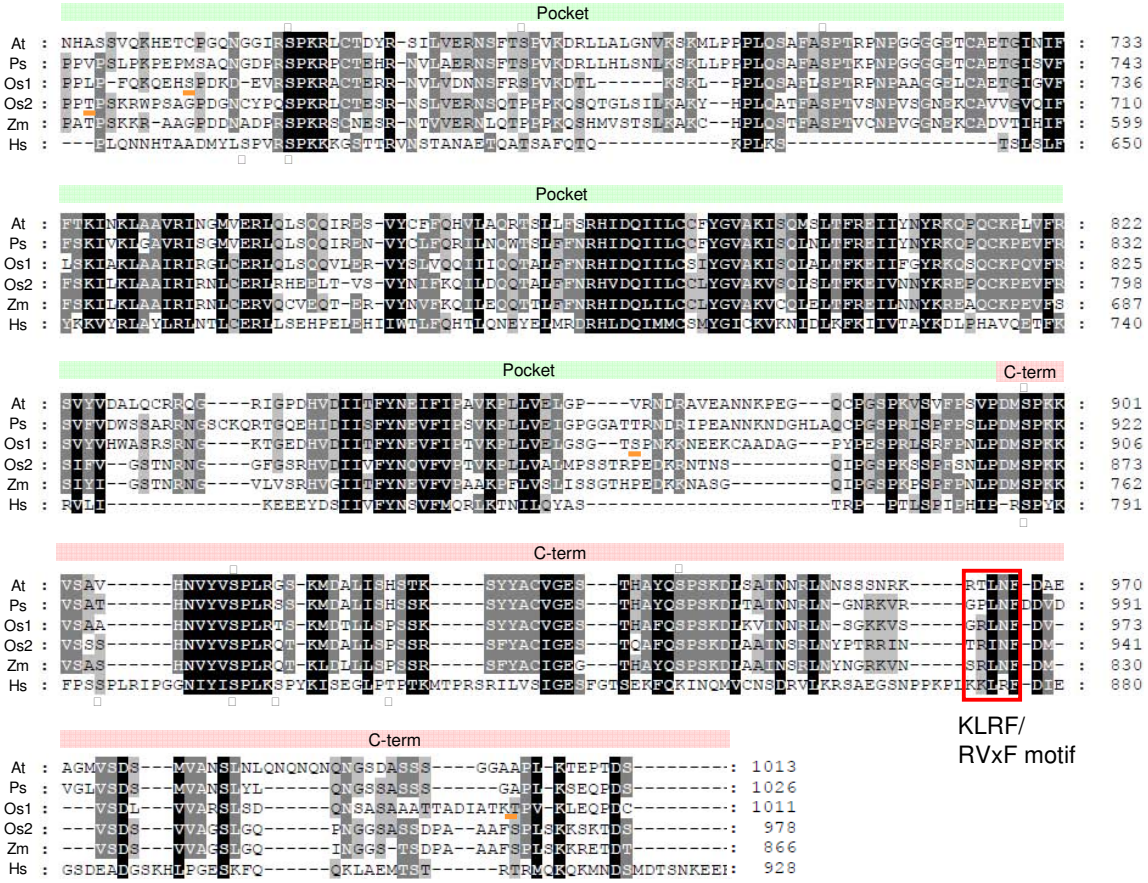

Supplementary Figure S15. PP1-binding KLRF/RVxF motifs and CDK phosphorylation sites are conserved among plant and human Rb proteins. Alignment of the amino acid sequences of Rb homologues (*Arabidopsis thaliana* [At: At3g12280], *Pisum sativum* [Ps: AB012024], *Oryza sativa* [Os1: Os08g0538700, Os2: Os11g0533500], *Zea mays* [Zm: AAB69649.1], *Homo sapiens* [Hs: NP_000312.2]). The N-terminal, Pocket, and C-terminal regions are shown by coloured bars. The PP1-binding KLRF motif[11] and the conserved RVxF motifs ([R/K]x$_{0-1}$[V/I]x[F/W]) are indicated by a red box. The manner of the binding of the KLRF motif to PP1 is essentially the same as that of other RVxF motifs: both interactions are dependent on the hydrogen bonds between the parallel ß-strands and on the hydrophobic side-chain interactions[16]. The positions of CDK phosphorylation sites ([S/T]PxK/R) conserved among plant Rb proteins, particularly in rice and maize Rb proteins, and in human Rb, are indicated by stars above the sequences, by underlines, and by stars below the sequences, respectively. The phosphorylation by CDKs at S608 and S612 in the pocket region, and at S788, S795, T821 and T826 in the C-terminal region of the human Rb is required for E2F-DP release[48,75]. Of the six phosphorylation sites, S608 in the pocket region and S788 in the C-terminal region are also conserved among the plant Rb proteins.

Supplementary Table S1. Primer sets used for the quantitative RT-PCR analysis.

Description	Gene	Direction	Sequence
Ubiquitin-conjugating enzyme E2	Os01g0673600	Forward	5'-ctaagtgcaccaaaccctgatg-3'
		Reverse	5'-tctgcttcattggctttcca-3'
PCNA	Os02g0805200	Forward	5'-ccgagtaccaggccatcgt-3'
		Reverse	5'-atgctgctcagatccttgca-3'
CycB2:1	Os04g0563700	Forward	5'-tccttgacgcgaaaagaacag-3'
		Reverse	5'-cagacgaccgctgcatca-3'
NHX1	Os07g0666900	Forward	5'-gatgctcatggcttacctttcata-3'
		Reverse	5'-ccacagaagaatacggtgagaatg-3'
DREB1A	Os09g0522200	Forward	5'-gacgtcccactctggagctact-3'
		Reverse	5'-gcatcggaagccagaaaaga-3'
CKX2	Os01g0197600	Forward	5'-tgccaatctctggccaaga-3'
		Reverse	5'-cgggacaggatggccttt-3'
OsRR1	Os04g0442300	Forward	5'-ccgatcccagtgatcgtcat-3'
		Reverse	5'-tgaaatcttcagcaccagcatt-3'

Supplementary Methods

Microscopy For microscopic observation, roots were incubated in a chloral hydrate solution (8 g of chloral hydrate, 1 mL of glycerol, and 2 mL of water). For confocal laser scanning microscopic observation, root samples were prepared by a modified pseudo-Schiff propidium iodide staining technique[76]. The tissues were treated with a fixative (50% methanol and 10% acetic acid) at 4°C for 12 h, transferred to 80% ethanol, and then incubated at 80°C for 5 min. The tissues were then rinsed twice with water, incubated in 1% SDS and 0.2 N NaOH for 24 h, rinsed twice with water, incubated in 1% periodic acid at room temperature for 40 min, rinsed twice with water, and then incubated in 50 μg/mL propidium iodide in 100 mM sodium disulphite and 0.15 N HCl for 3 h. After rinsing with water, the tissues were incubated in a chloral hydrate solution (4 g of chloral hydrate, 1 mL of glycerol, and 2 mL of water) for 12 h. For DNA synthesis inhibitor treatment, seedlings were grown in MS medium containing 150 mM NaCl in the presence of 10 mg/L aphidicolin for 5 days. The cell numbers in the MZ and EZ were measured by counting the epidermal cells in the primary root of seedlings grown in the absence of NaCl for 4 days or in the presence of 150 mM NaCl for 5 days. Variation between the scores in the same root, according to cell arrays, was less than 10%. For the analysis of RSS1-GFP localisation, *RSS1* cDNA was subcloned into a GFP fusion vector. The resultant plasmid was introduced into onion epidermal cells by particle bombardment, and the GFP signals were observed under a fluorescence microscope.

Measurement of the Na^+ and K^+ contents Above-ground tissues from two-week-old seedlings grown in the presence or absence of 150 mM NaCl were cut into small pieces, dipped in water in 1.5-ml tubes, and heated at 100°C for 10 min. After vigorous vortexing and centrifugation twice at 20,400 *g* for 5 min each, the crude extracts were filtered

through 0.45-μm PTFE membranes (Millex-LH, #SLLHH04NL; Millipore, Tokyo, Japan) for ion-exchange column chromatography (IC-C3; Shimadzu, Kyoto, Japan).

Transformation of rice and complementation testing A *Bam*HI-*Sal*I fragment (1.2 kb) of the *RSS1* cDNA in pADGAL4-2.1 was subcloned into pBluescriptII SK+ (Stratagene, La Jolla, CA), resulting in pSK*RSS1*. An *Spe*I-digested fragment (1.1 kb) of *RSS1* cDNA was then subcloned directionally between the *actin* promoter fragment and a terminator at the *Xba*I site of pActnos-Hm2 (courtesy of Dr. M. Matsuoka, Nagoya University, Nagoya, Japan). For construction of the *RSS1* promoter::*RSS1-GFP* fusion construct, genomic DNA and cDNA for *RSS1* were fused at the *Hin*dIII site, 12 bp upstream of the first codon in the second exon. A *Sal*I-*Hin*dIII fragment (3.2 kb) carrying the *RSS1* promoter and 5'-UTR sequences was ligated with a *Hin*dIII-*Eco*RI fragment (1.7 kb) carrying *RSS1* cDNA fused to *GFP* and the *Nos* terminator, and subcloned into pBluescriptII SK+ with an *Eco*RI-*Sal*I-*Bam*HI linker. A *Sal*I fragment carrying the chimeric *RSS1-GFP* fusion was introduced into a pBI-based binary vector. Deletion constructs based on the *RSS1* promoter::*RSS1-GFP* were prepared by swapping DNA fragments. For the C-terminal deletion series (1-208, 1-175, 1-130, and 1-104), truncated *RSS1* cDNA fragments were amplified by PCR using a forward primer (5'-ggatgtatccaacattggcaa-3') and respective reverse primers (5'-ccaaccatggcagccagcttcattttatca-3', 5'-ccaaccatggctgctaccacctcagatgga-3', 5'-ccaaccatggcattcccagtgaagtgggtg-3', and 5'-ccaaccatggcagtcttcttcactgggttc-3'), digested with *Pst*I and *Nco*I, and inserted between the *Pst*I site in the *RSS1* cDNA and the *Nco*I site at the first ATG in *GFP*. For the N-terminal deletion series (59-243, 164-243, and 209-243), *RSS1* fragments were amplified by PCR using respective forward primers (5'-ggggaagcttatgccggtgtctacgcggaagcc-3', 5'-ggggaagcttatggatcatgtgctttttccatctgagg-3', and 5'-

ggggaagcttatggatcctttcacggaagacgagcttg-3') and a reverse primer (5'-aacagctcctcgcccttgct-3'), digested with *Hin*dIII and *Nco*I, and inserted between the *Hin*dIII site, 12 bp upstream of the first codon in *RSS1* and the *Nco*I site at the first ATG in *GFP*. The resultant pBI-based constructs were used for *Agrobacterium*-mediated transformation. Transformed calli were selected with 50 mg/L hygromycin. For the complementation assays, regenerated plants were grown *in vitro* on MS medium supplemented with 500 mg/L carbenicillin (Wako Pure Chemicals, Tokyo, Japan), excised, and examined for salt tolerance by growth in a medium containing 150 mM NaCl.

Protein stability assay Rice calli expressing the RSS1-GFP fusion protein were cultured in a N6-based liquid medium (2 g/L glycine, 0.5 mg/L nicotine acid, 0.5 mg/L pyridoxine acid, 1 mg/L thiamine acid, 100 mg/L myo-inositol, 0.3 g/L casamino acid, 2,878 mg/L proline, 2 mg/L 2,4-dichlorophenoxy acetic acid, 3.981 g/L CHU[N6] [C1416; Sigma, St. Louis, MO], and 30 g/L sucrose) at 80 rpm at 28°C and then treated with 50 μM MG132 (Enzo Life Sciences, Plymouth Meeting, PA) plus 1% DMSO or with DMSO alone for 6 h. Total soluble proteins were extracted with extraction buffer (20 mM Tris-HCl [pH 7.5], 2 mM EDTA, 2 mM EGTA, 50 mM β-glycerophosphate, 100 μM Na_3VO_4, 2 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 250 mM sucrose, 0.1% Tween-20, and a protease inhibitor cocktail [Complete EDTA-free; Roche, Penzberg, Germany]) and analysed by immunoblotting. The amount of protein was determined using the Bio-Rad Extraction Assay system (Bio-Rad Laboratories, Richmond, CA). The proteins were then transferred to a membrane in a blotting buffer (25 mM Tris-[hydroxymethyl] aminomethane, 192 mM glycine, and 10% methanol). Equal loading was confirmed by the staining of proteins in the gels or on the blotted membranes.

Northern blot and microarray analysis Total RNA isolation, gel blotting, and hybridisation for northern blot analysis were performed as described[77]. A *Bam*HI-*Sal*I fragment (1.2 kb) of the *RSS1* cDNA was used as the probe. For microarray analysis, total RNA was extracted using the RNeasy Mini kit (Qiagen, Hilden, Germany) from 1-cm-long shoot basal tissues of 1-week-old seedlings grown on MS-based medium in the presence or absence of 150 mM NaCl. The RNAs were labelled with a Low RNA Input Linear Amplification/Labelling kit (Agilent Technologies, Palo Alto, CA). Four different aliquots (WT[–NaCl], WT[+NaCl], *rss1-2*[–NaCl], and *rss1-2*[+NaCl]) of Cy-3-labelled cRNA (825 ng) and a control aliquot (WT[–NaCl]) of Cy-5-labelled cRNA (825 ng) were prepared. Each aliquot of Cy-3-labelled cRNA was mixed with the control Cy-5-labelled cRNA, and used for hybridisation with an Agilent Rice Oligo Microarray (44K, custom-made; Agilent Technologies). The scanned images were analyzed with Feature Extraction Software v9.5.3.1 (Agilent). Our raw microarray data from three biological replicates were normalised by the variance stabilisation normalisation method[78]. After normalisation, the relative expression levels of the genes in each sample were calculated by dividing the Cy-3 value by the Cy-5 value. Significant differences were examined using the Rank product[79] with the R-software and two-way ANOVA with the TIGR Multiexperiment Viewer (MeV; http://www.tm4.org/mev/)[80]. The analysed data of all the genes that were differentially expressed are shown in the Supplementary Data 1.

Categorisation of the genes analysed using the 44K Agilent microarray The number of genes in the respective categories and the term accession of Gene Ontology (GO) (http://www.gramene.org/plant_ontology/) are listed in Supplementary Data 2. For

extraction of genes from public data, raw Affimetryx microarray data were normalised with GCRMA (GC Robust Multi-Array)[81]. The lists of the genes in the categories that were classified by the expression pattern, and the genes in the GO term "cell cycle" and "DNA replication," are provided in the Supplementary Data 2.

Cytokinin treatment Shoot explants without roots, 2.5 cm long, were excised from seven-day-old seedlings and transferred to MS medium containing 150 mM NaCl in the presence or absence of kinetin and then incubated for 19 days.

Quantitative RT-PCR Reverse transcription was carried out using an Omniscript Reverse Transcription kit (Qiagen). cDNA was then amplified by real time-PCR using an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA) with Power SYBR Green PCR master mix (Applied Biosystems) and the primer sets listed in Supplementary Table S1. The quantified expression levels of the tested genes were normalised with that of *ubiquitin E2*.

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