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Antagonistic Effects of Abscisic Acid and Jasmonates on Salt Stress–Inducible Transcripts in Rice Roots

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Abscisic acid (ABA) and jasmonates have been implicated in responses to water deficit and wounding. We compared the molecular and physiological effects of jasmonic acid (JA) (≤10 μM), ABA, and salt stress in roots of rice. JA markedly induced a cationic peroxidase, two novel 32- and 28-kD proteins, acidic PR-1 and PR-10 pathogenesis-related proteins, and the salt stress–responsive SalT protein in roots. Most JA-responsive proteins (JIPs) from roots also accumulated when plants were subjected to salt stress. None of the JIPs accumulated when plants were treated with ABA. JA did not induce an ABA-responsive group 3 late-embryogenesis abundant (LEA) protein. Salt stress and ABA but not JA induced *oslea3* transcript accumulation. By contrast, JA, ABA, and salt stress induced transcript accumulation of *salT* and *osdrr*, which encodes a rice PR-10 protein. However, ABA also negatively affected *salT* transcript accumulation, whereas JA negatively affected ABA-induced *oslea3* transcript levels. Endogenous root ABA and methyl jasmonate levels showed a differential increase with the dose and the duration of salt stress. The results indicate that ABA and jasmonates antagonistically regulated the expression of salt stress–inducible proteins associated with water deficit or defense responses.

INTRODUCTION

Plants respond to abiotic and biotic stresses by inducing the expression of a set of genes whose products are thought to help overcome these adverse conditions. Different plant growth regulators, such as abscisic acid (ABA) and jasmonic acid (JA), are involved in regulating these changes in gene expression.

The plant growth regulator JA and its methyl ester, methyl jasmonate (MeJA), are ubiquitously present in plants (Meyer et al., 1984) and involved in signaling the plant's response to wounding and pathogen attack (Creelman et al., 1992; Farmer and Ryan, 1992; Staswick, 1992; Mueller et al., 1993). Jasmonates accumulate rapidly and transiently when plants are wounded or treated with an elicitor (Creelman et al., 1992; Gundlach et al., 1992). Spraying plants with JA results in enhanced pathogen resistance (Schweizer et al., 1993). JA and MeJA induce the expression of genes encoding proteins that are typically involved in wounding and pathogen attack responses, such as different types of proteinase inhibitors (Farmer and Ryan, 1992; Hildmann et al., 1992), thionins (Andresen et al., 1992), a proline-rich cell wall protein (Creelman et al., 1992), enzymes involved in phytoalexin biosynthesis, for example, phenylalanine ammonia–lyase (Gundlach et al., 1992) and chalcone synthase (Lee et al., 1996), and different pathogenesis-related (PR) proteins (La Rosa et al., 1992; Xu et al., 1994; Schweizer et al., 1997). Jasmonates were also found to induce a ribosome-inactivating protein in barley leaves (Becker and Apel, 1992; Chaudry et al., 1994; Reinbothe et al., 1994) and different types of lipoxygenase (LOX) isoforms involved in JA biosynthesis in various plants (Grimes et al., 1992; Melan et al., 1993; Bell et al., 1995). Other JA-induced proteins from barley and cotton have unknown functions (Reinbothe et al., 1992; Leopold et al., 1996).

Jasmonates induce the expression of wounding-responsive genes at the transcriptional and post-transcriptional level (Reinbothe et al., 1994). MeJA-responsive regions in the promoter of wounding-responsive genes have been identified (Mason et al., 1993; Rouster et al., 1997). However, JA and MeJA have also been found to exert negative post-transcriptional effects on the expression of photosynthetic genes through alternative RNA processing and an inhibition of translation initiation (Reinbothe et al., 1994). Involvement of the JA-induced ribosome-inactivating protein in the differential control of translation initiation that is governed by JA has been proposed (Chaudry et al., 1994).

The plant hormone ABA, which endogenously increases when tissues become dehydrated, is an important signal for the physiological and molecular responses to water-limiting

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stresses, such as desiccation, salt stress, and cold (Zeevaart and Creelman, 1988; Hetherington and Quatrano, 1991). ABA and water deficit induce the expression of a large set of genes that are thought to ameliorate desiccation tolerance, from which the different classes of late-embryogenesis abundant (LEA) proteins form a substantial proportion (reviewed in Bray, 1994). Based on structural properties, LEA proteins have been implicated in membrane protection, water replacement, or ion scavenging (Dure, 1993). Water-limiting stresses induce the transcription of *LEA* genes via ABA-dependent and ABA-independent pathways, which has been studied extensively (Chandler and Robertson, 1994; Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 1997). Little is known about post-transcriptional effects governed by ABA.

ABA has been demonstrated to be involved in gene activation in response to wounding (Peña-Cortés and Willmitzer, 1995). Endogenous ABA levels rise in plants after mechanical damage, both locally and systemically. By using ABA-deficient mutants, researchers have established a correlation between the increase of ABA after wounding and the expression of different proteinase inhibitors (Peña-Cortés et al., 1989; Hildmann et al., 1992). The ABA response of the wound-responsive *pin2* gene was found to require cytosolic protein synthesis (Peña-Cortés and Willmitzer, 1995). A *LOX* gene of Arabidopsis was found to be induced by ABA (Melan et al., 1993), suggesting interactions of both plant growth regulators.

Similarities in ABA- and JA-induced changes of unidentified in vitro translation products in cotton and barley (Reinbothe et al., 1992) and the detection of increased jasmonate production in plant tissue exposed to water stress (Creelman and Mullet, 1995; Harms et al., 1995; Kramell et al., 1995; Lehmann et al., 1995) led to the proposal of a role for JA in the water deficit response (Sembdner and Parthier, 1993). Moreover, JA and water stress induce the expression of vegetative storage protein genes of soybean (Mason and Mullet, 1990), the rape storage protein genes napin and cruciferin (Wilen et al., 1991), and a gene encoding a 37-kD protein of barley (Leopold et al., 1996). However, other genes that are expressed in response to JA and ABA were found to be induced when plants were wounded but not when plants were deprived of water, for example, *proteinase inhibitor* (Hildmann et al., 1992) or *LOX* (Melan et al., 1993) genes.

In an attempt to distinguish the function of both plant growth regulators in plant stress responses, gene expression, and physiological effects of ABA, JA and salt stress were analyzed in roots of rice. ABA and JA were found to differentially affect the root development of seedlings. Changes in endogenous ABA and jasmonate concentrations were determined in roots during salt stress. Eight stress-induced proteins, which were characterized by partial amino acid analysis, were compared for their ABA and/or JA response. In addition, the effect of JA, ABA, and salt stress on the transcript levels of *osdrr*, *salT*, and *oslea3*, encoding a PR-10 protein, a salt stress–responsive protein, and a group 3 LEA protein, respectively, were analyzed. ABA and JA were found to exert antagonistic effects on the transcript and/or protein accumulation of two classes of salt stress–responsive genes.

RESULTS

Effects of ABA, JA, and Salt Stress on Rice Seedling Growth and Root Morphology

Extensive similarities but also clear differences in the physiological effects of ABA and JA have been reported (Sembdner and Parthier, 1993; Yeh et al., 1995). We compared the effect of low physiological JA and ABA concentrations (1, 3, and 9 μM) and moderate salt stress (50 and 75 mM NaCl) on the growth of 5-day-old rice seedlings after a 6-day treatment. These JA amounts (<10 μM) did not cause chlorosis or any other visible symptoms of senescence and provoked a significantly less pronounced relative growth inhibition of the seedling shoot than did equal concentrations of ABA (Figure 1A, plants 1, 2, and 3, and Figure 1C). ABA and NaCl markedly inhibited the growth of the first leaf (Figure 1C) but had less (ABA) or no (NaCl) inhibitory effect on the elongation of the primary root (Figure 1B, plants 1, 3, and 4, and Figure 1D), which resulted in an increased root-to-shoot ratio (Figure 1E). Conversely, JA did not differentially inhibit the shoot-versus-root growth of the seedlings (Figure 1E).

In addition, ABA, JA, and NaCl differentially affected root development (Figure 1B). Low concentrations of JA (≤3 μM) stimulated the development of adventitious roots compared with the average number in the root system of 11-day-old control seedlings (Figure 1B, plants 1 and 2, and Figure 1F). By contrast, ABA strictly inhibited adventitious root development, which was not affected by mild salt stress (Figure 1B, plants 3 and 4, and Figure 1F).

Endogenous ABA and Jasmonate Accumulations in Roots of Rice Plants Subjected to Salt Stress

In view of the proposed roles of JA and MeJA in plants exposed to water-limiting stresses, changes in endogenous jasmonate concentrations were compared with the well-established increase in endogenous ABA in plants subjected to salt stress. The ABA and jasmonate concentrations were quantitated using heavy isotope–labeled internal standards coupled with gas chromatography–mass spectrometry (GC-MS). Salt shock (150 mM NaCl) induced a rapid increase in the ABA content in roots of 10-day-old seedlings, reaching a maximum at 8 hr of stress and decreasing to near control levels after 12 hr (Figure 2A). By contrast, endogenous jasmonates, in particular MeJA concentrations, exhibited a delayed and gradual increase of approximately fourfold after 12 hr of stress. The MeJA accumulation occurred when ABA levels were decreasing.

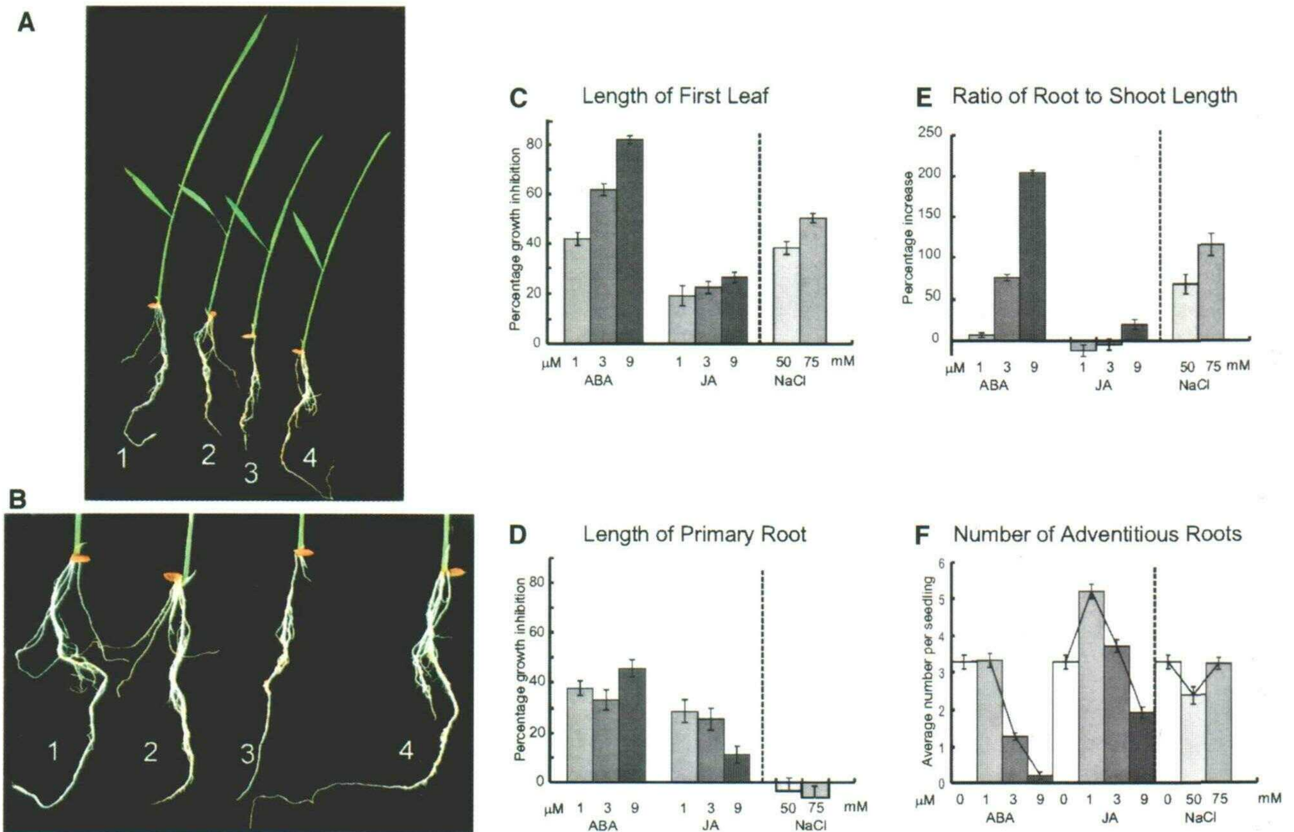

Figure 1. Differential Effect of JA, ABA, and Salt Stress on Rice Seedling Growth and Root Development.

The effects of a 6-day application of 1, 3, and 9 µM ABA or JA and 50 or 75 mM NaCl on rice seedling growth were tested.
(A) and **(B)** Eleven-day-old seedlings grown on control medium (plant 1), 3 µM JA (plant 2), 3 µM ABA (plant 3), and 75 mM NaCl (plant 4).
(C) and **(D)** Percentage of growth inhibition of the first leaf and primary root, respectively.
(E) Percentage of increase of the root-to-shoot ratio.
(F) Average number of adventitious roots.
The values are averages of two independent sets of 100 seedlings.

Changes in ABA and MeJA concentrations were also examined in roots of seedlings exposed to increasing NaCl concentrations for 48 hr (Figure 2B). Consistent with the time-course accumulation pattern (Figure 2A), the endogenous ABA levels had already returned to relatively low values at that time for all NaCl doses. The endogenous MeJA concentrations only exhibited a marked cumulative increase when plants were exposed to high NaCl concentrations (>150 mM), correlated with a decrease in shoot water content of >10% (Figure 2B, inset).

Characterization of JA-Induced Proteins from Rice Roots

JA exerts many of its effects through the induction of changes in gene expression, whether applied externally or released internally. The exogenous application of JA was found to significantly alter gene expression in roots of rice seedlings, as determined by the changes in two-dimensional protein patterns (Figures 3A and 3B). In roots of rice seedlings incubated on growth medium supplemented with JA concentrations of 10 µM for 60 hr, a marked accumulation of a set of proteins, including a 40-kD protein with a pI of 8, a 34-kD protein, pI 8, a 32-kD protein, pI 8, a 28-kD protein, pI 5, an 18-kD protein, pI 5, a 16.5-kD protein, pI 4.5, and a 14.5-kD protein, pI 5.5, was observed as well as a general decrease in the abundance of most other proteins (Figures 3A and 3B).

Five JA-induced proteins (JIPs) from rice roots were further characterized by partial amino acid analysis of internal peptides (Table 1). The peptide of the 40-kD JIP, pI 8 (Figures 3A to 3C, protein 5), exhibited 53% identity and 73 to 80% similarity to expressed sequence tag–derived peroxidase sequences of Arabidopsis (Table 1 and Figure 4A). Internal peptides from the 32-kD JIP, pI 8 (Figures 3A to 3C,

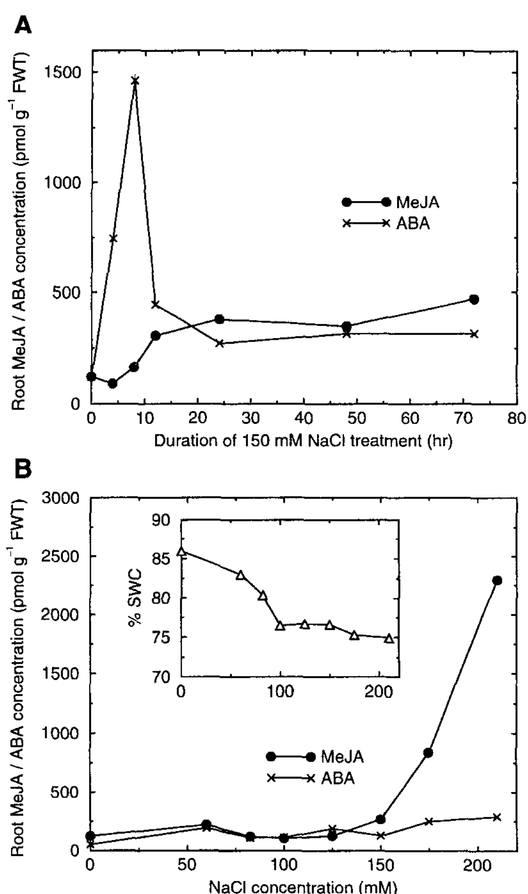

Figure 2. Endogenous ABA and MeJA Levels in Seedling Roots upon Salt Shock.

(A) Time-course analysis of the ABA and MeJA levels (in picomoles per gram fresh weight of tissue [FWT]) in roots of seedlings exposed to osmotic shock (150 mM NaCl).

(B) Endogenous root ABA and MeJA concentrations (in picomoles per gram fresh weight of tissue [FWT]) and shoot water content (% SWC, inset) of rice seedlings exposed to increasing concentrations of NaCl for 48 hr.

protein 4), did not show homology to the proteins that are present in the available databanks (PIR release 53.9; Swiss-Prot release 35) (Table 1). The two peptide sequences of an 18-kD JIP, pI 5 (Figures 3B and 3C, protein 3), corresponded to an intracellular PR-10 protein of rice (Midoh and Iwata, 1996) related to a stress-induced protein of soybean (Crowell et al., 1992), a disease resistance response protein of pea (Daniels et al., 1987), and the major pollen allergen of trees (Breiteneder et al., 1993) (Table 1 and Figure 4A). The partial amino acid sequence of the 16.5-kD JIP, pI 4.5 (Figures 3A to 3C, protein 2), exhibited 60% sequence identity to the class 1 PR-1 proteins of maize and tobacco (Cornelissen et al., 1986). Remarkably, the tryptic peptide sequence from the 14.5-kD JIP, pI 5.5 (Figures 3B and 3C, protein 1), completely matched the SalT protein, which is induced by high salt concentrations in roots of rice (Claes et al., 1990).

Comparison of JA- and ABA-Induced Changes in Protein Patterns in Roots of Rice

Similarities in ABA- and JA-induced gene expression have been highlighted, suggesting overlapping functions for the plant growth regulators. We compared the changes in two-dimensional protein patterns in response to different concentrations of ABA and JA in rice roots. The application of 20 (Figure 3C) and 40 μM JA (data not shown) for 60 hr induced an even stronger, dose-dependent accumulation of the JIPs characterized above. In contrast, none of the identified JIPs accumulated in response to ABA. Peroxidase (Figures 3B and 3C, protein 5), the 32-kD protein (Figures 3A to 3E, protein 4), the 28-kD protein (Figures 3B to 3E, protein 8), the PR-10 protein (Figures 3B to 3E, protein 3), the PR-1 protein (Figures 3A to 3E, protein 2), and the SalT protein (Figures 3B to 3E, protein 1) were only detected at low abundance in ABA-treated roots and did not increase with the ABA dose (10, 20, or 40 μM). Two prominent, previously characterized ABA-responsive proteins from rice roots, a 26-kD group 3 LEA protein (Figures 3D and 3E, protein 6) and a 40-kD protein (OSR40c1; Figures 3D and 3E, protein 7) (Moons et al., 1995, 1997b), did accumulate in the ABA-treated roots in a dose-dependent manner.

The expression of the group 3 LEA protein was examined further by protein gel blot analysis, using an antiserum raised against a wheat group 3 LEA protein (Ried and Walker-Simmons, 1993). Figure 5 shows the immunoblot detection of the de novo–induced group 3 LEA protein in ABA-treated seedling roots, whereas no specific cross-reactivity was found in the roots of the seedlings treated with JA concentrations as high as 40 μM for the same duration. In summary, five prevalent JA-induced proteins of rice roots did not accumulate in response to ABA, whereas two prominent ABA-responsive proteins, including a LEA protein, were not induced by JA.

Similarities in JA- and Salt Stress–Responsive Gene Expression in Rice Roots

The JA-induced accumulation of the SalT protein suggested similarities in the response of rice roots to salt stress and JA, as have been observed in barley seedlings (Maslenkova et al., 1992). Indeed, the JIPs from rice roots, that is, the SalT protein (Figure 3F, protein 1), the PR-1 protein (Figure 3F, protein 2), the PR-10 protein (Figure 3F, protein 3), the 28-kD protein (Figure 3F, protein 8), the 32-kD JIP (Figure 3F, protein 4), and the 34-kD JIP, were also induced in roots of rice seedlings exposed to 175 mM NaCl for 60 hr. The 28-kD protein that markedly accumulated in roots exposed to salt stress (Figure 3F, protein 8), and to a lesser extent also

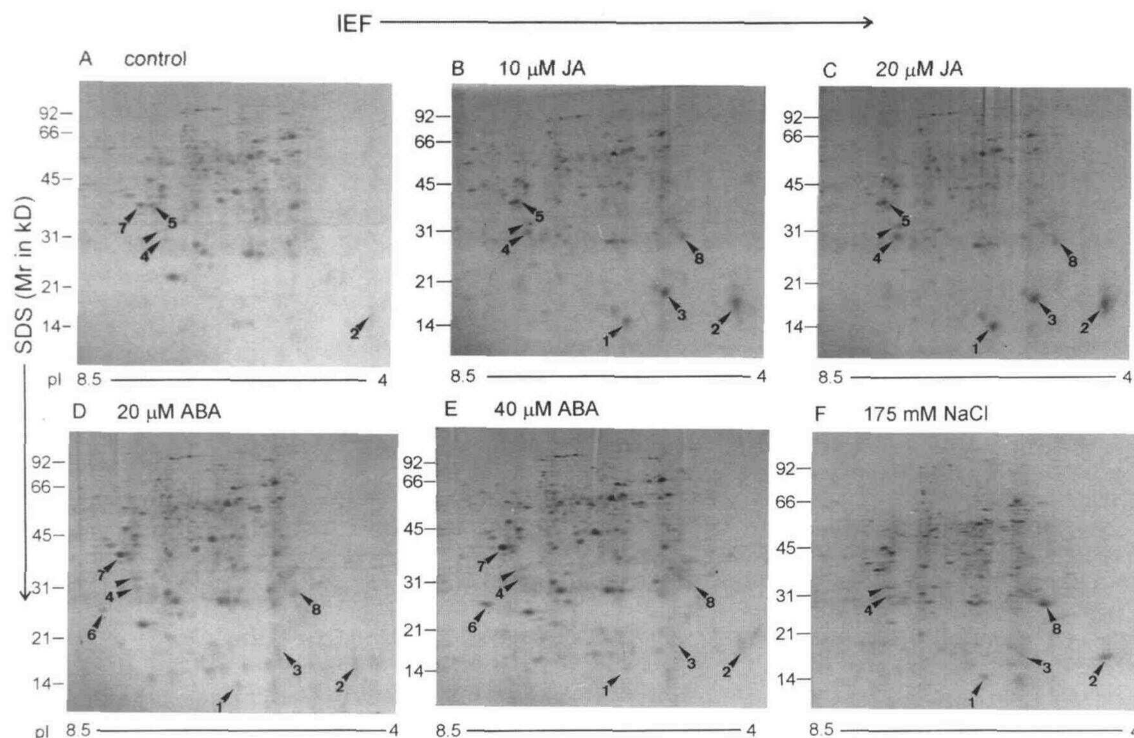

Figure 3. Comparison of the Changes in Two-Dimensional Protein Patterns in Response to Exogenously Applied JA and ABA and to Salt Stress in Roots of Rice Seedlings.

Proteins were isolated from roots of 12-day-old seedlings.
(A) Incubation on growth solution.
(B) to **(F)** Incubation on the same solution as given in **(A)** supplemented with 10 μM JA, 20 μM JA, 20 μM ABA, 40 μM ABA, and 175 mM NaCl solutions, respectively, for 60 hr.
The isoelectric focusing (IEF) gels have been stained with Coomassie Brilliant Blue R 250. The position of proteins that were markedly induced by JA (proteins 1 to 5 and 8) or by ABA (proteins 6 and 7) are indicated with arrowheads. Numbers correspond to those indicated in Tables 1 and 2.

responded to JA (Figures 3B and 3C, protein 8), was also characterized by microsequencing. Two internal peptide sequences did not exhibit homology to known proteins from the available databanks (Table 1). The abundance of all salt stress–induced proteins increased with the NaCl concentration and the duration of the stress (data not shown). The OSLEA3 protein was also expressed de novo in the salt-stressed roots, as was evident upon protein blot analysis (data not shown).

Organ-Specific Aspects of JA- and ABA-Induced Gene Expression in Rice Seedlings

The ABA- and JA-induced changes in gene expression were also analyzed in the sheath, the lower part of the shoot of the seedlings, which revealed organ-specific patterns. The 32-kD protein (Figures 6A to 6C, protein 4), the 28-kD protein (Figures 6A to 6C, protein 8), and SalT (Figures 6B and 6C, protein 1) accumulated in shoot tissue in response to JA in a dose-dependent manner. However, neither the peroxidase nor the PR-10 and PR-1 proteins were induced in response to JA in shoot tissue in contrast to their strong accumulation in roots. Different concentrations of ABA did not trigger the accumulation of any of the JIPs, including the 32-kD JIP (Figures 6E and 6F, protein 4), the 28-kD protein (Figures 6E and 6F, protein 8), and the SalT protein (Figures 6E and 6F, protein 1) in shoot tissue. No accumulation of the OSR40c1 and the OSLEA3 proteins were observed in shoots in response to ABA due to root-specific (Moons et al., 1997b) and presumably developmental (Hong et al., 1992) regulations, respectively.

JA, ABA, and Salt Stress Induce *salT* Transcript Accumulation

In contrast to the observations at the protein level, ABA did induce *salT* transcript accumulation in the root and most

Table 1. Partial Amino Acid Sequence Analysis of JA-Induced Proteins from Rice Roots

Molecular Mass (kD)	pI	No.[a]	Peptide Sequence[b]	Identification[c]
40	8	5	YFDLIAI AIVASGFPYFDVPLG	Peroxidase
32	8	4	V–YSTVDPGHMMTAP DSIVQLPQR	Novel
28	5	8	EVYPFYTGAPMVVLR DGASSQEILFSTGSNGAAPNTVR	Novel
18	5	3	L–PAAGVGSTYK MIEDYLVAHPAEY	PR-10 protein
16.5	4.5	2	SVEGVGEVVWDDAVAAYAEN	PR-1 protein
14.5	5.5	1	EISGTHGPVYDLADIVTYLK	SalT

[a] The numbers refer to those indicated in Figure 3.
[b] The internal peptides were generated by trypsin digestion. Unidentified positions are indicated with a (–).
[c] Identifications based on similarities with known proteins are given.

extensively in the shoot (Figure 6D) of the same seedlings, as had been demonstrated previously (Claes et al., 1990). We compared the effects of low physiological JA and ABA concentrations and salt stress on *salT* transcript levels in the seedling roots and shoot after a 24-hr treatment. JA was found to induce the expression of *salT* most extensively in the roots but also in shoot tissue (Figure 7). ABA concentrations of <10 μM and salt stress induced marked *salT* transcript

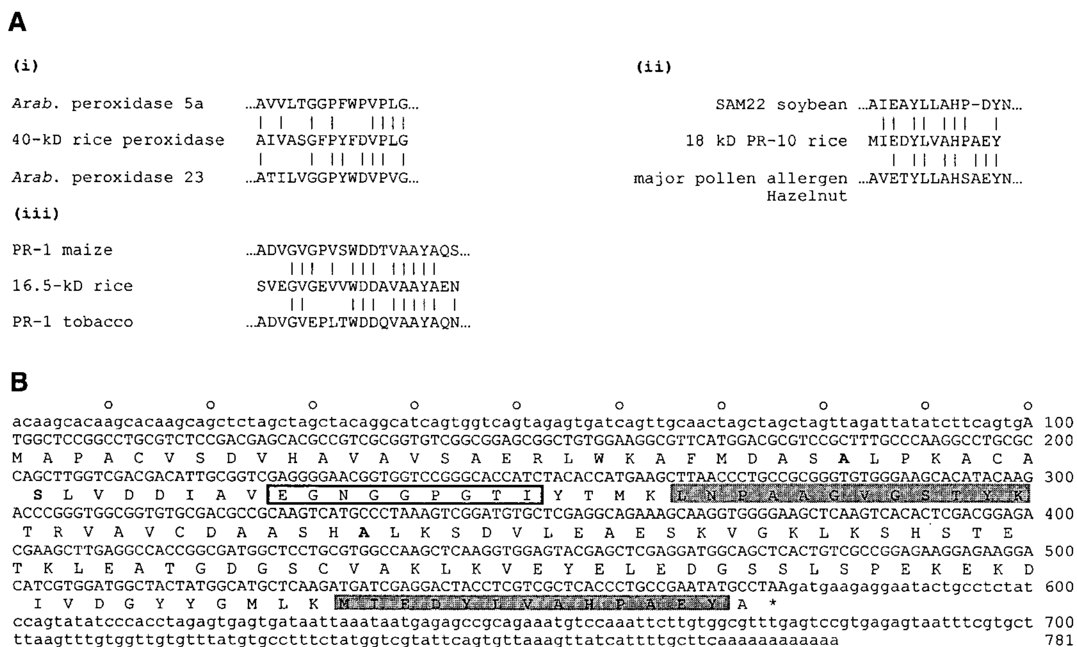

Figure 4. Homology of JA-Induced Proteins from Rice Roots to Other Plant Proteins and *osdrr* cDNA and Amino Acid Sequence.

(A) Shown are the alignment of an internal peptide from the 40-kD JIP (protein 5) to expressed sequence tag–deduced peroxidases of Arabidopsis (*Arab.*) in (i), the alignment of a peptide of the 18-kD JIP (protein 3) to the C terminus of a stress-responsive PR-10 protein of soybean (Crowell et al., 1992) and the major pollen allergen of hazelnut trees (Breiteneder et al., 1993) in (ii), and the alignment of an internal peptide from the 16.5-kD JIP (protein 2) to PR-1 proteins of maize and tobacco (Cornelissen et al., 1986) in (iii). Bars indicate identical amino acids.

(B) The cDNA sequence of *osdrr,* encoding a rice PR-10 protein, is shown. The internal peptides determined by microsequencing are shaded. An amino acid sequence conserved in the PR-10 proteins of various plants is boxed. Amino acid differences to a probenazole-inducible rice cDNA (Midoh and Iwata, 1996) are indicated in boldface. The stop codon is indicated by an asterisk. Lowercase letters indicate 5′ and 3′ untranslated regions.

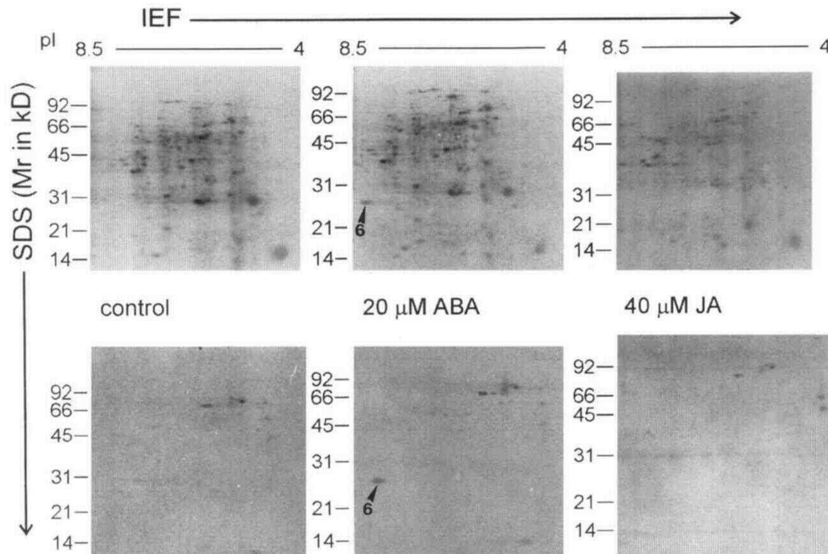

Figure 5. Immunodetection of Group 3 LEA Proteins in Rice Seedling Roots.

Proteins were isolated from seedling roots that were incubated on control medium, on 20 μM ABA solution, or on 40 μM JA solution for 60 hr.
(Top) Amido black staining of two-dimensional isoelectric focusing (IEF) blots.
(Bottom) Immunodetection using an antiserum raised against a wheat group 3 LEA protein (arrowhead, number 6).
IEF, isoelectric focusing.

accumulation in the shoot, which was less pronounced in the roots of the seedlings (Figure 7).

ABA, JA, and Salt Stress Induce the *osdrr* Transcript Encoding a PR-10 Protein

A cDNA encoding the rice PR-10 protein was isolated using a reverse transcriptase–polymerase chain reaction (RT-PCR)–based approach (see Methods). The *osdrr* cDNA (Figure 4B) contained two peptides that fully corresponded to the two tryptic peptide sequences of the 18-kD JIP, pI 5 (Figures 3B and 3C, protein 3). JA, ABA, and salt stress were found to induce marked *osdrr* transcript accumulation in the shoot (Figure 8) and, fully analogously, in the root of seedlings (data not shown). Hence, as for *salT*, ABA-induced *osdrr* transcript accumulation (Figure 8) but no PR-10 protein accumulation was found (Figures 3D and 3E, protein 3, and Figures 6E and 6F). Moreover, the root-specific JA response of the PR-10 protein (Figures 3B and 3C, protein 3, and Figures 6B and 6C) contrasted with the observed JA-induced *osdrr* transcript accumulation (Figure 8), indicating post-transcriptional regulation of the root-specific JA response.

Because of its classification as an intracellular PR protein, *osdrr* expression was also examined in response to salicylic acid (50 μM) and ethylene (35 and 350 μM ethephon). Neither induced an *osdrr* mRNA increase (Figure 8). Also, the *salT* transcript was not induced by these treatments (data not shown).

Time-Course Inductions of the *salT* and the *oslea3* Transcripts in Response to ABA and/or JA

Significant differences in the timing of ABA-induced transcript accumulation of *salT* and *oslea3*, encoding a rice group 3 LEA protein (Moons et al., 1997a), were found. The *oslea3* transcript accumulated to high levels in rice roots within 2 hr, reaching maximal concentrations after 4 hr. The *salT* mRNA was first detected 4 hr after ABA application and only reached high levels after 8 hr in the roots of the same seedlings (Figure 9). JA-induced *salT* transcript accumulation was detected after 4 to 6 hr, reaching high levels after 8 hr. JA did not induce *oslea3* transcript accumulation.

Antagonistic Effects of ABA and JA on *salT* and *oslea3* Transcript Levels in Roots of Rice

After salt stress, ABA and jasmonate concentrations were found to increase endogenously in rice roots in a proportion that depended on the dose and duration of the salt stress (Figures 2A and 2B). Therefore, we assessed the effect of a simultaneous application of different concentrations of ABA and JA on *salT* and *oslea3* transcript levels in roots (Figure 10). JA (5 and 10 μM) induced marked *salT* transcript accumulation, whereas ABA concentrations of 10, 20, and 40 μM induced a moderate *salT* transcript increase in the roots of seedlings after 48 hr. A simultaneous application of 5 or 10 μM JA combined with 10 or 20 μM ABA synergistically

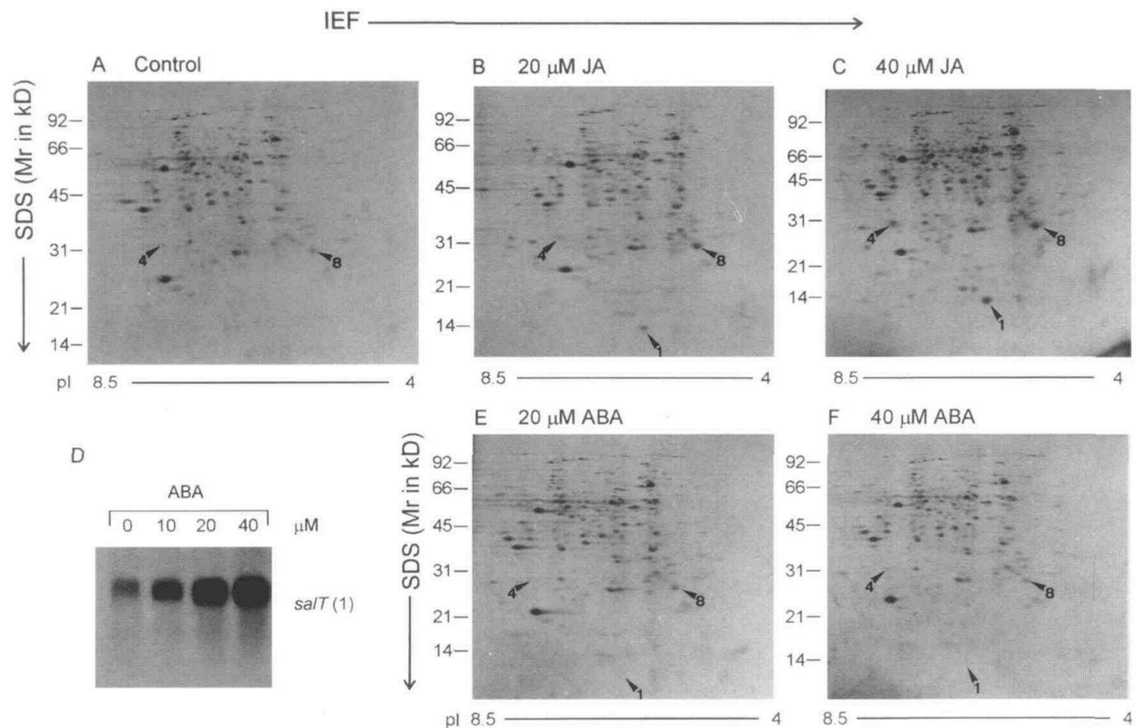

Figure 6. Comparison of the Changes in Two-Dimensional Protein Patterns in Response to Exogenously Applied JA and ABA in the Shoot of Rice Seedlings.

(A) Proteins were isolated from the sheath of 12-day-old seedlings that were incubated on growth medium.

(B), **(C)**, **(E)**, and **(F)** Seedlings incubated on the same solution as given in **(A)** supplemented with 20 μM JA, 40 μM JA, 20 μM ABA, and 40 μM ABA for 60 hr, respectively.

(D) RNA gel blot analysis of *salT* transcript levels in the sheath of the ABA-treated seedlings.

IEF, isoelectric focusing.

affected *salT* transcript accumulation, inducing higher mRNA levels than did ABA on its own (Figure 10). However, an excess of ABA versus JA, for example, 40 μM ABA to 5 or 10 μM JA, reduced *salT* transcript accumulation.

For comparison, the RNA gel blot was probed with *oslea3*, a representative of the *LEA* genes. JA did not induce *oslea3* transcript accumulation, whereas ABA concentrations as low as 10 μM were sufficient to trigger a maximal transcript increase (Figure 10). A simultaneous application of 10 μM JA drastically decreased ABA-induced *oslea3* transcript levels (Figure 10). However, 5 μM JA only inhibited *oslea3* transcript accumulation in the presence of 10 and 20 μM ABA, whereas 40 μM ABA, representing an eightfold excess of ABA versus JA, could prevent the JA-induced decrease of the *oslea3* transcript.

Time Course of *salT* and *oslea3* Transcript Accumulation in Roots When Salt Shocked

We compared the induction of *salT* and *oslea3* transcripts in roots of seedlings exposed to salt stress for different times (Figure 11A). NaCl (150 mM) induced rapid *oslea3* transcript accumulation that reached high levels within 2 to 4 hr and decreased to low levels after 24 hr. A delayed and moderate *salT* transcript increase was found, maximal after 24 hr of stress, when the drop in shoot water content was more pronounced.

Antagonistic Effects of ABA and JA on the Salinity-Induced Expression of *salT* in Seedling Roots

We also examined the effect of JA and ABA on *salT* expression during salt stress. A pretreatment and simultaneous treatment with the LOX inhibitors indoprofen and phenylbutazone were performed to eliminate effects of endogenous JA. Exposure to high salt concentrations (175 mM) markedly induced the *salT* transcript in roots, despite the LOX inhibitors (Figure 11B). A simultaneous application of 5 μM JA had a moderate, synergistic effect on *salT* transcript accumulation (Figure 11B) but caused strong accumulation of the SalT protein and all other JIPs on two-dimensional protein

gels (data not shown). Application of 25 μM ABA, which is an inducer of *salT* expression (Figure 7), caused the disappearance of the *salT* messenger in roots exposed to salt stress in the presence of JA biosynthesis inhibitors (Figure 11B).

DISCUSSION

ABA is not only an important signal for the plant's molecular and physiological response to a water deficit but also plays an essential role in triggering gene expression upon wounding and pathogen attack, whereas JA, an intracellular signal for the plant's pathogen defense, has been suggested to be involved in controlling gene expression in response to water-limiting stresses. Intrigued by this apparent functional redundancy, we compared the effects of exogenous JA and ABA in roots of rice seedlings with respect to molecular and physiological responses to salt stress. Our results indicate distinct roles for both plant growth regulators.

Distinct Morphological Effects of JA, ABA, and Salt Stress

JA and ABA were found to exert different effects on rice seedling growth and root development (Figure 1). Physiological JA concentrations of <10 μM did not provoke chlorosis or other symptoms of senescence (Figure 1A), in contrast to 20 and 40 μM concentrations. Exogenous application of ABA but not JA mimicked the differential inhibition of shoot versus root growth imposed by salt stress (Figure 1E), which is regarded as a morphological adaptation to water-limiting stresses (Creelman et al., 1990; Saab et al., 1990). Low JA concentrations (<3 μM) were found to stimulate the development of adventitious roots, strictly inhibited by ABA (Figure 1F), which suggested JA-induced activation of cell division and an ABA-imposed dormancy in the adventitious root meristems. The elongated primary root and the presence of adventitious roots on salt-stressed seedlings (Figure 1B, plant 4) indicated effects of ABA as well as the activity of antagonists, which could be jasmonates, that are abundant in young organs (Creelman and Mullet, 1995) and capable of breaking ABA-induced "dormancy" of the adventitious root meristems.

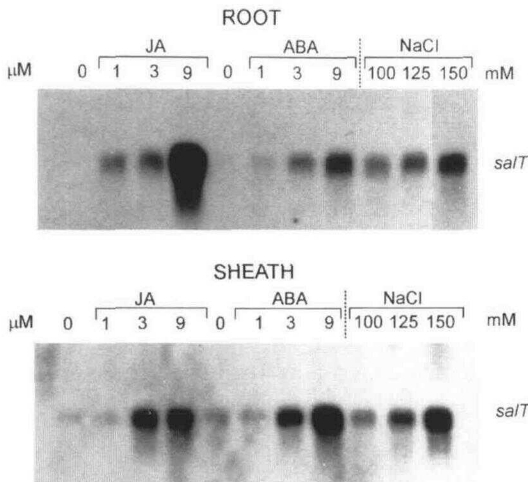

Figure 7. JA, ABA, and Salt Stress Induce *salT* Transcript Accumulation in the Roots and Shoots of Rice Seedlings.

Total RNA was extracted from the roots and sheaths of 12-day-old seedlings that were incubated on growth solution (0) or on growth solution supplemented with 1, 3, and 9 μM ABA or JA and with 100, 125, and 150 mM NaCl for 24 hr. The RNA blots contain 9 μg of total RNA in each lane. Hybridization was with a *salT* cDNA probe.

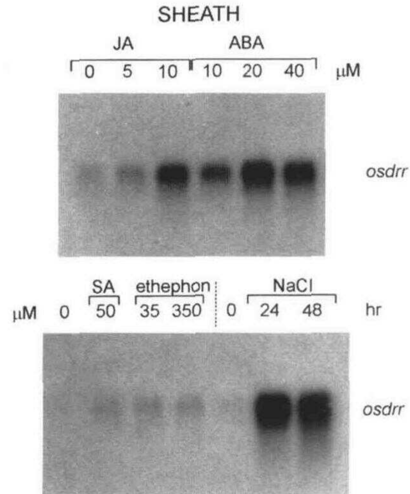

Figure 8. JA, ABA, and Salt Stress Induce *osdrr* Transcript Accumulation.

RNA was extracted from the sheaths of 12-day-old seedlings that were incubated for 24 hr in the presence of JA (5 and 10 μM), ABA (10, 20, and 40 μM), combinations of JA and ABA, 50 μM salicylic acid (SA), and ethephon (35 and 350 μM) or exposed to 175 mM NaCl for 24 and 48 hr. The RNA blots were hybridized with *osdrr* riboprobes.

The Salt Stress Response Encompasses Defense and Water Deficit Responses

Low JA concentrations (10 μM) were found to significantly affect gene expression in rice, inducing the abundant accumulation of more than seven proteins in seedling roots and approximately three proteins in shoots (Figures 3B, 3C, 6B, and 6C). At least three of the six most prominent JIPs from roots that were characterized by partial amino acid analysis (Table 1 and Figure 4A), that is, a peroxidase, a PR-1 protein, and a PR-10 protein, are typically part of plant defense

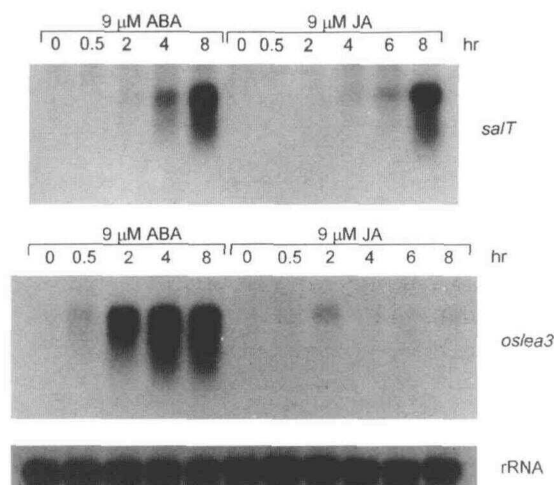

Figure 9. Time-Course RNA Blot Analysis of *salT* and *oslea3* Transcript Levels in Response to ABA and JA in Rice Roots.

RNA was extracted from roots of 12-day-old control seedlings (0) and seedlings incubated on 9 μM ABA or 9 μM JA solution for 0.5, 2, 4, 6, or 8 hr. The RNA gel blot contains 10 μg of RNA in each lane and was hybridized either with the *salT* cDNA probe or with *oslea3* riboprobes. The gel at bottom represents nonspecific hybridization to 18S rRNA in the lanes.

responses to wounding and pathogen attack. PR-1 expression has been associated with fungal resistance of barley varieties and was demonstrated to increase tolerance to fungi in transgenic tobacco (Alexander et al., 1993). PR-10 proteins have been associated with resistance to *Fusarium* spp and *Pseudomonas* spp in pea (Daniels et al., 1987).

The finding of a JA response of the rice SalT protein (Figures 3B, 3C, 6B, and 6C), originally identified as being induced by salt stress (Claes et al., 1990), could indicate a possible involvement in the plant's pathogen defense response as well. The SalT protein shares 45% identity and 65% similarity to the C-terminal domain of a MeJA-induced protein from barley leaves (Lee et al., 1996). This protein was reported to show homology to lectins and to myrosinase binding proteins (Taipalensuu et al., 1997). Myrosinase binding proteins could have a role during the myrosinase defense response, which is activated upon damage and decomposes glucosinolates into potentially toxic products in Brassica seeds (Falk et al., 1995; Taipalensuu et al., 1996).

When assessed at the protein level, ABA- and JA-induced changes in gene expression in rice seedlings were essentially distinct (Table 2). ABA was insufficient to induce an accumulation of all prominent JA-responsive proteins, including the peroxidase, the novel 32-kD JIP, the novel 28-kD JIP, the PR-1 and PR-10 proteins, and the SalT protein in rice roots (Figures 3D and 3E) or shoots (Figures 6E and 6F). On the other hand, two prevalent, ABA-induced proteins from rice roots, OSR40C1 and a LEA protein, OSLEA3, did not accumulate in response to JA (Figures 3B to 3E and 5). The salt stress response of rice roots was found to comprise most JA-induced proteins, for example, the 32-kD JIP, the 28-kD JIP, the PR-10 and PR-1 proteins, and the SalT protein (Figure 3F), but also ABA-responsive proteins, such as OSLEA3 (Table 2). So, salt stress induces water deficit responses and a defense response reminiscent of aspects of the plant's response to wounding and pathogen attack.

JA Does Not Induce *oslea3* Encoding a Group 3 LEA Protein

JA did not induce a transcript accumulation of *oslea3* (Figure 9), consistent with the absence of a protein response (Figure 5). There is little ground to assume that LEA proteins would be expressed in response to JA, although this has been stated (Reinbothe et al., 1992, 1994). Jasmonates do not induce the expression of the barley gene encoding a group 3 LEA protein, or rice *rab21* and tomato *tas-14*, encoding representatives of the dehydrin/Rab/group 2 LEA protein family (Hildmann et al., 1992; Harms et al., 1995; Rouster et al., 1997). Moreover, ABA, salt, and osmotic stress but not wounding were found to induce the *tas-14* transcript (Godoy et al., 1990).

JA Induces *salT* and *osdrr* Transcript Encoding a PR-10 Protein

JA was found to induce *salT* transcript accumulation predominantly in the roots but also in the shoot of rice seed-

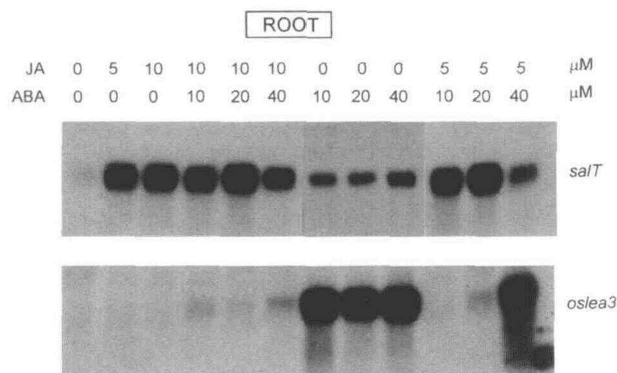

Figure 10. Antagonistic Effect of ABA and JA on *salT* and *oslea3* Transcript Levels in Seedling Roots.

RNA was extracted from the roots of 12-day-old seedlings that were incubated on growth solution supplemented with JA (5 and 10 μM), ABA (10, 20, and 40 μM), or combinations of JA and ABA as indicated for 48 hr. The blot contains 9 μg of total RNA in each lane. Hybridization was with the *salT* cDNA probe and *oslea3* riboprobes.

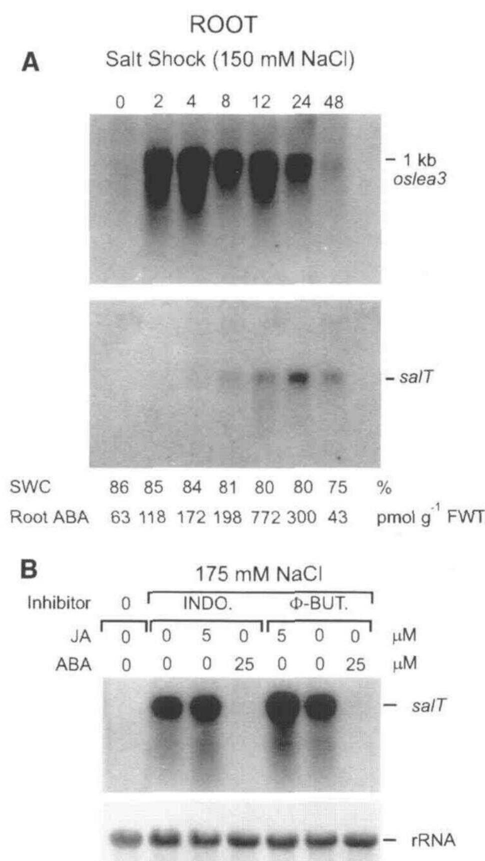

Figure 11. Salt Stress Response of *oslea3* and *salT* and Effects of JA and ABA on Salinity-Induced *salT* Transcript Levels in Rice Roots.

(A) RNA was extracted from roots of 12-day-old control seedlings (0) and seedlings that were incubated on 150 mM NaCl solution for 2, 4, 8, 12, 24, and 48 hr. The blot contains 9 μg of total RNA in each lane. Hybridization was with *oslea3* riboprobes and with the *salT* cDNA probe. The increase in endogenous root ABA levels (in picomoles per gram fresh weight of tissue [FWT]) and the decrease in shoot water content (% SWC) are depicted below.

(B) RNA was extracted from the roots of control seedlings (0) and seedlings incubated on 175 mM NaCl solution and 175 mM NaCl solution supplemented with 5 μM JA or 25 μM ABA for 48 hr. Except for the control seedlings, all seedlings were incubated on growth solution containing LOX inhibitors (50 μM indoprofen [INDO.] or 50 μM phenylbutazone [Φ-BUT.]) for 24 hr before transfer to the induction solutions, which also contained the same amounts of LOX inhibitors.

lings (Figure 7), which is consistent with the observed protein response. JA-induced expression of *salT* was not very fast (Figure 9), as has been demonstrated for JA-induced protein accumulation (Schweizer et al., 1997). JA also triggered an accumulation of the *osdrr* transcript in roots and shoots (Figure 8). Expression of this intracellular PR protein (Figure 4B) has been associated with the disease resistance response in rice (Midoh and Iwata, 1996). The root-specific JA response of the PR-10 protein (Figures 3B and 3C) is apparently post-transcriptionally regulated.

Distinct Features of the ABA Response of *salT*, *osdrr*, and *oslea3*

ABA did induce *salT* transcript accumulation in the roots and most extensively in the sheath of rice seedlings (Figures 6D and 7), as demonstrated previously (Claes et al., 1990). However, the SalT protein did not accumulate in response to ABA in the roots (Figures 3D and 3E) or in the seedling sheath (Figures 6E and 6F). ABA also triggered *osdrr* transcript (Figure 8) accumulation but no PR-10 protein accumulation (Figures 3D and 3E). Analogously, ABA has been demonstrated to induce transcript but no protein accumulation of the osmotin gene, which is also responsive to salt stress as well as jasmonates and is involved in plant pathogen defense responses (La Rosa et al., 1992; Liu et al., 1994). Besides ABA, additional stress-induced regulators are apparently required to trigger protein expression.

By contrast, ABA induced abundant *oslea3* transcript (Figure 9) and protein accumulation (Figures 3D, 3E, and 5), which is a characteristic of ABA-responsive *LEA* gene expression (Dure, 1993). ABA-induced transcript accumulation without protein expression could be typical for stress-responsive genes that are also part of the wounding response, as opposed to the typical water deficit–associated *LEA* genes (Bray, 1994) (Figure 12).

Furthermore, the differences in timing of ABA-induced transcript accumulation of *oslea3*, which was rapid, and of *salT*, which was delayed (Figure 9), suggested a different ABA response pathway (Shinozaki and Yamaguchi-Shinozaki, 1997). The ABA induction of the wound-responsive *pin2* gene requires protein synthesis (Peña-Cortéz and Willmitzer, 1995), in contrast to the ABA response of the water stress–inducible *rab21* gene encoding a group 2 LEA protein (Mundy and Chua, 1988).

ABA Exerts a Negative Effect on *salT* Transcript and Protein Accumulation in Rice Roots

ABA was not merely insufficient to induce an accumulation of the SalT protein. An excess of ABA over JA was found to reduce *salT* transcript accumulation in roots (Figure 10). In addition, ABA application in the presence of inhibitors of JA biosynthesis caused a complete disappearance of the abundant salinity-induced *salT* transcript in roots (Figure 11B). However, ABA had been demonstrated to induce the *salT* transcript (Figures 7 and 9; Claes et al., 1990), which occurs through a transcriptional activation (Garcia et al., 1995). Therefore, ABA could possibly exert this negative effect on *salT* transcript accumulation post-transcriptionally by affecting mRNA stability and/or turnover, although transcriptional

Table 2. Effect of JA, ABA, and Salt Stress on the Abundance of Eight Proteins and Four Corresponding Transcripts in Rice Roots

Molecular Mass (kD)	pI	No.[a]	Protein[b] JA	ABA	NaCl	Transcript[b] JA	ABA	NaCl	Identification
40	8.5	7	−	+	+[c]	−	+	+	OSR40c1[d]
40	8	5	+	−	+[c]				Peroxidase
32	8	4	+	−	+				Novel
28	5	8	+	−	+				Novel
26	8.5	6	−	+	+[c]	−	+	+	Group 3 LEA
18	5	3	+	−	+	+	+	+	PR-10
16.5	4	2	+	−	+				PR-1
14.5	5.5	1	+	−	+	+	+	+	SalT

[a] The numbers refer to those indicated in Figures 3 and 5.
[b] (+), induced; (−), not induced. Blank spaces indicate no data for these transcripts.
[c] Accumulation was evident when analyzed by protein gel blotting or multiple experiments.
[d] Moons et al. (1997b).

effects cannot be strictly excluded. The negative ABA effect on *salT* transcript accumulation in roots seemed to be enhanced during stress, which in the case of post-transcriptional events has been associated with the presence of aberrant transcripts (Matzke and Matzke, 1995) that are more frequent in stressed tissue. The disappearance of the *salT* messenger when two inducers were present was reminiscent of the phenomenon of post-transcriptional gene silencing, which often involves genes of the wounding and pathogen response, for example, β-glucanase, chalcone synthase, and LOX. Finally, the absence of ABA-induced SalT accumulation, despite high transcript levels (Figures 6D to 6F), does not exclude possible negative post-transcriptional effects governed by ABA. On the other hand, ABA has been reported to exert positive post-transcriptional effects on the expression of a group 1 LEA protein, which is typically part of water deficit responses (Hetherington and Quatrano, 1991) (Figure 12).

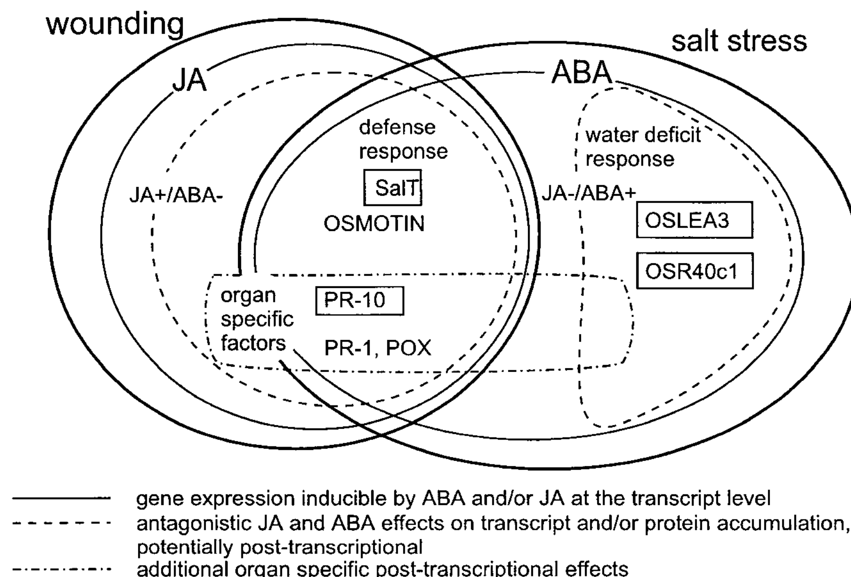

Figure 12. Proposed Model for the Effects of ABA and Jasmonates on the Expression of Distinct Classes of Salt Stress–Inducible Genes.

JA and ABA indicate gene expression inducible by JA and/or ABA at the transcript level. JA−/ABA+ indicates ABA-responsive protein accumulation but negative, possibly post-transcriptional JA effects on transcript and protein accumulation. JA+/ABA− indicates JA-responsive protein accumulation but negative, possibly post-transcriptional ABA effects on transcript and/or protein accumulation. OSMOTIN, based on La Rosa et al. (1992); OSR40c1, based on Moons et al. (1997b); POX, peroxidase; PR-10, *osdrr* gene product.

JA Exerts a Negative Effect on *oslea3* Transcript Accumulation

Application of JA markedly reduced the abundant ABA-induced *oslea3* transcript accumulation in rice roots, unless ABA was present in great excess (Figure 10). Because the remaining amounts of the de novo ABA–induced *oslea3* transcript were detected, JA possibly exerted a negative post-transcriptional effect on the expression of the *LEA* gene by affecting mRNA stability and/or turnover, although effects on transcription cannot be excluded.

By contrast, JA had a slight, synergistic effect on ABA- and salinity-induced *salT* transcript accumulation (Figures 10 and 11B) and provoked markedly abundant SalT protein accumulation (Figures 3 and 6), which suggested a combination of transcriptional but possibly also positive post-transcriptional effects. The strong JA-induced expression of all JIPs, many of which are associated with the wounding response (Table 2), was concomitant with a reduced expression of most other proteins from rice roots (Figures 3B and 3C). JA has been demonstrated to promote the expression of wounding-responsive genes at the transcriptional and post-transcriptional levels, the latter through a selective stimulation of translation (Reinbothe et al., 1994).

Differential Increase of ABA and MeJA in Roots upon Salt Stress

Endogenous ABA concentrations were found to increase rapidly in roots upon salt stress, and they returned to low levels upon extended stress (Figure 2A). Conversely, jasmonates, particularly MeJA, only accumulated after longer durations of salt stress (Figure 2A) or when high salt concentrations were applied (Figure 2B), two factors that determine the severity of salt stress. The increase in MeJA levels was associated with the observed stress effects, monitored as a decrease in shoot water content (Figure 2B). Presumably, a significant decrease in turgor and ion toxic effects could have generated membrane damage and hence triggered the release of the lipid precursors for jasmonate synthesis in roots exposed to salt stress.

Differential *oslea3* and *salT* Expression in Roots upon Salt Stress

Salt stress is a complex stress provoking osmotic and ion toxic effects (Greenway and Munns, 1980). Besides a biphasic change in endogenous ABA and MeJA concentrations (Figures 2A and 2B), a biphasic molecular salt stress response in rice roots was evident from the different timing of *oslea3* and *salT* transcript accumulation (Figure 11A). Moreover, the *salT* transcript only accumulated to high levels in roots of plants exposed to high-dose salt stress (Figures 11A and 11B) in contrast to *oslea3* (Figure 11A).

LOX inhibitors did not inhibit the salinity-induced *salT* transcript accumulation (Figure 11B), suggesting that the endogenous increase of MeJA had no effect on salt stress–responsive gene expression at the transcriptional level. Transcription activation most likely involves different pathways and stress-induced signals (Shinozaki and Yamaguchi-Shinozaki, 1997). However, post-transcriptional JA effects might have an important impact on the regulation of gene expression during salt stress. Salt stress (175 mM NaCl) generally induced abundant *oslea3* and *salT* transcript accumulation in roots, whereas high levels of the SalT protein and low levels of the OSLEA3 protein were detected (Table 2), suggesting differential post-transcriptional effects. Furthermore, the decrease of the *oslea3* transcript with the duration of stress (Figure 11A) could at least in part be due to post-transcriptional effects.

Antagonistic Effects of ABA and JA on the Expression of Two Classes of Salt Stress–Responsive Genes in Rice Roots

Salt stress–induced *oslea3* and *osr40c1* (Moons et al., 1997b) expression was found to be part of a rapid primary response reminiscent of the water deficit response (Table 2), inducible by ABA at the transcript and protein level, and negatively affected by JA at the transcript level (Figure 12). On the other hand, *salT* and *osdrr* expression seemed to be part of a second response reminiscent of wounding and pathogen defense (Table 2). The *salT* and *osdrr* genes were found to be induced by ABA and JA, but ABA exerted a negative effect on the *salT* and *osdrr* transcript and/or protein accumulation unless JA was present in sufficient concentrations to trigger an accumulation of the SalT and PR-10 proteins (Figure 12). Therefore, the endogenous balance of MeJA and ABA, correlated with the dose and the duration of salt stress, might determine the abundance of proteins from the water deficit and defense response. The results suggest that this fine-tuning of the salt stress response could take place in part at the post-transcriptional level.

METHODS

Plant Material, Growth Conditions, and Plant Treatments

Rice seeds (*Oryza sativa* subsp Indica cv Taichung N1) were supplied by the International Rice Research Institute (Manila, The Philippines). The rice seeds were sown, germinated, and grown on grids placed above pots containing hydroponic culture solution, pH 5.6, for rice (Yoshida et al., 1976). Seedlings were grown at 27°C in a 16-hr-light/8-hr-dark regime for 9 days. The treatments were performed by transferring the grids holding 9-day-old seedlings to pots containing growth solution supplemented with jasmonic acid (JA) (1, 3, 5, 9, 10, 20, or 40 μM; mixed isomers; Apex Organix, Devon, UK), abscisic acid (ABA) (1, 3, 9, 10, 20, or 40 μM; mixed isomers; Janssens Chimica,

Beerse, Belgium), NaCl (60, 80, 100, 125, 150, 175, and 210 mM), combinations of ABA, JA, and NaCl as indicated, 50 μM salicylic acid (Sigma), and 35 or 350 μM 2-chloroethylphosphonic acid (ethephon; Sigma), all adjusted to pH 5.6. At the same time, relevant control seedlings were transferred to fresh growth medium. The seedlings were incubated on these media for the indicated durations. Seedlings treated with 150 mM NaCl, 9 μM ABA, or 9 μM JA for different times were harvested simultaneously. Indoprofen (4-[1,3-dihydro-1-oxo-2H-isoindol-2-yl]-α-methylbenzeneacetic acid) and phenylbutazone (4-butyl-1,2-diphenyl-3,5-pyrolidinedione) were applied at concentrations of 50 μM 24 hr before and during the subsequent treatment with 175 mM NaCl and 5 μm JA or 25 μM ABA, as indicated.

Measuring ABA-, JA-, and NaCl-Induced Growth Inhibition and Root Development

Rice seeds were germinated and grown on autoclaved vermiculite soaked with control medium (Yoshida et al., 1976). After 5 days, the vermiculite was thoroughly drained and rewatered with either control medium or the same growth solution supplemented with 1, 3, and 9 μM ABA or JA and 50 or 75 mM NaCl, pH 5.6, for 6 additional days. The first leaf and the primary root were measured, and the number of adventitious roots >1 cm were determined for 100 seedlings in two independent experiments. The percentage of growth inhibition of the first leaf and the primary root and the percentage of increase of root-to-shoot ratio were calculated relative to the values of the control seedlings.

Synthesis of 1-$^{18}O_1$-JA and 1-$^{18}O_1$-MeJA

$^{18}O_1$-JA was synthesized from ^{18}O-H_2O, as described for the synthesis of 1-$^{18}O_1$-ABA by Gray et al. (1974). 1-$^{18}O_1$-MeJA was obtained from synthesized 1-$^{18}O_1$-JA after methylation using ethereal diazomethane, according to the method of Schlenk and Gellerman (1960). Stock solutions were calibrated using an ES^- MRM-MS/MS (Quatro II mass spectrometer equipped with an electrospray interface; Micromass, Manchester, UK). Samples (10 μL) were injected in methanol–ammonium acetate 0.01 M (50/50 [v/v]) at 0.1 mL/min with a source temperature of 80°C, capillary voltage of +3.5 kV, and cone voltage of 20 V. Collision-activated dissociation of the protonated molecular ion ($[MH]^+$) was obtained at a collision energy of 20 eV (where 1 eV = 1.6 10^{-19} J) and a P_{Ar} of 4.10^{-3} mbar (argon pressure, 1 bar = 10^5 Pa). Quantitation was done by multiple reaction monitoring of $[MH]^+$ (dwell time 0.1 sec, span 0) and the appropriate production resulting in the diagnostic transitions 211→61 and 209→59 for 1-$^{18}O_1$-JA and unlabeled JA, respectively. To avoid recovery losses caused by derivatization, gas chromatography–mass spectrometry (GC-MS) was not used for calibration purposes.

Quantification of Endogenous JA and MeJA

Frozen plant material was homogenized in liquid nitrogen. JA and MeJA were extracted with 90% MeOH (10 mL/g fresh weight) overnight. For recovery and quantification purposes, 1-$^{18}O_1$-JA and 1-$^{18}O_1$-MeJA were added to the extract (100 ng each). Taking into account the volatility of MeJA, we performed evaporation under a continuous nitrogen stream at 35°C (TurboVap LV evaporator; Zymark, Hopkinton, MA). Extracts were purified for JA by using DEAE–Sephadex (Pharmacia) and C_{18}-RP (Varian, Palo Alto, CA) cartridges, as described for the purification of indole-3-acetic acid and ABA, using ammonium acetate 40 mM, pH 6.5, as a mobile phase (Prinsen et al., 1995a, 1995b). The DEAE–Sephadex effluent containing MeJA was concentrated on a C_{18}-RP cartridge coupled underneath. MeJA was eluted from the C_{18} column by using 1 mL of diethyl ether. JA was methylated by diazomethane before GC-MS. The endogenous MeJA and the endogenous JA after methylation were measured in these separate fractions by using electron impact GC-MS in selective ion response mode ([+] EI GC-SIR-MS) (Prinsen et al., 1995b) using 224 and 226 as diagnostic ions for MeJA and ^{18}O-MeJA, respectively. The experimental conditions were as follows: HP 5890 series II coupled to a quadrupole mass spectrometer (Trio 2000; Micromass), column 15mBD-XLB, 0.25-mm i.d., 0.25-μm film diameter (J & W Scientific, Folsom, CA), gas phase He, injector temperature gradient 150 to 325°C over 12°C/sec, temperature gradient over column 100 to 275°C in 25 min, interchannel delay 0.02, dwell time 0.2 sec, span 0.1 atomic mass units. Corrections were made for the abundance of the unlabeled isotope in the $^{18}O_1$-JA tracer. All data were processed by Masslynx software and expressed as picomoles per gram fresh weight.

Two-Dimensional Protein Analysis and Microsequencing

Proteins were extracted and separated by mini-two-dimensional gel electrophoresis, as described previously (Moons et al., 1995), and run in capillary pipettes of 100 μL in the first dimension and on 17.5% (w/v) SDS–polyacrylamide gels of 90 × 100 mm in the second dimension. Partial amino acid analysis was performed on tryptic peptides from the combined JIPs of six preparative two-dimensional protein blots (Immobilon; Millipore, Bedford, MA), as described elsewhere (Bauw et al., 1989; Moons et al., 1995). The PIR data bank (release number 53.9) and the University of Geneva Protein Sequence data bank (SwissProt release number 35) were screened using software supplied by Genetics Computer Group (Madison, WI).

Protein Gel Blot Analysis

The mini-two-dimensional protein blots (Hybond-C; Amersham International) were stained with amido black and destained in 10 mM NaOH before immunodetection. Protein gel blot detection was performed using an antiserum produced against a group 3 LEA protein of wheat (Ried and Walker-Simmons, 1993), an anti–rabbit Ig alkaline phosphatase conjugate (Sigma), and the substrates 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt and *p*-nitro blue tetrazolium chloride, according to the manufacturer's instructions (Bio-Rad).

Reverse Transcriptase–Polymerase Chain Reaction—Based cDNA Cloning and Sequence Analysis

The *osddr* cDNA was isolated using a polymerase chain reaction (PCR)–based approach. A 250-bp 3′ cDNA fragment was cloned using the rapid amplification of cDNA ends technique (Gibco BRL), according to the manufacturer's protocol, using poly(A^+) RNA templates (Oligotex mRNA purification system; Qiagen, Surrey, UK) isolated from JA-treated rice roots and a degenerated amplification primer 5′-AAA/GATGATIGAA/GGAC/TTAC/TCTIGT-3′ based on the tryptic

peptide KMIEDYLVAHPAEYA. Within the 3′ untranslated region sequence, a gene-specific antisense reverse transcriptase (RT) primer 5′-TTATCACTCACTCTAG-3′ and a 22-bp nested amplification primer were chosen. 5′ rapid amplification of cDNA ends resulted in the amplification of a 594-bp cDNA fragment, of which >66 bp were identical to the 3′ cDNA fragment. The 781-bp *osdrr* cDNA clone has an open reading frame of 477 bp and encodes a 158–amino acid protein with a calculated molecular mass of 16.5 kD, pI 4.5 (Figure 4B). Compared with the probenazole-inducible cDNA of rice (cv Jikkoku) (Midoh and Iwata, 1996), *osdrr* contains an additional 17 bp of the 5′ untranslated region, the poly(A) addition occurs at a different site, and the deduced protein differs in three amino acids (Figure 4B).

The PCR mixture contained 1/10 of the RT products, 1 × PCR buffer (10 × PCR buffer is 100 mM Tris HCl, pH 8.4, 15 mM $MgCl_2$, 500 mM KCl, 0.1% gelatine), 1.5 pmol of each primer, 0.2 mM of each dNTP, and 1.5 units of Taq polymerase (Beckman, Fullerton, CA). Standard PCR conditions were a hot start of 5 min at 94°C followed by 30 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C. The amplified PCR fragments were cloned in pGEM-T (Promega, Madison, WI). DNA sequencing was performed using a T7 sequencing kit (Pharmacia, Uppsala, Sweden).

RNA Preparation and Analysis

Total RNA was prepared by a standard phenol extraction method. The RNA was quantified spectrophotometrically, normalized to equal concentrations, and verified by gel electrophoresis. Equal quantities of RNA were separated on 6% formaldehyde gels, transferred to Hybond-N membranes (Amersham International), and stained with methylene blue to visualize rRNA bands. Hybridization was performed overnight in 50% formamide, 5 × SSC (1 × SSC is 0.15 M NaCl, 0.015 sodium citrate), 0.5% SDS, 10% dextrane sulfate, and 20 $\mu g\ mL^{-1}$ denatured shearing herring sperm DNA at 65°C by using α-^{32}P-CTP–labeled riboprobes generated from the 603-bp *oslea3* coding region (Moons et al., 1997a) and from the 594-bp 5′ *osdrr* fragment cloned in pGEM-T (Promega), using the Boehringer Mannheim transcription kit. Filters were washed twice for 15 min at 65°C in 3 × SSC, 0.5% SDS when using the *osdrr* riboprobe and twice more for 15 min at 70°C in 1 × SSC, 0.5% SDS when using the *oslea3* riboprobe. Alternatively, hybridization was performed in the same buffer at 42°C by using an α-^{32}P–labeled PCR-amplified cDNA insert of *salT* as a probe (Multiprime labeling kit; Amersham International), and washing was performed three times for 15 min at 55°C in 3 × SSC, 0.5% SDS. Filters were exposed overnight.

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