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Research Note

LSD1 Regulates Salicylic Acid Induction of Copper Zinc Superoxide Dismutase in *Arabidopsis thaliana*

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We characterized the accumulation patterns of *Arabidopsis thaliana* proteins, two CuZnSODs, FeSOD, MnSOD, PR1, PR5, and GST1, in response to various pathogen-associated treatments. These treatments included inoculation with virulent and avirulent *Pseudomonas syringae* strains, spontaneous lesion formation in the *lsd1* mutant, and treatment with the salicylic acid (SA) analogs INA (2,6-dichloroisonicotinic acid) and BTH (benzothiadiazole). The PR1, PR5, and GST1 proteins were inducible by all treatments tested, as expected from previous mRNA blot analysis. The two CuZnSOD proteins were induced by SA analogs and in conjunction with *lsd1*-mediated spreading cell death. Additionally, *LSD1* is a part of a signaling pathway for the induction of the CuZnSOD proteins in response to SA but not in *lsd1*-mediated cell death. We suggest that the spreading lesion phenotype of *lsd1* results from a lack of up-regulation of a CuZnSOD responsible for detoxification of accumulating superoxide before the reactive oxygen species can trigger a cell death cascade.

Additional keyword: phx21.

Plants are continuously subjected to potentially destructive reactive oxygen species (ROS) whose concentrations can be increased by environmental stimuli. Superoxide (O_2^-), which is formed by univalent electron transfer to O_2, is an abundant ROS that can contribute to the synthesis of highly reactive hydroxyl radicals (Cadenas 1989; Halliwell and Gutteridge 1990). Pathogen invasion, as well as exposure to photoinhibitory light and ozone, increases O_2^- levels in plants (Yruela et al. 1996; Lamb and Dixon 1997; Runeckles and Vaartnou 1997). Hence, control of O_2^- accumulation is essential.

Plants contain numerous antioxidant systems capable of detoxifying O_2^- and limiting potential damage. Superoxide dismutase (SOD, E.C. 1.15.1.1), which catalyzes the conversion of O_2^- to hydrogen peroxide (H_2O_2), is a major antioxidant. Plants contain three classes of SOD activity that differ by the active site metal cofactors (iron [FeSOD], manganese [MnSOD], or copper and zinc [CuZnSOD]). SOD is also believed to function with NADPH oxidase to generate an H_2O_2 signal during the pathogen-induced oxidative burst (Desikan et al. 1996; Lamb and Dixon 1997). These ROS then work in conjunction with nitric oxide to induce hypersensitive response (HR) cell death in response to avirulent pathogens (Delledonne et al. 1998; Durner et al. 1998).

We previously identified seven SOD genes in Arabidopsis and showed that they are differentially regulated in response to high light, ozone, or UV-B exposure (Kliebenstein et al. 1998). Because pathogen attack can generate O_2^- and hence oxidative stress, we investigated the regulation of SOD proteins and other pathogenesis-related (PR) proteins to various pathogen or pathogen-like treatments in *Arabidopsis thaliana*. We compared protein accumulation after infection with virulent and avirulent strains of *Pseudomonas syringae* pv. *maculicola* (Psm) during spontaneous lesion formation in the *lsd1* mutant and following inoculation with salicylic acid (SA) analogs.

Polyclonal antibodies were previously generated against cytosolic CuZnSOD (CSD1), plastidic CuZnSOD (CSD2), FeSOD, MnSOD, glutathione-S transferase 1 (GST1, E.C. 2.5.1.18), PR1, and PR5 from *A. thaliana* (Kliebenstein et al. 1998; Zhao et al. 1998; M. K. Pelletier and R. L. Last, *unpublished data*). All protein extractions, immunoblots, and immunodetections were as previously described except anti-PR1 and anti-PR5 were used at respective dilutions of 1:250 and 1:500 (Zhao and Last 1995; Kliebenstein et al. 1998; Zhao et al. 1998). The specificity of anti-PR1 and anti-PR5 was confirmed by several criteria. First, anti-PR1 and anti-PR5 detected the purified bacterial fusion protein and protein from Arabidopsis tissue extracts of approximately the predicted molecular weights. Notably, induction of the detected polypeptides by pathogen infection was blocked in plants ex-

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pressing the salicylate hydroxylase transgene (Cao et al. 1994) and in *npr1* mutants (Delaney et al. 1994). This finding correlates with RNA hybridization data with the PR1 and PR5 cDNAs in these mutants (M. K. Pelletier, D. J. Kliebenstein, A. C. Martin, and R. L. Last, *data not shown*). These data suggest that the antisera are specific for the PR1 and PR5 proteins.

To characterize the regulation of SOD proteins in response to a virulent pathogen, Arabidopsis ecotype Columbia (Col-0) leaves were infiltrated with Psm ES4326 (OD_{600} = 0.01) as previously described (Zhao and Last 1996). Tissue samples for protein analysis were taken at 1-day intervals for 3 days. As shown in Figure 1, PR1, PR5, and GST1 exhibited large and rapid increases in protein accumulation that reached a maximum within 24 h of the Psm infiltration. In contrast, CSD1 protein accumulation gradually increased from day one to day three (Fig. 1). The delayed CSD1 kinetics in comparison to the well-established pathogen response markers PR1, PR5, and GST1 suggests that the gradual CSD1 induction may reflect an indirect effect of gradually increasing cell death levels or Psm population density. The CSD2, FeSOD, and MnSOD proteins did not show any significant or reproducible changes in response to infection with the virulent strain of Psm (Fig. 1).

To test whether cell death induces CSD1 protein accumulation, Arabidopsis ecotype Col-0 was infiltrated with Psm expressing the *avrRpm1* gene (OD_{600} = 0.01). This pathovar generates HR cell death on Col-0 via the action of the *RPM1* disease resistance gene (Grant et al. 1995). CSD1 protein showed a rapid induction within 1 h of infiltration with avirulent Psm and further induction by the 11-h time point (Fig. 2). The first induction of CSD1 occurred before the detection of visible HR lesions, while the second induction at 11 h corresponded to the formation of visible HR lesions (D. J. Kliebenstein and R. L. Last, *data not shown*). CSD2 protein was also induced in response to the avirulent Psm strain whereas it was not induced in response to the virulent Psm strain (Figs. 1 and 2). This suggests that CSD1 and CSD2 protein are upregulated in response to the avirulent Psm strain, but infection with the virulent Psm strain only induces CSD1. GST1 protein was also induced by the avirulent Psm strain but not until 4 h after infiltration (Fig. 2). Within the time frame of the experiment, the FeSOD, MnSOD, PR1, and PR5 proteins did not show any increased accumulation in response to the avirulent Psm strain (Fig. 2). Control infiltrations with $MgSO_4$ caused a slight induction of GST1, CSD1, and CSD2 after 11 h that was lower than in the sample of leaves infiltrated with the avirulent Psm strain (Fig. 2). The differences in basal accumulation for the various proteins in the different experiments are due to differing film exposure times. The exposure times were adjusted to maintain induced signals within the linear range of the X-ray film.

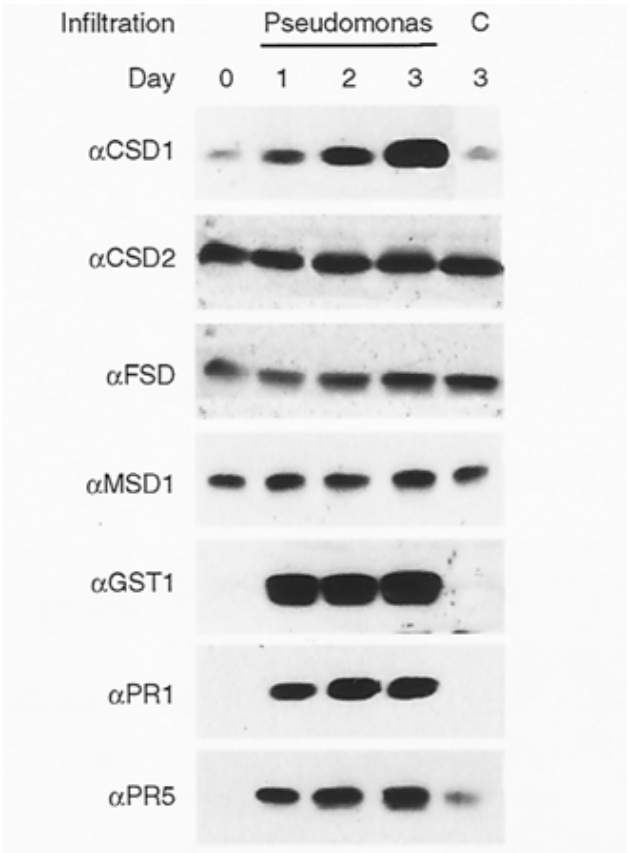

Fig. 1. Immunoblot analysis showing protein induction over time in extracts from Arabidopsis wild-type ecotype Col-0 leaves infiltrated with 0.01 OD_{600} of the virulent strain of *Pseudomonas syringae* pv. *malicula* ES4326. Antisera used are listed on left. C = $MgSO_4$ control infiltration. Each lane contains 20 µg of total protein.

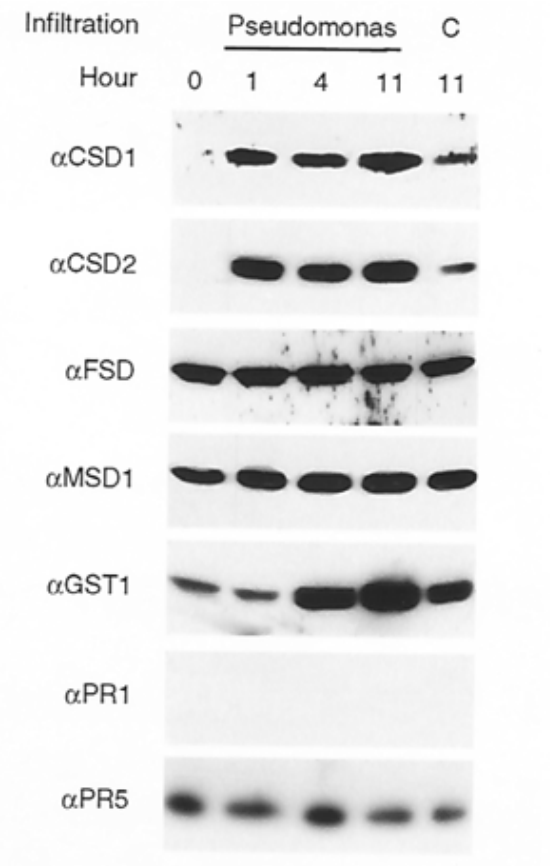

Fig. 2. Immunoblot analysis showing protein induction over time in extracts from Arabidopsis wild-type ecotype Col-0 leaves infiltrated with 0.01 OD_{600} of the avirulent strain of *Pseudomonas syringae* pv. *malicula* ES4326 *avrRPM1*. Antisera used are listed on left. C = $MgSO_4$ control infiltration. Each lane contains 20 µg of total protein.

We further studied the regulation of CuZnSOD protein accumulation in response to cell death in the spontaneous lesion mimic mutant *lsd1*. *LSD1* encodes a putative zinc finger protein responsible for preventing cell death in response to an O_2^--derived signal (Jabs et al. 1996; Dietrich et al. 1997). The current model for spreading lesion formation in *lsd1* is that once a cell death occurs, a signal produced at the border of the lesion initiates cell death in the surrounding cells. This leads to another round of O_2^- production and generates spreading lesions, which eventually envelop the entire leaf. Lesions in *lsd1* can be induced by moving the plants from low to high light environments, or by exposure to pathogens or SA or SA analogs that can induce systemic acquired resistance (SAR; Jabs et al. 1996; Dietrich et al. 1997).

Wild-type Arabidopsis, homozygous *lsd1* mutants, and *lsd1* homozygous for the *phx21* suppressor mutation, which blocks lesion formation in *lsd1* (Jabs et al. 1996), were grown under short day conditions and shifted to long days to initiate lesion formation (Dietrich et al. 1994). Leaf tissue was collected from all lines and the *lsd1* leaves were separated into pools based on the presence or absence of visible lesions. CSD1 and CSD2 protein accumulation increased in parallel with lesion formation, indicating that they may be induced in conjunction with cell death (Fig. 3). As predicted from previous mRNA analysis (Jabs et al. 1996), lesion formation in *lsd1* induced the accumulation of PR1, PR5, and GST1 proteins (Fig. 3). There was also a slight induction in the accumulation of all three proteins in the lesion-minus *lsd1* tissue, suggesting either the presence of non-macroscopic lesions in this tissue or a role for *LSD1* in repressing PR1, PR5, and GST1 accumulation in lesion-minus tissue (Fig. 3). Finally, FeSOD and MnSOD protein levels did not change in response to lesion formation (Fig. 3).

To further analyze the relationship between CSD protein accumulation and cell death, we induced lesions in *lsd1* under short day conditions by spraying with either INA (2,6-dichloroisonicotinic acid) or BTH (benzothiadiazole) (Dietrich et al. 1994). Tissue was collected for protein analysis 24 and 72 h after spraying with the SA analog or buffer control. Wild-type Ws-0 and *phx21/lsd1* double homozygotes did not form lesions during the experiment while some *lsd1* leaves displayed visible lesions at 72 h. At this time the *lsd1* tissue was separated into pools based on the presence or absence of visible lesions.

Figure 4 shows results from only the INA spraying: the BTH results were nearly identical. At 24 h after either INA or BTH treatment, only GST1 showed an increase in protein accumulation (data not shown). Within 72 h of INA treatment, both CSD1 and CSD2 protein levels increased in wild type (Fig. 4). However, CSD1 and CSD2 protein did not accumulate in the 72-h, INA-sprayed, lesion-minus *lsd1* mutant samples (Fig. 4). This suggests that *lsd1* blocks the induction of

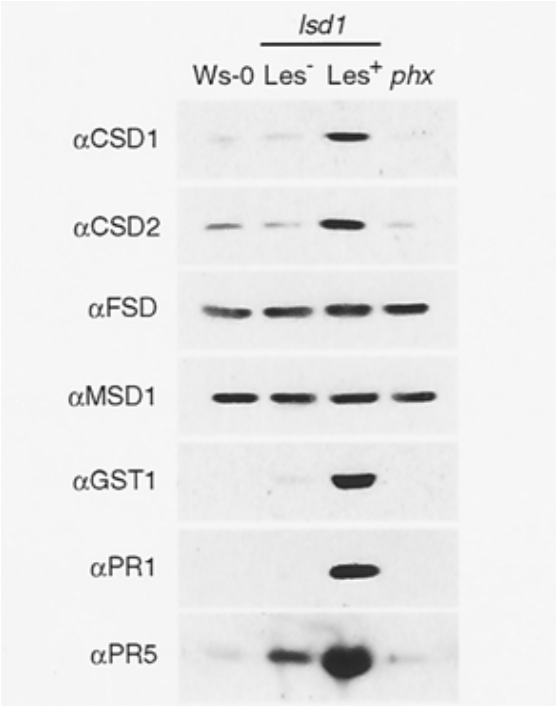

Fig. 3. Immunoblot analysis showing protein induction in wild-type Arabidopsis and *lsd1* plants. Plants were shifted from short to long days during growth to induce spontaneous lesion formation in *lsd1* plants. Ws-0 = wild-type Arabidopsis ecotype Wassilewska. Les-= lesion-minus leaves of *lsd1*. Les+ = lesion-plus leaves of *lsd1*. *phx* = lines homozygous for both *phx21* and *lsd1*. Antisera used are listed on left. Each lane contains 20 µg of total protein.

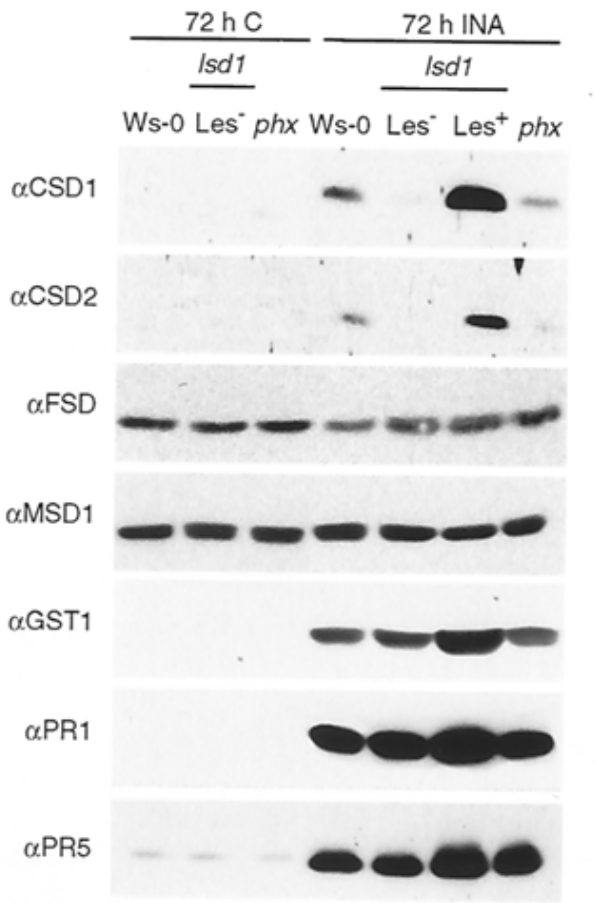

Fig. 4. Immunoblot analysis showing protein induction in wild-type Arabidopsis and *lsd1* plants treated with INA for 72 h. Ws-0 = wild-type Arabidopsis ecotype Wassilewska. Les-= lesion-minus leaves of *lsd1*. Les+ = lesion-plus leaves of *lsd1*. *phx* = lines homozygous for both *phx21* and *lsd1*. C = buffer control. Antisera used are listed on left. Each lane contains 20 µg of total protein.

CSD1 and CSD2 by INA. Hence, the LSD1 zinc finger protein is required downstream of SA (or INA) in the induction of CSD1 and CSD2 protein accumulation in response to SAR-inducing chemicals. In contrast, we observed large increases in PR1, PR5, and GST1 protein accumulation in the 72-h, INA-sprayed, wild-type and lesion-minus *lsd1* samples (Fig. 4). This indicates that the lesion-minus *lsd1* samples had perceived the SA analogs and that LSD1 is not required for the induction of PR1, PR5, or GST1 protein in response to SA.

In comparison to INA-sprayed, lesion-minus *lsd1* or wild-type leaves, INA-sprayed, lesion-plus *lsd1* leaves showed a consistent superinduction of CSD1, CSD2, GST1, PR1, and PR5 proteins (Fig. 4). This result supports the hypothesis that CSD1 and CSD2 protein levels are induced in conjunction with cell death. This result further suggests that *LSD1* is not required for the induction of CSD1 and CSD2 protein after the onset of cell death. The hyperaccumulation of these proteins in *lsd1* lesions could be due to elevated endogenous SA levels present in the *lsd1* lesions, or another, unidentified signal. As expected the FeSOD and MnSOD proteins did not show a significant response to either the SA analogs or the lesion formation.

The induction of PR1, PR5, and GST1 protein accumulation by virulent and avirulent strains of *P. syringae*, lesion formation in *lsd1*, and treatment with SA analogs is consistent with previous observations of the changes in mRNA levels for corresponding genes in Arabidopsis and other species (Jabs et al. 1996). Thus, these antisera are useful reagents to test pathogen stress in Arabidopsis.

Our results suggest that there are two pathways for CuZnSOD protein induction in response to pathogen infection. As shown for the wild type in Figure 5A, one pathway utilizes LSD1 to convey an SA analog-dependent signal that induces CuZnSOD protein accumulation. We propose that this increase in CuZnSOD protein accumulation would dismutate any accumulating O_2^- before the ROS signals a transition to cell death. In *lsd1*, SA analogs do not induce CuZnSOD, allowing O_2^- and subsequent ROS to accumulate and inappropriately initiate a cell death (Fig. 5B). Because the two CuZnSODs tested are cytosolic or plastidic and the O_2^- is believed to be extracellular, this hypothesis predicts the existence of an extracellular CuZnSOD that is co-regulated with CSD1 and CSD2 or an intracellular O_2^- pool that can initiate cell death. One potential intracellular O_2^- generator is nitric oxide synthase, which was recently identified in Arabidopsis and other plants (Delledonne et al. 1998; Durner et al. 1998). Recent studies have also identified a link between CuZnSOD deficiency and inappropriate initiation of cell death in human cell culture lines (Troy et al. 1996). Additionally, an Arabidopsis mutant with reduced CuZnSOD accumulation exhibited increased levels of cell death in response to infection with virulent or avirulent strains of *Peronospora parasitica* (D. J. Kliebenstein, A. C. Martin, P. Conklin, and R. L. Last, *unpublished results*). Alternatively, the SA analog-dependent induction of cytosolic and plastidic CuZnSOD may occur as a protectant mechanism, relevant for down-regulation of the overall local response to ROS-derived signals. Future work is required to determine if SA-analog-induced *lsd1* cell death is dependent upon the lack of CuZnSOD induction.

In addition to the *LSD1*/SA-dependent pathway for CuZnSOD induction there is another pathway that allows CuZnSOD induction in *lsd1*. This pathway occurs in lesion-plus *lsd1* leaves as they show a strong accumulation of CuZnSOD protein (Figs. 3 and 4). The presence of CuZnSOD induction in lesion-plus *lsd1* leaves suggests that cell death per se induces CuZnSOD protein accumulation by an LSD1-independent pathway possibly independent of SA analogs (Fig. 5B). The fact that the hyperinduction of CuZnSOD protein does not inhibit the spreading lesion phenotype of *lsd1* may be because the CuZnSOD is accumulating behind the progressing O_2^- front, or because the arsenal of CuZnSODs induced in lesion-plus tissue is not identical to the collection of CuZnSODs induced by the lesion-minus SA/LSD1 pathway. Further experiments are needed to address the mechanisms involved in the cell-death-mediated induction of CuZnSOD.

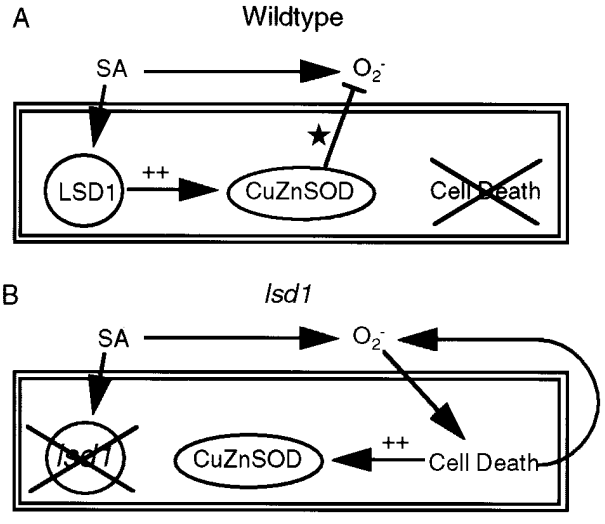

Fig. 5. LSD1/CuZnSOD/cell death progression model. SA = salicylic acid. ++ = increase in protein accumulation. ★ = theoretical role for CuZnSOD in regulating the superoxide cell death signal. **A,** Model for wild-type regulation of CuZnSOD and cell death. **B,** Model for *lsd1* mutant regulation of CuZnSOD and cell death.

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