JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Aluminum-Induced Cell Wall Peroxidase Activity and Lignin Synthesis Are Differentially Regulated by Jasmonate and Nitric Oxide

YAO JUAN XUE, LING TAO, AND ZHI MIN YANG*

College of Life Science, Nanjing Agricultural University, Nanjing 210095, China

Cassia tora is an annual legume and cultivated as a traditional medicinal herb for multiple therapies including regulation of blood pressure and blood lipid. Because of naturally occurring acidic soils in southeastern China, this plant species may possess strategies for tolerance to low pH and aluminum toxicity. In the search for the regulatory basis of biochemical response to Al, cell wall-bound peroxidases, including lignin-generated peroxidases and NADH oxidases, were investigated in the root tips of *C. tora*. Activities of both types of peroxidases significantly increased with Al concentrations. Analysis with native PAGE also demonstrated the strong induction of cell wall peroxidases by Al. The Al-induced increasing activities of peroxidases were closely correlated with lignin accumulation and H_2O_2 production. The biochemical effect of exogenous nitric oxide (NO) and methyl jasmonic acid (MJ) was examined to investigate signal properties and lignin synthesis under Al stress. Application of MJ at 10 μM promoted root sensitivity to Al by activating apoplastic peroxidase activity and accumulating H_2O_2 and lignin, whereas the opposite action was found for NO. The sensitivity of apoplastic peroxidases under Al stress was associated with the cross-talk of MJ and NO signals. The analysis reveals that the activity of lipoxygenase (an enzyme for MJ biosynthesis), with its transcripts increased in Al-exposed roots, was depressed by NO exposure. The effect of MJ on intracellular NO production was also investigated. It is shown that NO staining with 4,5-diaminofluorescein diacetate fluorescence was intensified by Al but was suppressed by MJ. These results suggest that NO and MJ may interplay in signaling the cell wall peroxidase activity and lignin synthesis in the roots exposed to Al.

KEYWORDS: Al; peroxidases; lignin; methyl jasmonate; *Cassia tora*

INTRODUCTION

Al toxicity is one of the most widespread problems for crop production. A number of studies have shown that inhibition of root growth under Al exposure is a major response. At the cellular level, apoplast is the first target of Al action. Distribution of a large proportion of initially absorbed Al in this section may trigger modification of the cell wall and thereby lead to the fast blocking of root growth. Thus far, several different mechanisms have been proposed for the Al toxicity. One mechanism is the Al disruption of cytoskeletal dynamics and interaction with both microtubules and actin filaments, which consequently lead to the blocking of root growth (*1*). Another refers to the rapid binding of Al to nucleic acids in the root meristem that arrests cell division (*2*). These suggest that either an apoplastic or a symplastic target site for Al is involved in Al-induced inhibition of root growth.

Modification of root cell wall properties by Al might be reversible, but the outcome of the process is linked to the cell wall stiffening and arrest of cell extension (*3*). It is observed that hemicellulosic polysaccharides accumulate in the cell wall of sensitive wheat root apex and that modifications of cell wall components cause cell wall thickness and rigidity (*4*). Also, the cross-linking of pectin substances was shown to block the cell extension under Al stress (*5*). However, it is still an open question whether the cell wall stiffening is the cause or consequence of root growth inhibition.

The process of cell wall rigidification triggered by Al is rather complex. A variety of enzymes and intermediates are shown to be involved in the mechanism (*6*). In normal conditions, cell wall loosening or cross-linking may depend on the status of growth and be regulated by cell wall-linked peroxidases (*7*). On the basis of their sequence and catalytic properties, the plant peroxidases can be classified into three groups (*8*). Group I contains peroxidases of prokaryotic origin involved in intracellular hydrogen peroxide removal. Group II peroxidases are extracellular fungal enzymes mainly involved in lignin degradation, and group III peroxidases refer to the classical secretory plant peroxidases, and this group is composed of a large number of isoforms, responsible for a variety of functions such as

* Corresponding author (telephone 86-25-84395057; e-mail zmyang@njau.edu.cn).

10.1021/jf802001v CCC: $40.75
Published on Web 09/20/2008

biosynthesis of secondary metabolites, stiffening of the cell wall, and cross-linking of matrix polysaccharides (*9*). The heme-containing group III peroxidases have complex relations with H_2O_2. They may catalyze the conversion of H_2O_2 into water by oxidizing various hydrogen donor molecules such as phenolic compounds and auxin metabolites (*10*). In this case, the peroxidase-mediated cross-linking of several compounds is believed to be the cause of the cell wall rigidification (*7*). On the other hand, the group III peroxidases may promote the generation of H_2O_2, provided that appropriate strong reductants are available. NAD(P)H, indoleacetic acid, and saturated fatty acids represent the important substrates oxidized by the peroxidase-mediated pathway (*11*). In this regard, the peroxidases appear to act as bifunctional enzymes that can not only oxidize various substrates in the presence of H_2O_2 but also generate H_2O_2.

Nitric oxide (NO) is a signaling molecule, mediating various plant physiological responses to biotic and environmental stresses. NO was reported to be involved in ethylene emission, biosynthesis of jasmonic acid, and salicylic acid (*12*). It is also believed to interact with reactive oxygen species (ROS) in plants (*13*). We previously described a role of exogenous NO in the enhancement of the tolerance of *Cassia tora* to Al toxicity (*14*). NO-treated roots exhibited the capability of antioxidation by reducing O_2^- and H_2O_2 accumulation in Al-treated roots. Similarly, a recent work with *Hibiscus moscheutos* has shown that endogenous NO improves the root growth under Al stress (*15*). However, the precise mechanisms for the NO improvement of the root tolerance to Al are poorly understood.

Jasmonic acid (JA) is a terminal product of the octadecanoid pathway (*16*) and belongs to a family of lipid-derived signaling molecules that regulate environmental stresses such as ozone (*17*), heavy metals (*18*), and pathogen attack (*16*). Recent studies have shown that heavy metals may serve as inductive factors regulating plant resistance to pathogens (*19, 20*). Also, Al exposure activated lipoxygenases (LOXs) in plant roots (*14*). LOXs catalyze oxygenation of fatty acids to their hydroperoxy derivatives and are considered to be one of the key enzymes in the octadecanoid pathway upstream controlling JA biosynthesis (*21*). However, whether Al regulates the process is largely unknown. In this study, we used *C. tora*, an annual legume, as a test material. *C. tora* is cultivated as a Chinese traditional medicinal herbs for regulating blood pressure and improving eyesight. Because of naturally occurring acidic soils in southeastern China, this plant species is rather tolerant to low pH and aluminum toxicity. Therefore, we were interested in investigating whether jasmonate mediates the cell wall-bound peroxidase activity and lignin accumulation independently or in the way of cross-talk with NO in this species. The outcome of the work may improve our understanding of the regulatory role of both NO and JA for plant tolerance to Al stress.

MATERIALS AND METHODS

Plant Materials and Growth. Seeds of *C. tora* L. were germinated on a mesh tray floating on 1.5 L of a solution containing 0.5 mM $CaCl_2$ (pH 4.5). After germination, seedlings grew for 3 days at 22 °C, with a photosynthetic photon flux density of 100 μmol m^{-2} s^{-1} and a 14 h photoperiod. The solution was changed daily. When the average root length was about 5.5 cm, the seedlings were exposed to various treatment solutions.

Sodium nitroprusside (SNP) was used as a NO donor and 2-(4-carboxy-2-phenyl)-4,4,5,5-tetramethylinidazoline-1-oxyl-3-oxide (cPTIO) as a NO scavenger. Seedlings were incubated in the solution of 0.5 mM $CaCl_2$ (pH 4.5) containing SNP and/or cPTIO for pretreatment for 12 h. Afterward, the root was rinsed with 0.5 mM $CaCl_2$ (pH 4.5) several times and exposed to various concentrations of $AlCl_3$. For jasmonate treatment, seedlings were incubated in 0.5 mM $CaCl_2$ (pH 4.5) containing Al and methyl jasmonate (MJ). After treatment, root apexes (5 mm) were excised from seedlings. The samples were immediately frozen in liquid nitrogen or stored at −80 °C for analysis.

Isolation of Cell Wall Proteins. Extraction of cell wall-bound proteins was performed by the method described previously (*22*), with some modifications. Root tips (1 g) were homogenized in 3 mL of 10 mM potassium phosphate buffer (pH 6.4). After centrifugation (2000*g*, 4 °C, 6 min), the supernatant was discarded. The pellet was suspended in the same volume of extraction buffer. Then, the suspended sample was centrifuged again (2000*g*, 4 °C, 6 min). The washing step was repeated at least five times until no peroxidase activity in the supernatant was detected. The pellet was then incubated in 1 M NaCl at 30 °C with continuous shaking for 6 h and centrifuged at 13000*g* and 4 °C for 15 min. The supernatants were used as cell wall-bound enzyme samples.

Assay of Enzyme Activities. Cell wall peroxidase (CPX, EC 1.11.1.7) activity was assayed by measuring the oxidation rate of the substrate coniferyl alcohol at 260 nm in a reaction mixture consisting of 100 mM sodium phosphate buffer (pH 7.0), 0.1 mM coniferyl alcohol, 0.5 mM H_2O_2, and enzyme extract. H_2O_2 was added to the mixture finally. The molar extinction coefficient of coniferyl alcohol is 2.2 L/mmol/cm (*23*). NADH oxidase activity was assayed by measuring the oxidation rate of NADH at 340 nm in a reaction mixture containing 100 mM sodium acetate buffer (pH 6.5), 1 mM $MnCl_2$, 0.5 mM *p*-coumaric acid, 0.2 mM NADH, and enzyme extract. Enzyme extract was added to the mixture finally. The molar extinction coefficient of NADH is 6.2 L/mmol/cm (*24*).

Gel Electrophoresis of POD. Samples of root tips were homogenized with 10 mM potassium phosphate buffer (pH 6.0) containing 1.0% (w/v) polyvinylpyrrolidone (PVP). After centrifugation (2500*g*, 4 °C, 4 min), the supernatant was discarded. The pellet was suspended in the same extraction buffer. Then, the sample was centrifuged again (2500*g*, 4 °C, 4 min). This process was repeated four times. Cell wall peroxidase was then extracted with 0.2 M $CaCl_2$ in 4 mM $Na_2S_2O_5$ for 12 h with constant stirring at 4 °C. After centrifugation at 12000*g* for 30 min, the supernatant was collected and used for isoenzyme separation using discontinuous polyacrylamide gels (PAGE) under the nondenaturing conditions (*25, 26*).

In Vivo Detection of H_2O_2 and Peroxidase Activity. H_2O_2 was quantified according to the method described previously (*25*). In situ detection of H_2O_2 and peroxidase activity was detected by an endogenous peroxidase-dependent staining procedure using 3,3-diaminobenzidine (DAB) (*27*). Root tips were immersed in the DAB-HCl solution (1 mg mL^{-1}, pH 3.8) for 1 h. After several washings with distilled water, the root apex was visualized under a microscope (Axio Imager, A1, Zeiss).

Lignin Staining and Quantification. Tissues of root tips were homogenized in 1 mL of 10 mM sodium phosphate buffer (pH 6.4). After centrifugation (2000*g*, 4 °C, 6 min), the supernatant was removed. The pellet was suspended in the same buffer, and the mixture was centrifuged again at 2000*g* and 4 °C for 6 min. The washing step was repeated three times. After centrifugation (2000*g*, 4 °C, 6 min), the supernatant was discarded. The pellet was stained with 300 μL of 1% (w/v) phloroglucinol in 20% (v/v) HCl and immediately photographed. Quantitative determination of lignin in root tips was carried out by the method described previously (*28*).

Determination of Nitric Oxide. For cellular NO visualization, roots were pretreated with Al and/or MJ in darkness for 12 h and then loaded with 10 μM 4,5-diaminofluorescence (DAF-2DA). After incubation for 2 h, the roots were washed and immediately visualized by a fluorescence microscope (Axio Imager, A1, Zeiss). The intracellular fluorescence of the roots was excited at 492 nm, and the emission signals at 515 nm were collected (*14*).

Transcript Analysis by Quantitative RT-PCR. Total RNAs were extracted from root tips using Trizol (Invitrogen, Carlsbad, CA). Reverse transcription was first carried out in 14 μL of a solution containing 3 μg of RNA and 0.2 μg of oligo (dT) primer at 70 °C for 5 min. Then, 12.5 nmol of dNTPs, 25 units of RNase inhibitor, and 200 units of

M-MLV reverse transcriptase (Promega, Madison, WI) were added up to a total volume of 14 μL. The cDNA products were obtained after 42 °C for 1 h and then 95 °C for 5 min. The primers for the following genes of LOX, PAL, 4CL, and F5H were designed on the basis of nonhomologous conserved sequences of other plant species from the GenBank database (NCBI). The primers for *LOX* are 5′-TGG ATG ACT GAT GAG GAA TTT GC-3′ (forward) and 5′-TAT AAT GAA TAA AGA CAC AAG GA-3′ (reverse); for *PAL*, 5′-CAA CAC TCG CTT GGC TCT TGC TTC-3′ (forward) and 5′-GAG TTC ACA TCT TGG TTG TGT TGC TC-3′ (reverse); for *4CL*, 5′-GTT GCT GGA GTT GAT ACA GAG GC-3′ (forward) and 5′-ACT GGC CCT GCC TCA GTC AT-3′ (reverse); and for *F5H*, 5′-CAG GGG TTT TCC AAG TTA TTC GGT GC-3′ (forward) and 5′-TCC GTT CCT CCA AAC ATC ACG TCC-3′ (reverse). Expression of the *Tubulin* gene (forward, 5′-TGA ATC TCA TCC CTT TCC CTC G-3′; and reverse, 5′-TGC CTC TGT GAA CTC CAT CTC G-3′) was used as a control. RT-PCR was carried out in a 25 μL reaction volume containing 1 μL of the above cDNAs as the template, 25 mM $MgCl_2$, 5 mM dNTP, 1 units of Taq DNA polymerase (Takara), and 0.5 μM primers, under the following conditions: the first denaturation at 94 °C for 3 min, followed by 33 (LOX, 4CL and F5H)/36 (PAL) cycles of (1) denaturizing at 94 °C for 30 s, (2) annealing at 68 °C for 40 s (LOX, 4CL, and F5H) and 66 °C for 40 s (PAL), (3) extension at 72 °C for 50 s (LOX, 4CL, and F5H) and at 72 °C for 60 s, and a final extension at 72 °C for 10 min. The PCR products were applied to 1% (w/v) agarose gel electrophoresis and stained with ethidium bromide. The strength of the fluorescent signal derived from ethidium bromide in each lane was determined by the software GIS Gel-ID (Tanon Co., Shanghai, China).

Statistical Analysis. Each result shown in the figures was the mean of three replicated treatments, and each treatment contained at least 12 seedlings. The significant differences between treatments were statistically evaluated by standard deviation and Student's *t* test methods.

RESULTS

Effect of Al on the Activity of Cell Wall-Bound Peroxidase. To investigate the role of Al in regulating apoplastic peroxidase activity, we separated wall-bound proteins from those in cytoplasm and assayed the activity of peroxidase using specific substrates. This type of peroxidase is believed to be lignin-generated peroxidases (*7, 11*). Our results showed that activities of coniferyl alcohol-based peroxidases significantly increased in the root tips exposed to Al. The saturated activity appeared at 10 μM Al, with a 63.6% increase over the control (**Figure 1A**). In a time course experiment, no differences of peroxidase activities were observed in the initial 6 h between the Al-treated root (10 μM Al) and control. At 12 and 24 h, the significantly increased peroxidase activities were observed (**Figure 1B**). After that, however, the peroxidase activity decreased. Activities of syringaldazine-based peroxidases were examined, but they had a similar response to Al (data not shown). We further determined the cell wall-bound POD by performing nondenaturing PAGE. Although only one isoform of peroxidase was detected in the cell wall (**Figure 1C**), the activities of POD showed a general increase with Al compared to the control. This result was consistent with the data obtained from the spectrophotometric assay (**Figure 1A**).

We examined another cell wall-bound peroxidase, termed NADH oxidase. This enzyme has been suggested to contribute to the H_2O_2 formation required for lignification of cell wall (*7, 29*). Seedlings of *C. tora* showed an elevated activity of NADH oxidase in root tips with Al treatment (**Figure 2A**). Also, a time-dependent change in the activity was observed in the root tips exposed to 10 μM Al. The significant increase was observed around 12−36 h after Al exposure (**Figure 2B**). A prolonged exposure to Al did not promote further the NADH oxidase activity.

Effect of Al on Accumulation of Lignin and Hydrogen Peroxide. Lignins belong to the cell wall phenolic heteropolymers, which result from the oxidative coupling of three monolignols, *p*-coumaryl, coniferyl, and sinapyl alcohols, in a reaction regulated by cell wall peroxidases (*30*). In Al-treated root tips, the lignin accumulated with Al concentrations (**Figure 3A**). Treatments with Al at 10, 30, and 50 μM resulted in lignin accumulation by 27.9, 45.2, and 69.1%, respectively, as compared to the control. There was a time-dependent change in lignin accumulation. During the first 6 h, seedlings with 10 μM Al showed only a slight increase in lignin accumulation, but they continued to accumulate after that (**Figure 3B**). The remarkable accumulation lasted up to 24 h. Al-induced accumulation of lignin in root tips was confirmed by staining with phloroglucinol. As shown in **Figure 3C**, the root samples with Al appeared dark brown, whereas the control samples were light brown.

H_2O_2 has been proposed as a necessary substrate for the formation of cross-linking of cell wall polymers (*10*). To support the possibility that NADH oxidase-mediated H_2O_2 generation is responsible for lignin production in the root tips, we measured H_2O_2 content. As expected, accumulation of H_2O_2 was shown to increase with Al concentrations applied (**Figure 4A**). Concerning the formation of H_2O_2 in cell walls, a histochemical detection of H_2O_2 on the root tip epidermal cells was performed with DAB. DAB reacts with H_2O_2 in the presence of peroxidases

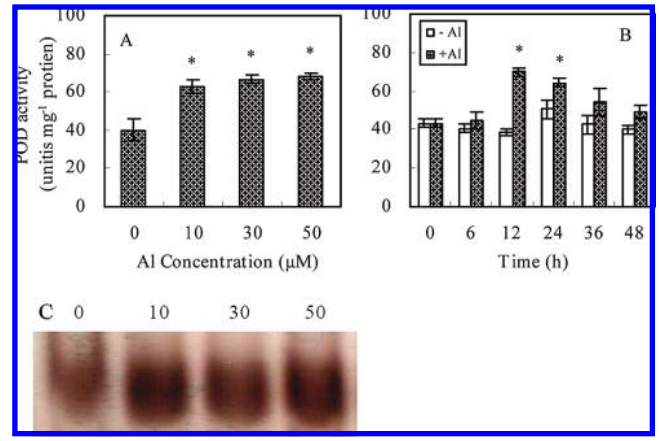

Figure 1. Effect of Al on the cell wall-bound peroxidase (POD) activities in the root tips of *C. tora*. Seedlings were exposed to 0.5 mM $CaCl_2$ (pH 4.5) solutions containing 0, 10, 30, and 50 μM Al for 12 h (**A**, **C**) or 10 μM Al for 0−48 h (**B**). Vertical bars represent standard deviation of the mean. Asterisks indicate that the mean values are significantly different between the Al treatments and controls ($p < 0.05$). For determination of POD activity in gel (**C**), 40 μg proteins from root tip extracts were loaded into the native PAGE gel. Following the electrophoresis, the gels were stained and photographed.

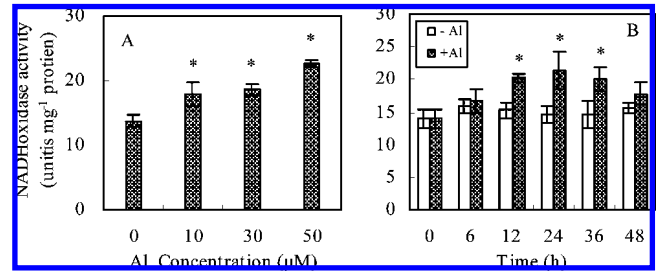

Figure 2. Effect of Al on the cell wall NADH oxidase activities in the root tips of *C. tora*. Seedlings were exposed to 0.5 mM $CaCl_2$ (pH 4.5) solutions containing the indicated concentrations of Al for 12 h (**A**) or 10 μM Al for 0−48 h (**B**). Vertical bars represent standard deviation of the mean. Asterisks indicate that the mean values are significantly different between the Al treatments and controls ($p < 0.05$).

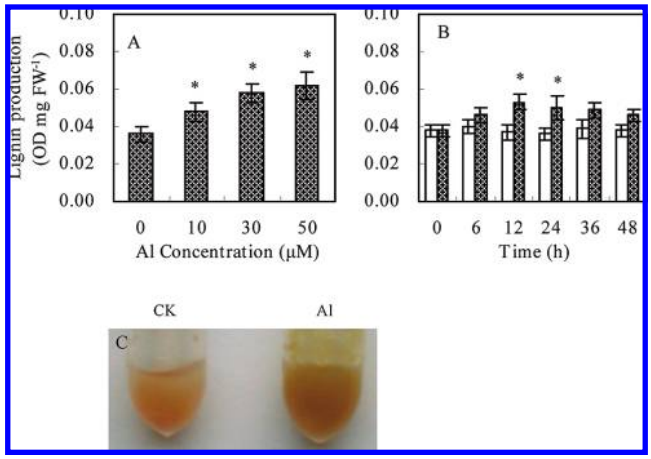

Figure 3. Effect of Al on the lignin accumulation in the root tips of *C. tora*. Seedlings were exposed to 0.5 mM $CaCl_2$ (pH 4.5) solutions containing the indicated concentrations of Al for 12 h (**A**) or 10 μM Al for 0−48 h (**B**). Vertical bars represent standard deviation of the mean. Asterisks indicate that the mean values are significantly different between the Al treatments and control ($p < 0.05$). In experiment **C**, seedlings were exposed to 10 μM Al for 24 h. The root tips were stained with 1% (w/v) phloroglucinol after homogenization in sodium phosphate buffer and photographed.

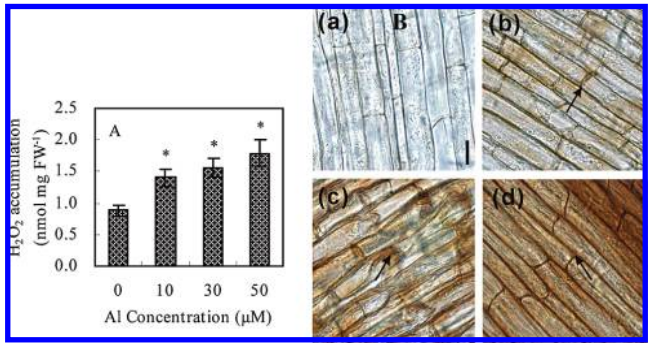

Figure 4. Effect of Al on the H_2O_2 production in the root tips of *C. tora*. Seedlings were exposed to 0.5 mM $CaCl_2$ (pH 4.5) solutions containing the indicated concentrations of Al for 12 h. After that, the accumulation of H_2O_2 in root tips was measured by spectrophotometric determination (**A**). Vertical bars represent standard deviation of the mean. Asterisks indicate that the mean values are significantly different between the Al treatments and control ($p < 0.05$). (**B**) distribution of H_2O_2 in the epidermal cells of root tips exposed to Al at 0 (a), 10 (b), 30 (c), and 50 (d) μM. Arrows indicate the site of H_2O_2 occurring on the epidermal cells. Bar is 10 μM for all images.

to produce a brown polymerization product. As shown in **Figure 4B**, the control root cells displayed very light intensity along the cell walls, whereas the roots exposed to Al were stained extensively around the entire region of the cells (**Figure 4Bb−d**).

NO and JA Regulation of Enzyme Activities under Al Stress. Because NO is able to attenuate Al-induced peroxidative stress (*15*), we hypothesized that NO might mediate cell wall peroxidase activity and lignin synthesis in the roots of *C. tora*. To test the possibility, we measured cell wall-bound activities of peroxidase and NADH oxidase in SNP-pretreated root tips. As shown in **Figure 5A**, pretreatment with a NO donor SNP at 0.4 mM, followed by 10 μM Al exposure, significantly reduced the peroxidase activities relative to the Al treatment alone. Activities of NADH oxidase showed similar changes in response to exogenous NO in the presence of Al (**Figure 5B**). However, simultaneous pretreatment with a NO scavenger, cPTIO,

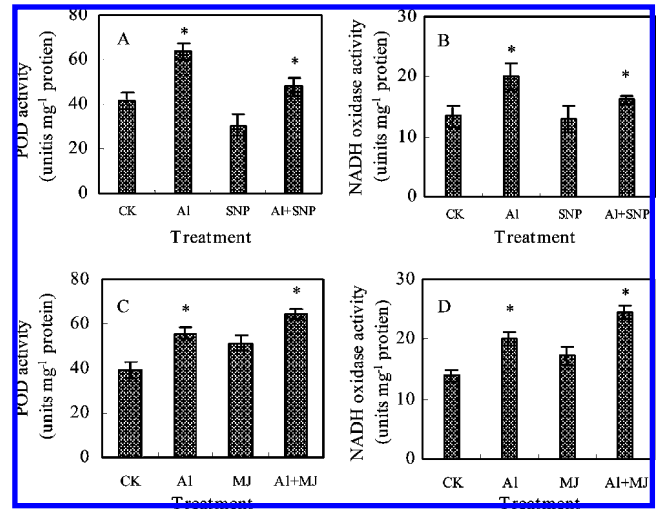

Figure 5. Effect of NO donor SNP and MJ on the cell wall peroxidase (**A**, **C**) and NADH oxidase (**B**, **D**) activities in the root tips of *C. tora*, respectively. Seedlings were pretreated with 0.4 mM SNP for 12 h and then exposed to 0.5 mM $CaCl_2$ (pH 4.5) solutions containing 10 μM Al for 12 h (**A**, **B**). For experiments **C** and **D**, seedlings were incubated in 0.5 mM $CaCl_2$ (pH 4.5) solutions containing 10 μM Al alone and 10 μM Al + 10 μM MJ for 12 h. Vertical bars represent standard deviation of the mean. Asterisks indicate that the mean values are significantly different between the treatments of Al and Al + SNP or Al + MJ ($p < 0.05$).

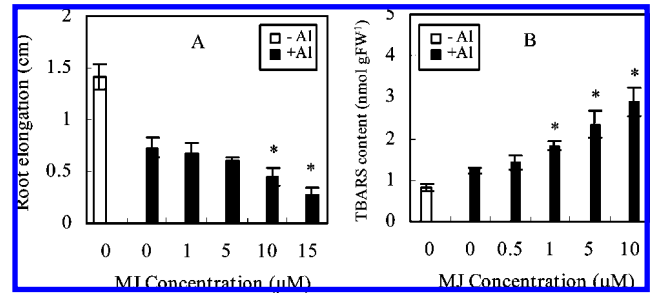

Figure 6. Effect of MJ concentrations on the elongation (**A**) and peroxidation (**B**) of *C. tora* root tips exposed to Al. Seedlings were exposed to 0.5 mM $CaCl_2$ (pH 4.5) solutions containing 10 μM Al and the indicated concentrations of MJ for 24 h. After that, the root elongation (**A**) and TBARS accumulation (**B**) were measured. Vertical bars represent standard deviation of the mean. Asterisks indicate that the mean values are significantly different between the treatments of Al + MJ and Al alone ($p < 0.05$).

completely canceled the NO suppression of peroxidase activities and, also, the SNP analogue potassium ferricyanide, which is unable to release NO, had no effect on the peroxidase activities (data not shown).

Because JA has been proposed to regulate cell wall component metabolism when plants are subjected to abiotic stress (*16*), we tested whether JA regulates peroxidase activities and root tolerance to Al. Our results showed that application of 10 μM MJ increased the activities of both peroxidase and NADH oxidase in the Al-treated root tips (**Figure 5C**,**D**). MJ-regulated increase in activities of peroxidase and NADH oxidase suggests that MJ might regulate lignin synthesis and root growth under Al stress. Our analysis revealed that within 1−15 μM, MJ was able to reduce progressively the root elongation under Al stress (**Figure 6A**). To evaluate further the effect of MJ on Al toxicity to the roots of *C. tora*, the oxidative damage to membranes was examined by measuring the content of thiobarbituric acid reactive substances (TBARS), an indicator of lipid peroxidation. As shown in **Figure 6B**, treatments with MJ in the presence of

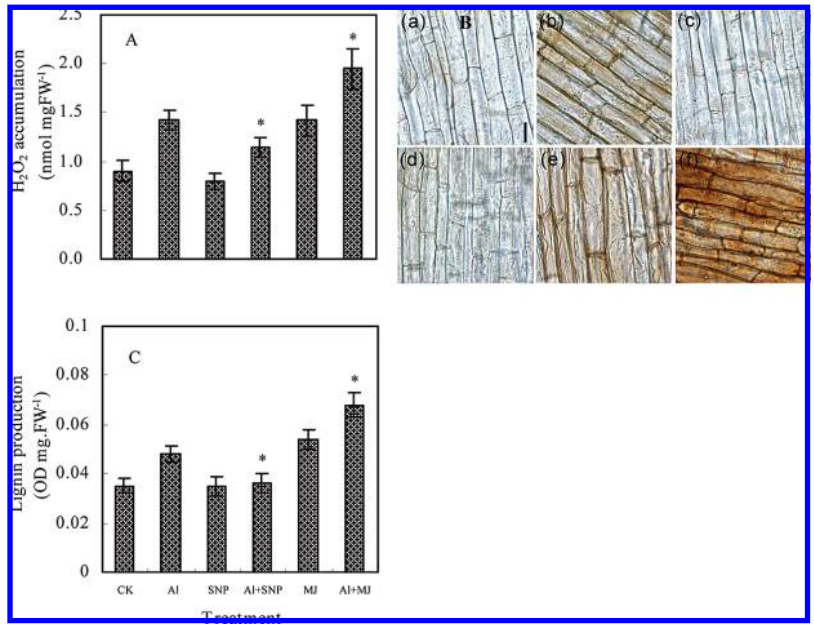

Figure 7. Effect of NO donor SNP and MJ on the production of cell wall H_2O_2 (**A**, **B**) and lignin (**C**) in root tips of *C. tora*. Seedlings were pretreated with 0.4 mM SNP for 12 h and then exposed to 0.5 mM $CaCl_2$ (pH 4.5) solutions containing 10 μM Al for 12 h. For MJ experiments, seedlings were incubated in 0.5 mM $CaCl_2$ (pH 4.5) solutions containing 10 μM Al alone and 10 μM Al + 10 μM MJ for 12 h. Vertical bars represent standard deviation of the mean. Asterisks indicate that the mean values are significantly different between the treatments of Al and Al + SNP or Al + MJ ($p < 0.05$). (**B**) Histochemical detection of H_2O_2 in the epidermal cell walls of root tips exposed to various treatments: (**a**) 0 μM Al (CK); (**b**) 10 μM Al; (**c**) 0.4 mM SNP; (**d**) 10 μM Al and 0.4 mM SNP; (**e**) 10 μM MJ; (**f**) 10 μM Al and 10 μM MJ. Bar is 10 μm for all images.

Al resulted in higher levels of TBARS accumulation than the treatment with Al alone, suggesting that JA might exert a positive effect of the Al toxicity on the roots.

We determined the effect of MJ on the production of H_2O_2 and lignin and show that whereas Al-induced generation of H_2O_2 and lignin accumulation in root tips were suppressed by SNP application, treatment with 10 μM MJ and with 10 μM Al increased the contents of H_2O_2 and lignin considerably (**Figure 7A**,**C**). To confirm the high level of H_2O_2 promoted by Al and/or MJ, a histochemical staining of H_2O_2 in the epidermal cell wall of root tips was performed with DAB. It is shown that Al at 10 μM induced intense staining (**Figure 7Bb**) as compared to the control (**Figure 7Ba**), indicating a higher level of H_2O_2 accumulated in the root tips. The roots pretreated with exogenous NO followed by Al showed a light staining (**Figure 7Bd**). In contrast, the roots treated with MJ in the presence of Al had substantial accumulation of H_2O_2 in both apoplastic and symplastic regions (**Figure 7Bf**).

Effect of NO and JA on Expression of Lignin Biosynthesis-Related Genes under Al Stress. L-Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) catalyzes β-elimination of ammonia from L-phenylalanine to *trans*-cinnamic acid and is a key enzyme in the phenylpropanoid pathway, from which lignins, avonoids, and coumarins are derived (*31*). 4-Coumarate: coenzyme A (CoA) ligase (4CL, EC 6.2.1.12), also located in the phenylpropanoid pathway, mediates the metabolism of some intermediates such as hydroxycinnamic acids 4-(*p*)-coumaric acid (PA), caffeic acid (CA), ferulic acid (FA), 5-hydroxyferulic acid (5HFA), and sinapic acid (SA) for biosynthesis of lignin (*32*). Ferulate 5-hydroxylase (F5H) is a rate-limiting monooxygenase that catalyzes the hydroxylation of ferulic acid, coniferaldehyde, and coniferyl alcohol in the pathway leading to sinapic acid and syringyl lignin biosynthesis (*33*). Because these genes control lignin biosynthesis, we performed a quantitative RT-PCR-based assay to analyze the transcript amounts of them. As shown in **Figure 8A**, the transcripts of *PAL*, *4CL*, and *F5H* increased considerably with Al concentrations at 30−50 μM, although they were shown to constitutively express in the root tips without Al exposure. Effects of NO and JA on the expression of *PAL*, *4CL*, and *F5H* were differently presented. It was shown that the expression of the three genes in Al-treated root tips were down-regulated by SNP treatment (**Figure 8B**), whereas treatment with MJ up-regulated the expression of *PAL* and *F5H* in the Al-treated root tips (**Figure 8C**). However, the Al-induced *4CL* expression was suppressed by MJ treatment.

Effect NO on Expression of LOX and JA Regulation of NO Production under Al Stress. Lipoxygenase is a class of enzymes that catalyze the oxygenation of polyunsaturated fatty acids (e.g., α-linolenic acids, 18:3) to form hydroperoxides (*34*). These components are highly reactive and rapidly degraded into metabolites that lead to the formation of JA (*16*). To understand further the LOX activity during Al stress, we analyzed the *LOX* transcripts. Our results showed that the expression of *LOX* was up-regulated by Al exposure, but down-regulated by SNP treatment in the Al-treated root tips (**Figure 9A**,**B**). Additionally, treatment with MJ induced the expression of *LOX* (**Figure 9C**), suggesting a feedback mechanism in the octadecanoid pathway.

The effect of MJ on NO production was investigated in root tips of *C. tora* under Al stress. Using DAF-2DA as a fluorescence indicator, we could detect the NO intensity of DAF-2DA-stained root tips. It is shown that DAF-2DA-dependent NO fluorescence intensity was enhanced by the exposure of Al ranging from 10 to 50 μM (**Figure 10B−D**). However, Al-induced NO fluorescence could be completely abolished by a NO scavenger cPTIO (data not shown). Application of 10 μM MJ to the roots resulted in the depression of NO production in Al-treated root tips (**Figure 10F**). These results suggest that JA negatively regulated synthesis of NO during the signaling of Al-induced stress in the roots of *C. tora*.

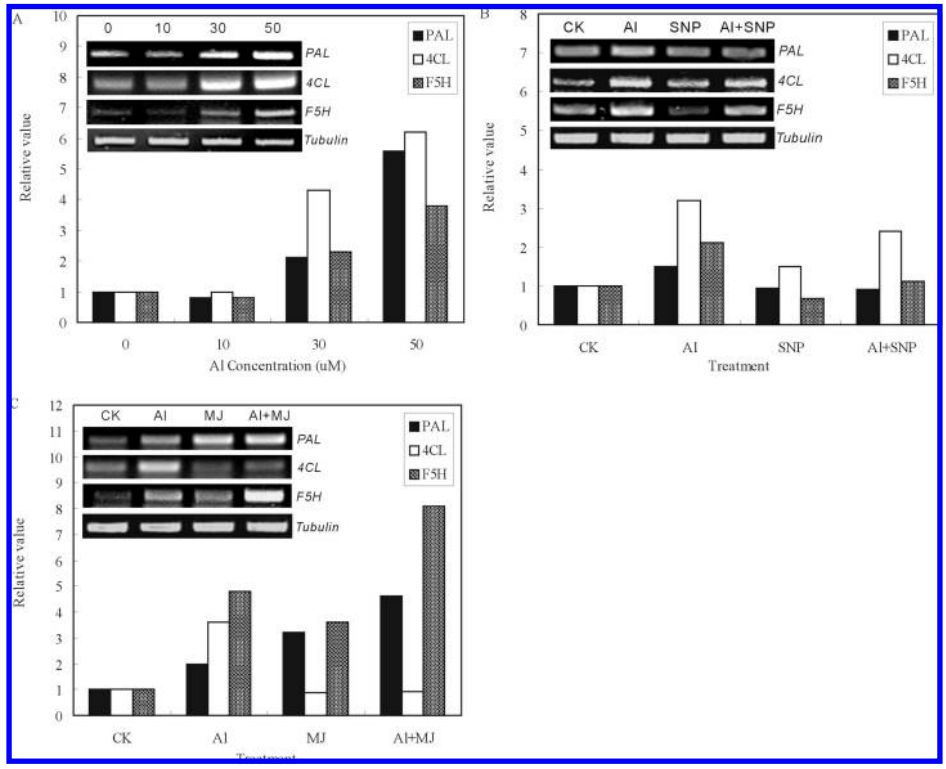

Figure 8. Analysis of transcript amounts of *PAL*, *4CL*, and *F5H* in the roots of *C. tora* exposed to Al with or without MJ and/or SNP: (**A**) seedlings were exposed to 0.5 mM $CaCl_2$ (pH 4.5) solutions containing 0, 10, 30, and 50 μM Al for 12 h; (**B**) seedlings were pretreated with 0.4 mM SNP for 12 h and then exposed to 0.5 mM $CaCl_2$ (pH 4.5) solutions containing 30 μM Al for 12 h; (**C**) seedlings were incubated in 0.5 mM $CaCl_2$ (pH 4.5) solutions containing 30 μM Al and/or 10 μM MJ for 12 h. After treatments, total RNAs were extracted from root tissues and quantitative RT-PCR was performed. Tubulin was used for cDNA normalization.

DISCUSSION

Plant apoplasts function as a barrier to Al entry to cytoplasm. Several lines of evidence have demonstrated that a majority of Al accumulates in apoplast when exposed to excess Al (*35*). Due to its high binding affinity to anionic components, Al has been proposed to modify chemical properties of cell wall (*5, 36*). Furthermore, the cell wall mechanical properties may be also modified by Al, thus resulting in cell wall stiffening and blocking of root elongation. However, the precise biochemical and molecular regulatory mechanisms are largely unknown. It is shown that a large number of enzymes, such as peroxidases, hydrolases, kinases, esterases, expansin, lyases, and transferase are located in the apoplast, and most of them participate in the metabolic process of cell wall organization (*37*). Within these enzymes, the cell wall-bound peroxidases may play a critical role in the formation of important components. Under the Al stress, the peroxidase-mediated modification of cell wall appears to be associated with cell wall stiffening (*21, 38*). It is noted that peroxidases belong to a superfamily, and only those localized in apoplast may modify cell wall properties (*7, 8*). The specific activity of peroxidase in the cell wall suggests that this type of enzyme is secreted outside root cells and plays roles in lignin biosynthesis. In this study, we examined the cell wall peroxidases in the Al-exposed root of *C. tora*. Our results indicated that the activity of wall-bound peroxidase increased in response to Al exposure. Although analysis of peroxidases with native PAGE revealed only one isoform in cell wall, it was strongly induced in the presence of Al. Simultaneously, the activity of NADH oxidase was also activated. The enhanced activity of NADH oxidase under Al stress was closely associated with the elevated level of hydrogen peroxide and accumulation of lignin. These results suggest that the cell wall peroxidases may play a role in promoting the lignin synthesis in the root of *C. tora*.

Although a close relationship between the apoplastic peroxidase and lignin synthesis can be established in Al-treated roots, the regulatory processes in relation to signaling properties are unknown. The NO-regulated suppression of oxidation triggered by Al was found to be correlated with reduced lipid peroxidation and improved root growth (*14*). Additionally, supporting evidence has been provided that endogenous NO is involved in the regulation of plant tolerance to Al toxicity (*15*). Despite this, questions with regard to the interactions or cross-talks with other novel signaling molecules need to be addressed. A number of plant species respond to Al stress by promoting the production of secondary metabolites (*5, 21, 39, 40*). This process is most likely to be mediated by signaling molecules. Some signaling molecules such as JA and ethylene are involved in the regulation of synthesis of secondary metabolites (*41*). For instance, when plants are subjected to pathogen attack, the overproduced JA induces many genes, some of which encode proteins located in the cell wall and responsible for cellulose syntheses (*41*). To support the hypothesis, we applied exogenous MJ to the root under Al stress and found that the root was rather sensitive to MJ. These results suggest that JA was involved in regulating the responses of root to Al.

JA biosynthesis also induces transcripts that regulate the synthesis of JA itself (*16*). One of the most dominant genes is *LOX*, coding lipoxygenase, which catalyzes peroxidation of polyunsaturated fatty acids (e.g., linoleic and linolenic acids) to produce a variety of intermediate metabolites. The intermediates, termed oxylipins, serve as messenger molecules, and

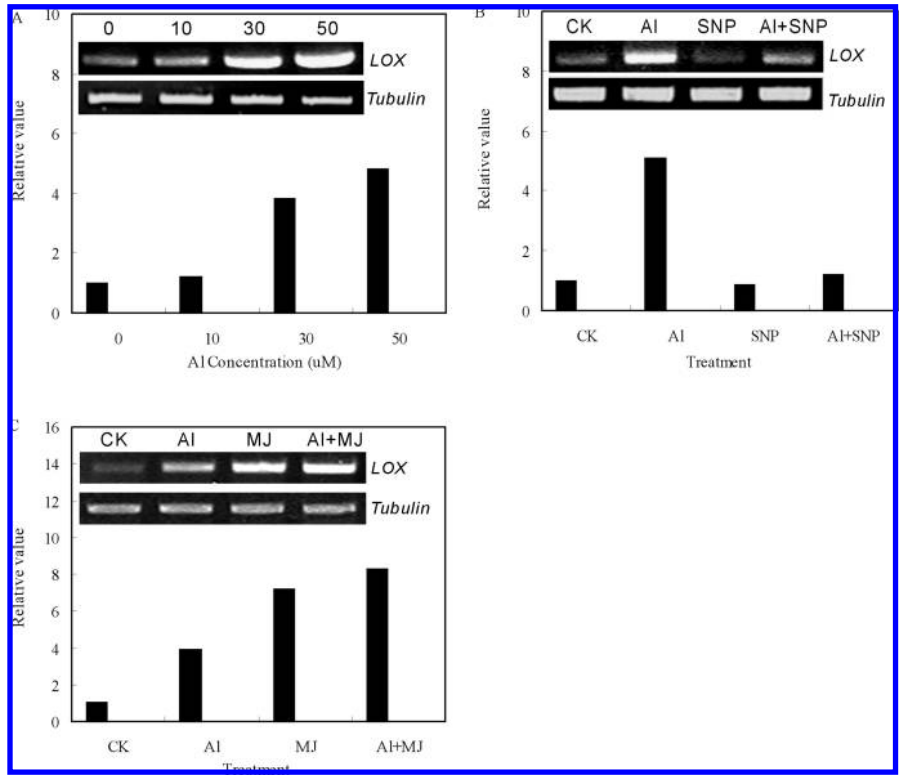

Figure 9. Analysis of transcript amounts of *LOX* in the root tips of *C. tora* exposed to Al with or without MJ and/or SNP: (**A**) seedlings were exposed to 0.5 mM $CaCl_2$ (pH 4.5) solutions containing 0, 10, 30, and 50 μM Al for 12 h; (**B**) seedlings were pretreated with 0.4 mM SNP for 12 h and then exposed to 0.5 mM $CaCl_2$ (pH 4.5) solutions containing 30 μM Al for 12 h; (**C**) seedlings were incubated in 0.5 mM $CaCl_2$ (pH 4.5) solutions containing 30 μM Al and/or 10 μM MJ for 12 h. After the treatments above, total RNAs were extracted from root tissues and quantitative RT-PCR was performed. Tubulin was used for cDNA normalization.

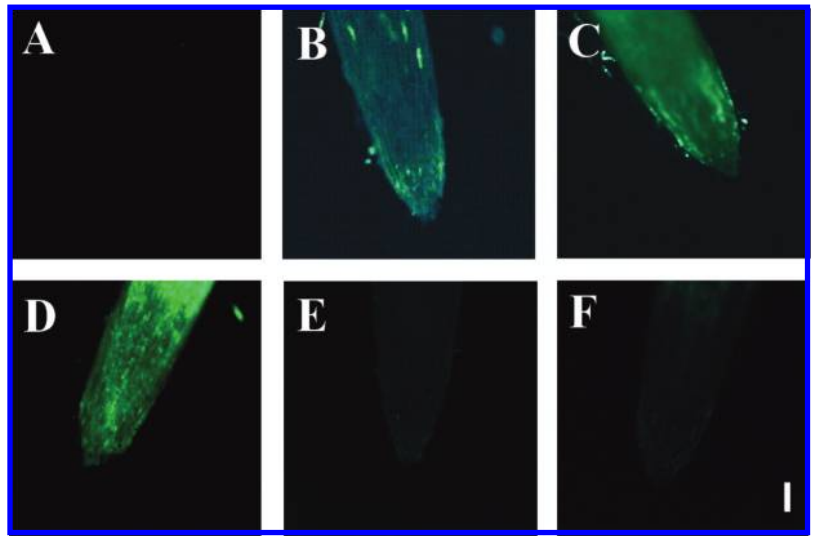

Figure 10. Visualization of in vivo NO generation in the root tips of *C. tora* exposed to Al and/or MJ. Seedlings were incubated in 0.5 mM $CaCl_2$ (pH 4.5) solutions containing 0 μM Al (**A**), 10 μM Al (**B**), 30 μM Al (**C**), 50 μM Al (**D**), 10 μM MJ (**E**), and 10 μM Al + 10 μM MJ (**F**) for 12 h. After that, the seedlings were loaded with 10 μM 4,5-diaminofluorescence (DAF-2DA) for 2 h. Finally, NO generated in the root tips was visualized under the fluorescence microscope. Bar indicates 1 mm.

activation of LOX activity is closely linked to JA biosynthesis under a variety of stressful conditions (*16*). Expression of *LOX* following infection by bacterial and fungal pathogens results in higher biosynthesis of JA and thus intensifies the response to the stress (*42*). The current results demonstrated that the expression of *LOX* was up-regulated by Al exposure. These results suggest that Al-induced LOX activity was possibly associated with jasmonate signal. Interestingly, NO was found to depress the LOX at the level of both enzymatic and transcriptional activities. On the other hand, seedlings treated with MJ showed the reduced NO production in Al-exposed root tips. In addition, MJ promoted the activity of cell wall peroxidase activity and lignin accumulation, whereas NO had a negative effect. All of these results indicate that JA appears to counteract with NO, suggesting a negative cross-talk in signaling plants to the Al exposure.

In conclusion, we provide evidence that Al exposure induces cell wall-bound peroxidases responsible for lignin

synthesis and hydrogen peroxide production in the roots of *C. tora*. The Al-activated apoplastic peroxidase activities can be negatively regulated by NO and positively regulated by methyl jasmonate. Thus, the sensitivity of the cell wall-bound peroxidases under Al stress is likely to be associated with the cross-talk between NO and JA signals. This finding, together with LOX activity, may allow further identification of the interaction between NO and JA in more detail and characterization of the interplay within other sets of signal elements, such as NO/ROS and JA/ROS, in regulating plant response to Al toxicity.

LITERATURE CITED

(1) Kochian, L. V.; Hoekenga, O. A.; Piñeros, M. A. How do crop plants tolerate acid soils? Mechanisms of aluminum tolerance and phosphorus efficiency. *Annu. Rev. Plant Biol.* **2004**, *55*, 459–493.

(2) Barceló, J.; Poschenrieder, C. Fast root growth responses, root exudates, and internal detoxification as clues to the mechanisms of aluminum toxicity and resistance: a review. *Environ. Exp. Bot.* **2002**, *48*, 75–92.

(3) Wehr, J. B.; Menzies, N. W.; Blamey, F. P. C. Model studies on the role of citrate, malate and pectin esterification on the enzymatic degradation of Al- and Ca-pectate gels:possible implications for Al-tolerance. *Plant Physiol. Biochem.* **2003**, *41*, 1007–1010.

(4) Tabuchi, A.; Matsumoto, H. Changes in cell wall properties of wheat (*Triticum aestivum*) roots during aluminum-induced growth inhibition. *Physiol. Plant.* **2001**, *112*, 353–358.

(5) Eticha, D.; Stass, A.; Horst, W. J. Cell-wall pectin and its degree of methylation in the maize root-apex: significance for genotypic differences in aluminium resistance. *Plant Cell Environ.* **2005**, *28*, 1410–1420.

(6) Tamá, L.; Huttová, J.; Mistrík, I. Inhibition of Al-induced root elongation and enhancement of Al-induced peroxidase activity in Al-sensitive and Al-resistant barley cultivars are positively correlated. *Plant Soil* **2003**, *250*, 193–200.

(7) Passardi, F.; Penel, C.; Dunand, C. Performing the paradoxical: how plant peroxidases modify the cell wall. *Trend Plant Sci.* **2004**, *9*, 534–540.

(8) De Gara, L. Class III peroxidases and ascorbate metabolism in plants. *Phytochem. Rev.* **2004**, *3*, 195–205.

(9) Pomar, F.; Caballero, N.; Pedreño, M. A.; Barcelò, A. H_2O_2 generation during the auto-oxidation of coniferyl alcohol drives the oxidase activity of a highly conserved class III peroxidase involved in lignin biosynthesis. *FEBS Lett.* **2002**, *529*, 198–202.

(10) Dunand, C.; Crèvecoeur, M.; Penel, C. Distribution of superoxide and hydrogen peroxide in *Arabidopsis* root and their influence on root development: possible interaction with peroxidases. *New Phytol.* **2007**, *174*, 332–341.

(11) Hirada, S.; Sasaki, K.; Ito, H.; Ohashi, Y.; Matsui, H. A large family of class III plant peroxidaes. *Plant Cell Physiol.* **2001**, *42*, 462–468.

(12) Huang, X.; Stettmaier, K.; Michel, C.; Hutzler, P.; Mueller, M. J.; Durner, J. Nitric oxide is induced by wounding and influences jasmonic acid signaling in *Arabidopsis thaliana*. *Planta* **2004**, *218*, 938–946.

(13) Beligni, M. V.; Lamattina, L. Nitric oxide counteracts cytotoxic processes mediated by reactive oxygen species in plant tissues. *Planta* **1999**, *208*, 337–344.

(14) Wang, Y. S.; Yang, Z. M. Nitric oxide reduces aluminum toxicity by preventing oxidative stress in the roots of *Cassia tora* L. *Plant Cell Physiol.* **2005**, *46*, 1915–1923.

(15) Tian, Q. Y.; Sun, D. H.; Zhao, M. G.; Zhang, W. H. Inhibition of nitric oxide synthase (NOS) underlies aluminum-induced inhibition of root elongation in *Hibiscus moscheutos*. *New Phytol.* **2007**, *174*, 322–331.

(16) Devoto, A.; Turner, J. G. Regulation of jasmonate-mediated plant responses in *Arabidopsis*. *Annu. Bot.* **2003**, *92*, 329–337.

(17) Rao, M. V.; Lee, H.; Creelman, R. A.; Mullet, J. E.; Davis, K. R. Jasmonic acid signaling modulates ozone-induced hypersensitive cell death. *Plant Cell* **2000**, *12*, 1633–1646.

(18) Xiang, C.; Oliver, D. J. Glutathione metabolic genes co-ordinatedly respond to heavy metals and jasmonic acid in *Arabidopsis*. *Plant Cell* **1998**, *10*, 1539–1550.

(19) Przymusiński, R.; Rucińska, R.; Gwóźdź, E. A. Increased accumulation of pathogenesis-related proteins in response of lupine roots to various abiotic stresses. *Envion. Exp. Bot* **2004**, *52*, 53–61.

(20) Maksymiec, W.; Krupa, Z. The effects of short-term exposition to Cd, excess Cu ions and jasmonate on oxidative stress appearing in *Arabidopsis thaliana*. *Envion. Exp. Bot.* **2006**, *57*, 187–194.

(21) Schaller, F. Enzymes of the biosynthesis of octadecanoid-derived signaling molecules. *J. Exp. Bot.* **2001**, *52*, 11–23.

(22) Lee, T. M.; Lin, Y. H. Changes in soluble and cell wall-bound peroxidase activities with growth in anoxia-treated rice (*Oryza sativa* L.) coleoptiles and roots. *Plant Sci.* **1995**, *106*, 1–7.

(23) Chaoui, A.; Jarrar, B.; El Ferjani, E. Effects of cadmium and copper on peroxidase, NADH oxidase and IAA oxidase activities in cell wall, soluble and microsomal membrane fractions of pea roots. *J. Plant Physiol.* **2004**, *161*, 1225–1234.

(24) Ishida, A.; Ookubo, K.; Ono, K. Formation of hydrogen peroxide by NAD(P)H oxidation with isolated cell wall-associated peroxidase from cultured liverwort cells *Marchantia polymorpha* L. *Plant Cell Physiol* **1987**, *28*, 723–726.

(25) Zhou, Z. S.; Huang, S. Q.; Guo, K.; Mehta, S. K.; Zhang, P. C.; Yang, Z. M. Metabolic adaptations to mercury-induced oxidative stress in roots of *Medicago sativa* L. *J. Inorg. Biochem.* **2007**, *101*, 1–9.

(26) Yin, X. L.; Jiang, L.; Song, N. H.; Yang, H. Toxic eeactivity of wheat (*Triticum aestivum*) plants to herbicide isoproturon. *J. Agric. Food Chem.* **2006**, *56*, 4825–4831.

(27) Song, N. H.; Yin, X. R.; Chen, G. F.; Yang, H. Metabolic adaptations to the herbicide chlorotoluron-induced oxidative stress in wheat (*Triticum aestivum*). *Chemosphere* **2007**, *68*, 1779–1787.

(28) Fukuda, H.; Komamine, A. Lignin synthesis and its related enzymes as markers of tracheary-element differentiation in single cells isolated from the mesophyll of *Zinnia elegans*. *Planta* **1982**, *155*, 423–430.

(29) Fecht-Christoffers, M. M.; Führs, H.; Braun, H.; Horst, W. J. The role of hydrogen peroxide-producing and hydrogen peroxide-consuming peroxidase in the leaf apoplast of cowpea in manganese tolerance. *Plant Physiol.* **2006**, *140*, 1451–1463.

(30) Barceló, A. R.; Gómez Ros, L. V.; Gabaldón, C.; López-Serrano, M.; Pomar, F.; Carrión, J. S.; Pedreño, M. A. Basic peroxidases: the gateway for lignin evolution. *Phytochem. Rev.* **2004**, *3*, 61–78.

(31) Alunni, S.; Cipiciani, A.; Fioroni, G.; Ottavi, L. Mechanisms of inhibition of phenylalanine ammonia-lyase by phenol inhibitors and phenol/glycine synergistic inhibitors. *Arch. Biochem. Biophys.* **2003**, *412*, 170–175.

(32) Harding, S. A.; Leshkevich, J.; Chiang, V. L.; Tsai, C. J. Differential substrate inhibition couples kinetically distinct 4-coumarate:coenzyme A ligases with spatially distinct metabolic roles in quaking aspen1. *Plant Physiol.* **2002**, *128*, 428–438.

(33) Franke, R.; McMichael, C. M.; Meyer, K.; Shirley, A. M.; Cusumano, J. C.; Chaplle, C. Modified lignin in tobacco and poplar plants over-expressing the *Arabidopsis* gene encoding ferulate 5-hydroxylase. *Plant Physiol.* **2000**, *22*, 223–234.

(34) Siedow, J. N. Plant lipoxygenase: structure and function. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1991**, *42*, 145–188.

(35) Taylor, G. J.; McDonald-Stephens, J. L.; Hunter, D. B.; Bertsch, P. M.; Elmore, D.; Rengel, Z.; Reid, R. J. Direct measurement of aluminum uptake and distribution in single cells of *Chara corallina*. *Plant Physiol.* **2000**, *123*, 987–996.

(36) Sasaki, M.; Yamamoto, Y.; Matsumoto, H. Lignin deposition induced by aluminum in wheat (Triticum aestium) roots. *Physiol. Plant.* **1996**, *96*, 193–198.

(37) Cosgrove, D. J. Expansive growth of plant cell walls. *Plant Physiol. Biochem.* **2000**, *38*, 109–124.

(38) Šimonovičová, M.; Huttová, J.; Mistrík Široká, B.; Tamás, L. Root growth inhibition by aluminum is probably caused by cell death due to peroxidase-mediated hydrogen peroxide production. *Protoplasma* **2004**, *224*, 91–98.

(39) Jones, D. L.; Blancaflor, E. B.; Kochian, L. V.; Gilroy, S. Spatial coordination of aluminum uptake, production of reactive oxygen species, callose production and wall rigidification in maize roots. *Plant Cell Environ.* **2006**, *29*, 1309–1318.

(40) Wang, P.; Bi, S.; Wang, S.; Ding, Q. Variation of wheat root exudates under aluminum stress. *J. Agric. Food Chem.* **2006**, *54*, 10040–10046.

(41) Ellis, C.; Turner, J. G. The *Arabidopsis* mutant cev1 has constitutively active jasmonate and ethylene signal pathway and enhanced resistance to pathogens. *Plant Cell* **2001**, *13*, 1025–1033.

(42) Wasternack, C. Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. *Ann. Bot.* **2007**, *100*, 681–697.

Received for review July 1, 2008. Revised manuscript received August 18, 2008. Accepted August 21, 2008.

JF802001V