LESION SIMULATING DISEASE1 Interacts with Catalases to Regulate Hypersensitive Cell Death in Arabidopsis[1][C][W]

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LESION SIMULATING DISEASE1 (LSD1) is an important negative regulator of programmed cell death (PCD) in Arabidopsis (*Arabidopsis thaliana*). The loss-of-function mutations in *LSD1* cause runaway cell death triggered by reactive oxygen species. *LSD1* encodes a novel zinc finger protein with unknown biochemical activities. Here, we report the identification of CATALASE3 (CAT3) as an LSD1-interacting protein by affinity purification and mass spectrometry-based proteomic analysis. The Arabidopsis genome contains three homologous catalase genes (*CAT1*, *CAT2*, and *CAT3*). Yeast two-hybrid and coimmunoprecipitation analyses demonstrated that LSD1 interacted with all three catalases both in vitro and in vivo, and the interaction required the zinc fingers of LSD1. We found that the catalase enzymatic activity was reduced in the *lsd1* mutant, indicating that the catalase enzyme activity was partially dependent on *LSD1*. Consistently, the *lsd1* mutant was more sensitive to the catalase inhibitor 3-amino-1,2,4-triazole than the wild type, suggesting that the interaction between LSD1 and catalases is involved in the regulation of the reactive oxygen species generated in the peroxisome. Genetic studies revealed that *LSD1* interacted with *CATALASE* genes to regulate light-dependent runaway cell death and hypersensitive-type cell death. Moreover, the accumulation of salicylic acid was required for PCD regulated by the interaction between LSD1 and catalases. These results suggest that the LSD1-catalase interaction plays an important role in regulating PCD in Arabidopsis.

Programmed cell death (PCD) is endogenously programmed, and its commencement and execution are strictly regulated by the physiological process (Cohen, 1993; Schwartz et al., 1993; Jacobson et al., 1997). In higher plants, PCD plays important roles in plant development, the stress response, and the defense response (Pennell and Lamb, 1997; Heath, 2000). The most studied PCD process in plants is the hypersensitive response (HR) to avirulent biotrophic pathogens (Dangl and Jones, 2001). The HR is characterized by the rapid death of cells in the local region surrounding an infection in order to restrict the growth and spread of pathogens to other parts of the plant. The HR is triggered by the plant when it recognizes a pathogen and is accompanied by the accumulations of specific signaling molecules, including ion fluxes, reactive oxygen species (ROS), salicylic acid (SA), and reactive nitrogen intermediates (Heath, 2000; Mur et al., 2008; Coll et al., 2011). The HR not only induces the local response but also systemic acquired resistance (Vlot et al., 2009).

In Arabidopsis (*Arabidopsis thaliana*), many lesion-mimic mutants have been isolated that show various defects in regulating PCD. One of the best characterized mutants is *lesion simulating disease1* (*lsd1*). The *lsd1* mutant shows abnormal cell death triggered by ROS and SA and presents a runaway cell death (RCD) phenotype under long photoperiods or after low-titer avirulent pathogen infection, indicating that LSD1 is a negative regulator of PCD (Dietrich et al., 1994; Jabs et al., 1996; Kliebenstein et al., 1999; Aviv et al., 2002). *LSD1* encodes a novel zinc finger protein with three LSD1-like zinc finger motifs (Dietrich et al., 1997). Genetic studies showed that *ENHANCED DISEASE SENSITIVITY1*, *PHYTOALEXIN DEFICIENT4*, and *NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1*, key regulators of specific pathogen resistance, are necessary for *LSD1*-regulated hypersensitive cell death (Rustérucci et al., 2001; Aviv et al., 2002). *LSD1* has also been shown to be involved in light acclimation to conditions that promote photooxidative stress, the regulation of lysigenous aerenchyma formation, and the regulation of low-temperature cell death (Mateo et al., 2004; Muhlenbock et al., 2007, 2008; Huang et al., 2010).

Despite extensive efforts in past decades, little is known about the biochemical activity of the LSD1

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protein. To explore the molecular mechanism of LSD1-regulated cell death, several LSD1-interacting proteins have been identified. The first LSD1-interacting protein was a basic region leucine zipper (bZIP) transcription factor, bZIP10, which plays a key role in response to environmental alterations, particularly in light and stress signaling (Kaminaka et al., 2006). The LSD1-bZIP10 interaction occurs in the cytoplasm, resulting in partial bZIP10 retention (Kaminaka et al., 2006). AtMC1, a type I Arabidopsis metacaspase containing a conserved LSD1-like zinc finger motif, was also found to interact with LSD1 via its zinc finger domain (Coll et al., 2010). AtMC1 is a positive regulator of cell death, and its caspase-like activity is required for both superoxide-dependent cell death and HR, mediated by an intracellular nucleotide-binding-leucine-rich repeat receptor (Coll et al., 2010). Recently, a lipopolysaccharide-induced tumor necrosis factor alpha factor domain protein, Arabidopsis GSH-induced LITAF domain protein, was identified to interact with LSD1 to negatively regulate hypersensitive cell death (He et al., 2011b). These findings provide important clues to understanding the LSD1 function in the regulation of PCD.

The *LSD1*-regulated cell death has long been associated with oxidative stress. In particular, whereas the RCD phenotype of *LSD1* is triggered by a superoxide-dependent signal (Jabs et al., 1996), *LSD1* is involved in a signaling pathway for the up-regulation of *COPPER-ZINC SUPEROXIDE DISMUTASE* to limit the spread of cell death (Kliebenstein et al., 1999). Moreover, the *lsd1* mutant showed a reduced level of peroxisomal catalase (CAT) activity and reduced stomatal conductance in short-day permissive conditions (Mateo et al., 2004). These observations led to the proposition that *LSD1* acts to monitor the intracellular level of ROS, thereby regulating distinctive types of cell death (Jabs et al., 1996; Dietrich et al., 1997; Coll et al., 2011).

Similar to other living organisms, plants have evolved complex machinery to regulate the homeostasis of the intracellular ROS level. Of the ROS-scavenging enzymes, catalase is a highly conserved enzyme catalyzing the conversion of hydrogen peroxide (H_2O_2) to water and oxygen and plays a key role in the removal of excessive amounts of H_2O_2 (Mhamdi et al., 2010). The Arabidopsis genome contains three catalase genes (*CAT1*, *CAT2*, and *CAT3*; Frugoli et al., 1996). These *CATALASE* genes exhibit different spatiotemporal expression patterns and expression levels (Zimmermann et al., 2006; Du et al., 2008; Mhamdi et al., 2010). Whereas *CAT1* is primarily expressed in the reproductive tissues and seeds, *CAT2* is strongly expressed in the photosynthetic tissue and *CAT3* is ubiquitously expressed, especially in roots and young leaves (Du et al., 2008). *CAT2* and *CAT3* are expressed at a significantly higher level than *CAT1* in the vegetative tissue. Biochemical studies reveal that CAT2 and CAT3 represent the major enzymatic activity in the vegetative tissues (Zimmermann et al., 2006; Mhamdi et al., 2010). The distinctive expression patterns of *CATALASE* genes and their activities imply a complex regulatory mechanism of three functionally redundant genes in response to internal and environmental signals. Consistent with these observations, the *cat2* mutant shows spreading necrotic lesions when grown in a long-day photoperiod (Mhamdi et al., 2010). In contrast to *cat2*, neither *cat1* nor *cat3* shows any obvious rosette phenotype under the same growth conditions. A slightly stronger lesion phenotype was observed in *cat2 cat3* and *cat1 cat2* double mutants in long days (Mhamdi et al., 2010). These observations indicate that catalase plays an important role in ROS-mediated PCD, particularly the HR.

In this study, we identified catalases as LSD1-interacting proteins. We found that LSD1 physically and genetically interacts with catalases in the light-dependent RCD and HR cell death processes in Arabidopsis, and the accumulation of SA is required for PCD regulated by LSD1 and catalases.

RESULTS

Identification of CAT3 as an LSD1-Interacting Protein

Previous studies showed that LSD1 is an important negative regulator of PCD in Arabidopsis (Dietrich et al., 1997). To further investigate the function of *LSD1*, we generated stable transgenic plants carrying an *LSD1-FLAG* transgene driven by a 1,520-bp native *LSD1* promoter in the *lsd1* null mutant background (Columbia-0 [Col-0] background; Rustérucci et al., 2001). Immunoblot analysis showed that whereas no LSD1 was detectable in the *lsd1* mutant using an anti-LSD1 antibody (Huang et al., 2010), the LSD1-FLAG fusion protein was expressed in *LSD1:LSD1-FLAG* transgenic plants (Fig. 1A; Supplemental Fig. S1, A and B). Analysis of multiple independent transgenic lines revealed that the *LSD1:LSD1-FLAG* transgenes fully rescued the *lsd1* mutant phenotype in long-day photoperiod (LD; 16/8 h of light/dark; Fig. 1A) and in continuous white-light growth conditions (Supplemental Fig. S1A). The expression level of *FLAVIN-DEPENDENT MONOOXYGENASE* (*FMO*), a marker gene for PCD, has been shown to increase in the *lsd1* mutant (Olszak et al., 2006). Analysis by reverse transcription (RT)-PCR showed that the increased expression of *FMO1* in the *lsd1* mutant was also rescued by the *LSD1:LSD1-FLAG* transgene (Fig. 1B). These data indicate that the LSD1-FLAG fusion protein was functional in planta.

To identify LSD1-interacting proteins, we performed affinity purification of the LSD1-containing protein complex followed by mass spectrometry-based proteomic analysis. Total soluble proteins prepared from 2-week-old seedlings of LSD1-FLAG or Col-0 in LD conditions were incubated with the anti-FLAG antibody-coupled agarose beads, and proteins eluted from the beads were separated by SDS-PAGE (Fig. 1C). Differential expressed bands specific to the LSD1-FLAG transgenic plants were identified and excised

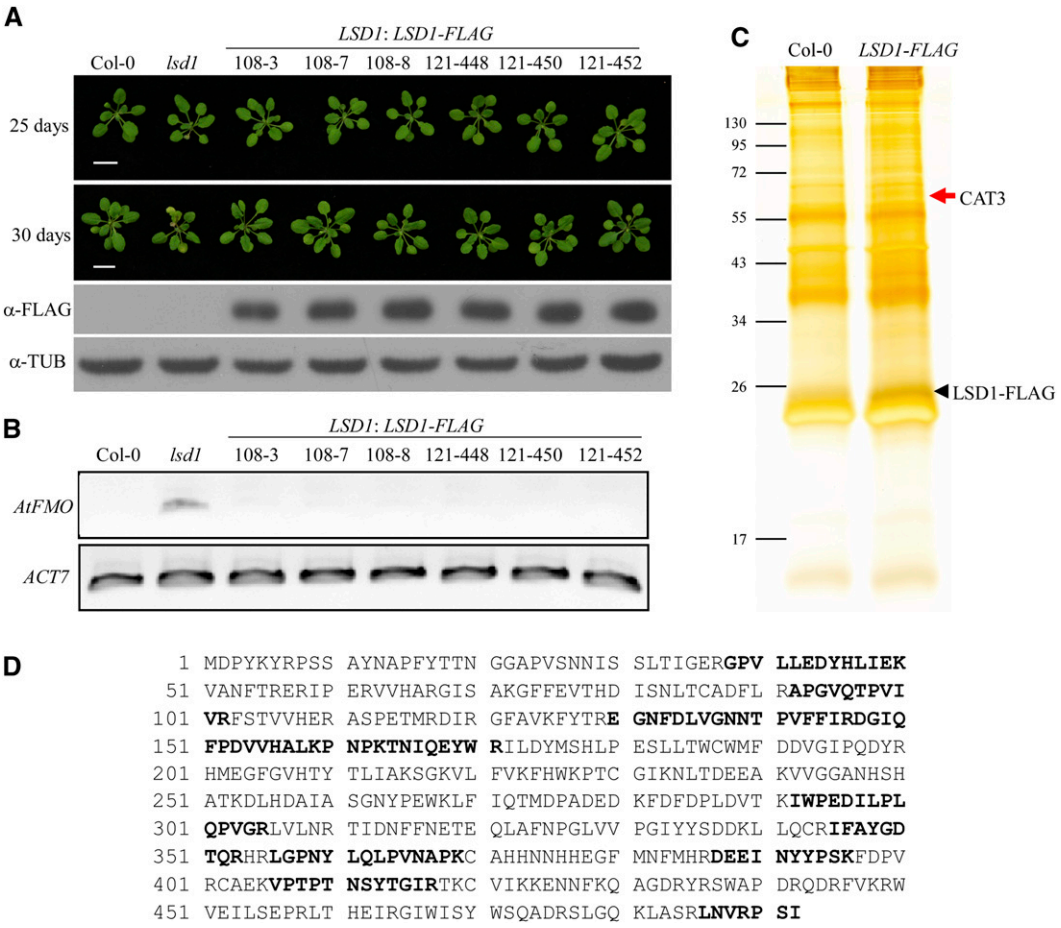

Figure 1. Identification of LSD1-interacting proteins in Arabidopsis. A, The *LSD1*:*LSD1-FLAG* transgene rescuing the phenotype of the *lsd1* mutant grown in LD conditions. Twenty-five-day-old (top panel) and 30-d-old (middle panel) Col-0, *lsd1*, and *LSD1*:*LSD1-FLAG* transgenic seedlings are shown. Bars = 1 cm. The bottom panels show immunoblot analysis of LSD1-FLAG fusion proteins in extracts prepared from 2-week-old seedlings using anti-FLAG antibody. Equal loading was verified by using anti-tubulin antibody. B, RT-PCR analysis of the expression of the *AtFMO* gene in Col-0, *lsd1* mutant, and *LSD1-FLAG* transgenic plants. RNA was prepared from about 4-week-old seedlings grown in LD (16 h of light/8 h of dark) conditions. *ACTIN7* (*At5g09810*) was used as an internal control. C, Isolation of LSD1-interacting proteins. LSD1-interacting proteins were isolated from the protein extracts prepared from 2-week-old seedlings with the indicated genotypes. The samples were separated by SDS-PAGE and stained with silver. Differential expressed bands (indicated by the red arrow) were excised and identified by mass spectrometry. D, Analysis of the protein bands isolated in C by CapLC Q-TOF mass spectrometry. The identified CAT3 peptides are shown in boldface. [See online article for color version of this figure.]

and then subjected to microcapillary liquid chromatography-quadrupole time-of-flight (CapLC Q-TOF) mass spectrometry. The experiment was repeated twice (biological repeats). In one experiment, 11 tryptic peptides of the CAT3 protein were identified, representing total protein coverage of 26% (Fig. 1D; Supplemental Fig. S2A). In a second experiment, eight tryptic peptides were obtained from CAT3, representing total protein coverage of 18% (Supplemental Fig. S2B). These results suggest that CAT3 is most likely an LSD1-interacting protein.

Previous studies have identified several LSD1-interacting proteins, including bZIP10, AtMC1, and Arabidopsis GSH-induced LITAF domain protein (Kaminaka et al., 2006; Coll et al., 2010; He et al., 2011b). None of these proteins was identified in our assay, presumably owing to the relatively low abundance of these proteins that were not recognized by silver staining under our assay conditions or other technical difficulties. Hereafter, we report our detailed analysis of the regulatory role of the LSD1-catalase interaction in hypersensitive cell death.

LSD1 Interacts with Catalases via Its Zinc Finger Domains

The affinity purification analysis identified CAT3 as an LSD1-interacting protein. To confirm this result, we investigated the protein-protein interaction by a yeast two-hybrid assay. The *CAT3* full-length

complementary DNA (cDNA) was fused to the transcription activation domain of *GAL4*, and the *LSD1* cDNA was fused to the DNA-binding domain of *GAL4*. We found that CAT3 interacts with LSD1 in yeast cells (Fig. 2A). In Arabidopsis, CAT3 has two close homologs (CAT1 and CAT2), and the amino acid sequences of the three catalases are 75% to 84% identical, considering conservative substitutions (Frugoli et al., 1996). Similar to CAT3, CAT1 and CAT2 also efficiently interact with LSD1 and CAT3 in the yeast two-hybrid assay (Fig. 2A).

LSD1 has three LSD1-like zinc finger motifs, which function to mediate protein-protein interactions (Kaminaka et al., 2006; Coll et al., 2010; He et al., 2011a; Fig. 2B). To determine whether the zinc finger structure is required for the interaction between LSD1 and catalases, we made various deletion mutants that lacked all three zinc fingers or individual zinc finger domains of LSD1 (Fig. 2B). The interactions between these mutated LSD1 and catalases were tested by the yeast two-hybrid assay. We found that mutations in any one of the three zinc finger domains completely abolished the interaction with catalases (Fig. 2C), indicating that intact zinc finger domains are essential for the interaction between LSD1 and catalases. However, the carboxyl region outside the zinc finger domains was dispensable for the LSD1-catalase interaction in yeast cells (Fig. 2C). These results indicate that LSD1 interacts with all three catalase proteins in a zinc finger-dependent manner.

LSD1 Interacts with Catalases in Planta

Because LSD1 physically interacts with catalase proteins in vitro, it is expected that LSD1 and catalase proteins may be present in the same protein complex in planta. In Arabidopsis, CAT2 and CAT3 were shown to represent major catalase activity in the vegetative tissues (Frugoli et al., 1996; Mhamdi et al., 2010; Supplemental Fig. S3). Therefore, we tested possible interactions of LSD1 with CAT2 and CAT3 in planta. To this end, we first constructed the *CAT2*:*FLAG-CAT2*

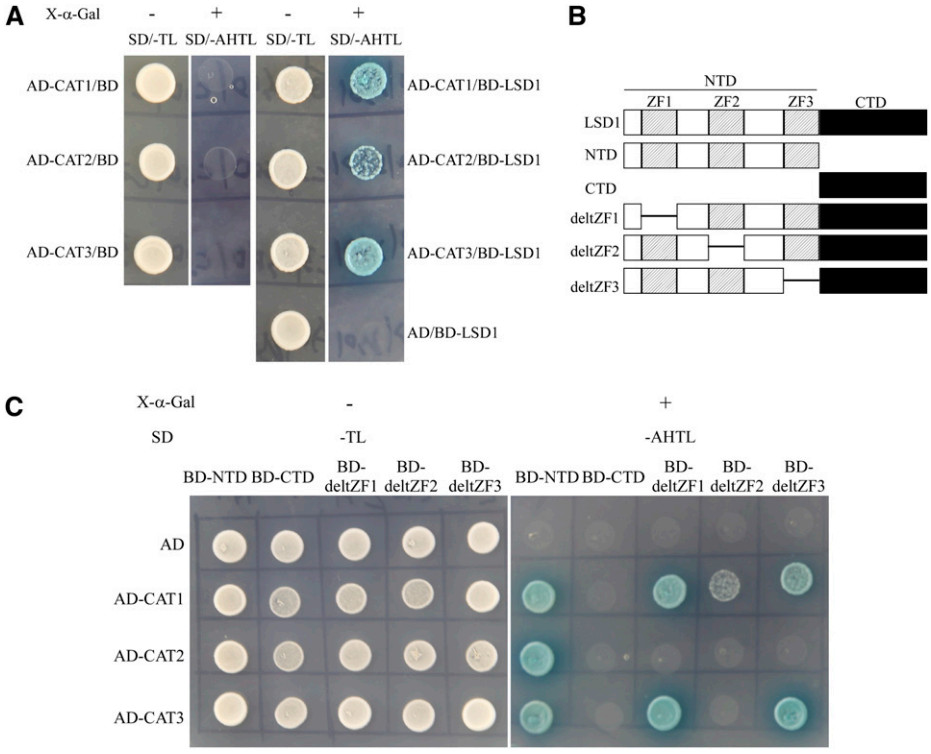

Figure 2. LSD1 interacts with catalases in yeast cells. A, Interactions between LSD1 and catalase proteins in the yeast two-hybrid assay. AD and BD represent the plasmids encoding the fusions to the GAL4 transcription activation domain and the DNA-binding domain, respectively. Cotransformed yeast colonies were spotted on selective synthetic dextrose medium minus Trp and Leu (SD-TL) and then grown on synthetic dextrose medium minus adenine, His, Trp, and Leu (SD-AHTL) supplemented with 40 μg mL^{-1} 5-Bromo-4-chloro-3-indoxyl-α-D-galactopyranoside (X-α-Gal) for β-galactosidase activity. B, Schematic diagram of LSD1 constructs. LSD1, Full-length protein (189 amino acid residues); NTD, N terminus of LSD1 contains three LSD1-like zinc fingers (residues 1–121); CTD, C terminus of LSD1 (residues 122–189); deltZF1, deletion of zinc finger 1 (residues 12–33); deltZF2, deletion of zinc finger 2 (residues 53–74); deltZF3, deletion of zinc finger 3 (residues 100–121). C, The interactions between LSD1 and catalases are dependent on the zinc fingers analyzed by the yeast two-hybrid system. For technical details, see A. [See online article for color version of this figure.]

and *CAT3*:*FLAG-CAT3* transgenes and transformed them into the *cat2-1* and *cat3-2* mutants, respectively. The *cat2-1* mutant (SALK_076998) was a null mutation and exhibited approximately 20% of the wild-type catalase enzymatic activity in leaves (Bueso et al., 2007). The *cat3-2* mutant was identified in this study, which carried a C-to-T transition at nucleotide 448 (where the first nucleotide of the CAT3 open reading frame is referred to as 1), resulting in the conversion of Gln-150 into a stop codon (Supplemental Fig. S4A). We also generated monoclonal anti-CAT2 and anti-CAT3 antibodies specifically recognizing CAT2 and CAT3 proteins, respectively (Supplemental Fig. S4B). Consistently, catalase activity was decreased in *cat2-1*, *cat3-2*, and *cat2-1 cat3-2* mutants (Supplemental Fig. S3B).

We performed a coimmunoprecipitation assay. In the extracts prepared from the *LSD1*:*LSD1-FLAG* transgenic seedlings, all three catalase proteins were efficiently precipitated by the anti-FLAG antibody-coupled agarose beads, as revealed by a polyclonal anticatalase antibody (Fig. 3A). Note that the anti-catalase antibody (Agrisera) used in this experiment was able to recognize all three CAT proteins (Supplemental Fig. S3, A and C). In a reverse experiment, LSD1 was also precipitated in protein extracts prepared from *CAT2*:*FLAG-CAT2* and *CAT3*:*FLAG-CAT3* transgenic plants by the use of the anti-FLAG antibody-coupled agarose beads (Fig. 3B). These results demonstrate that LSD1 and all three catalases were present in a protein complex in planta.

A previous study showed that LSD1 retained AtbZIP10 outside the nucleus, suggesting that LSD1 plays a role in the regulation of the subcellular localization of specific proteins (Kaminaka et al., 2006). To explore whether LSD1 also affects the subcellular localization of catalases, we analyzed the subcellular localization of CAT2 and CAT3 in wild-type (Col-0) and *lsd1* mutant plants by immunocytochemical staining with their specific antibodies. There were no differences in CAT2 or CAT3 subcellular localization between the wild type and the *lsd1* mutant (Supplemental Fig. S5, A and B). This result indicates that the interaction between LSD1 and catalase does not affect the subcellular localization of CAT2 and CAT3.

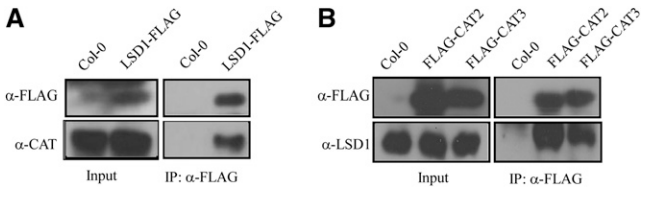

Figure 3. LSD1 physically interacts with catalases in planta. A, Co-immunoprecipitation of LSD1 and catalases. Proteins were extracted from Col-0 and *LSD1*:*LSD1-FLAG* transgenic plants and then immunoprecipitated (IP) with anti-FLAG agarose beads, followed by immunoblot detection with anti-catalase or anti-FLAG (Abmart) antibody. B, Coimmunoprecipitation of LSD1, CAT2, and CAT3. Proteins were extracted from Col-0, *CAT2*:*FLAG-CAT2*, and *CAT3*:*FLAG-CAT3* plants and then immunoprecipitated with anti-FLAG agarose beads. The sample was analyzed by immunoblotting with anti-LSD1 or anti-FLAG (Earthox) antibody.

The *lsd1* Mutant Is Hypersensitive to the Catalase Inhibitor 3-Amino-1,2,4-Triazole

In both plants and animals, 3-amino-1,2,4-triazole (3-AT) is widely used as an effective inhibitor of catalases (Middelkoop et al., 1991; Milton, 2001; Gechev et al., 2002; Shigeoka et al., 2002; Jannat et al., 2012). Exogenous application of 3-AT can significantly reduce the catalase activity, which can increase peroxisomal H_2O_2 concentrations and cause cell death in Arabidopsis (Gadjev et al., 2006). Whereas plants treated with 3-AT developed chlorosis and necrosis on leaves (Gechev and Hille, 2005), reduced catalase activity was found in *LSD1* plants in short-day permissive conditions (Mateo et al., 2004). Under our assay condition (both short and long days), the catalase activity was also decreased in the *lsd1* mutant (Supplemental Fig. S6, A and B). The reduced catalase activity of the *lsd1* mutant may lead to an altered sensitivity to 3-AT. To test this hypothesis, Col-0 and *lsd1* were germinated and grown in the absence or presence of 3-AT under the LD condition. In the absence of 3-AT, no difference was observed between wild-type and *lsd1* mutant seedlings (Fig. 4A). However, in the presence of 4 μM 3-AT, approximately 10% Col-0 and 18% *lsd1* seedlings became chlorotic (Fig. 4). When treated with a higher concentration of 3-AT (5 μM), approximately 90% *lsd1* and 80% Col-0 seedlings were chlorotic (Fig. 4). These results suggest that LSD1-regulated PCD is tightly coupled with the 3-AT-sensitive catalase activity.

LSD1 Genetically Interacts with Catalase Genes in a Light-Dependent RCD Process

To define genetic interactions between *LSD1* and catalase genes, we generated various multiple mutants, including the *lsd1 cat2-1*, *lsd1 cat3-2*, and *lsd1 cat2-1 cat3-2* mutants. When grown in LD conditions, *cat* (including *cat2-1*, *cat3-2*, and *cat2-1 cat3-2*) young seedlings showed a similar growth phenotype to the wild type (Fig. 5A). However, *lsd1*, the *lsd1 cat2-1* and *lsd1 cat3-2* double mutants, and the *lsd1 cat2-1 cat3-2* triple mutant displayed a leaf lesion-mimic phenotype (Fig. 5A). Except for the RCD phenotype, the *lsd1*, *lsd1 cat2-1*, *lsd1 cat3-2*, and *lsd1 cat2-1 cat3-2* mutants also showed small and dwarf phenotypes compared with *cat2-1*, *cat3-2*, *cat2-1 cat3-2*, and the wild type in mature plants (Supplemental Fig. S7).

The RCD of *lsd1* can be induced by LD conditions (Dietrich et al., 1994). In short-day photoperiod (SD) conditions (8 h of light/16 h of dark), no obvious

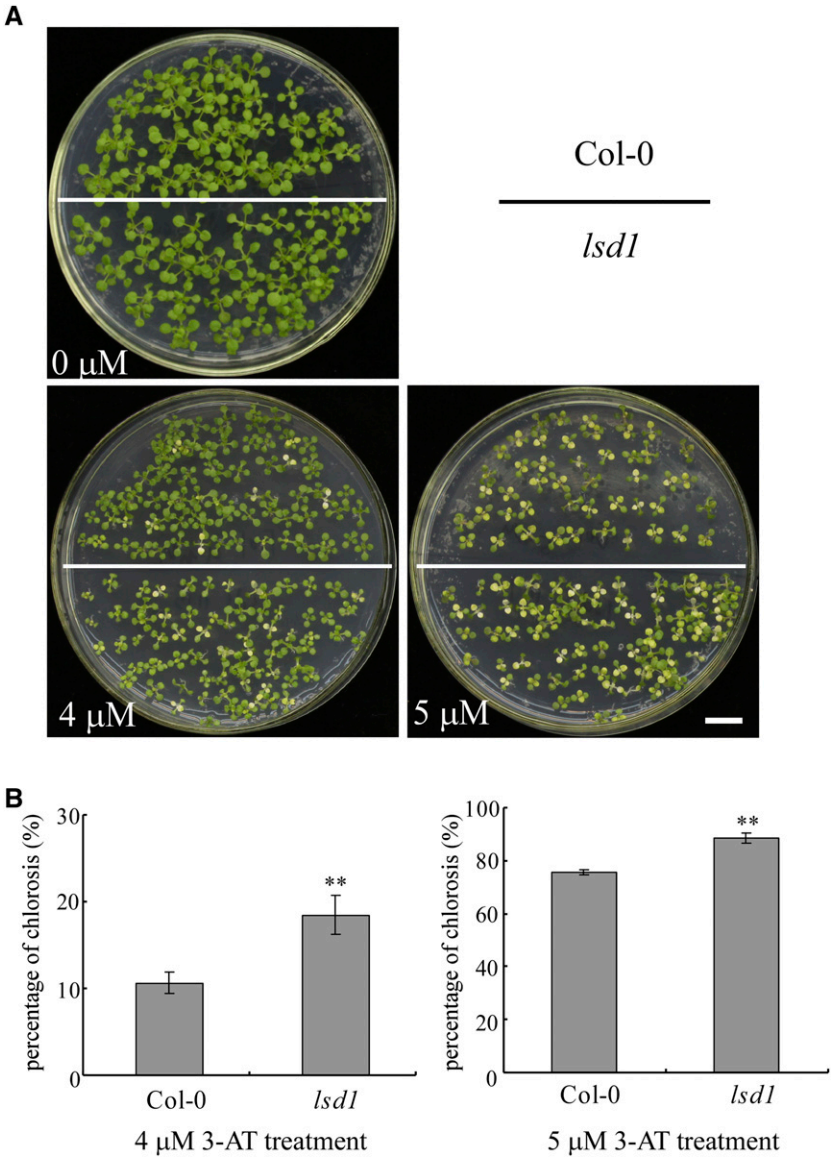

Figure 4. The *lsd1* mutant is hypersensitive to 3-AT. A, Two-week-old seedlings of Col-0 and *lsd1* germinated and grown in one-half-strength Murashige and Skoog medium containing various concentrations of 3-AT in LD conditions. Bar = 1 cm. B, Quantitative analysis of the number of plants showing chlorosis with the indicated genotypes. Means of three replicates ± SD are shown. Asterisks indicate significant differences (**$P < 0.01$, Student's *t* test). [See online article for color version of this figure.]

lesions were observed in all tested mutants (Supplemental Fig. S8). When grown in LD conditions, approximately 3.38% of leaf area showed spreading necrotic lesions in *lsd1* mutants. However, about 22.38%, 21.12%, and 28.02% of leaf area displayed lesions in *lsd1 cat2-1*, *lsd1 cat3-2*, and *lsd1 cat2-1 cat3-2*, respectively (Fig. 5B). Compared with *lsd1*, the lesions emerged earlier and were more severe in *lsd1 cat2-1*, *lsd1 cat3-2*, and *lsd1 cat2-1 cat3-2* (Fig. 5A). These results indicate that *lsd1* and *cat* were able to enhance lesion penetrance and the severity of the mutant phenotype in the light-dependent RCD process.

We also generated stable transgenic lines *35S:FLAG-CAT2* and *35S:FLAG-CAT3* in the *lsd1* mutant background to explore the function of the overexpression of catalase genes in *lsd1*. However, despite the substantially increased expression level and increased catalase enzyme activity (Supplemental Fig. S9, A and B), all these transgenic plants showed a similar phenotype to the *lsd1* mutant in LD conditions (Supplemental Fig. S9C), indicating that the overexpression of catalase genes does not reduce the RCD phenotype of *lsd1* in LD conditions.

LSD1 Genetically Interacts with the Catalase Genes in Cell Death Induced by Pathogen Infection

It has been shown that the induction of spreading cell death in *lsd1* was triggered by inoculation with a low-concentration bacterial suspension of avirulent *Pseudomonas syringae* pv. *tomato* (*Pst*), which had no visible effect on the wild type (Dietrich et al., 1994). To explore the function of catalases in this process, approximately 7-week-old Col-0, *cat2-1*, *cat3-2*, *cat2-1 cat3-2*, *lsd1*, *lsd1 cat2-1*, *lsd1 cat3-2*, and *lsd1 cat2-1 cat3-2*

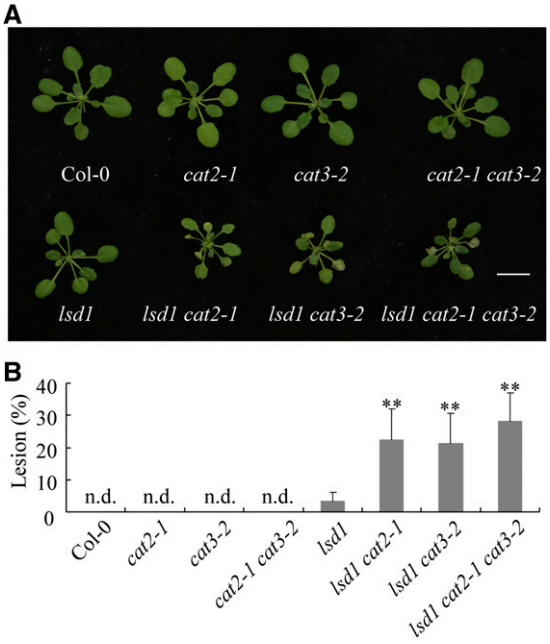

Figure 5. LSD1 genetically interacts with *CAT2* and *CAT3*. A, Twenty-five-day-old plants grown in LD conditions. Bar = 1 cm. B, Quantitative analysis of lesion areas in leaves with the indicated genotypes (percentage of rosette leaf areas). n.d., Not detected. Asterisks indicate significant differences (**$P < 0.01$, Student's t test). [See online article for color version of this figure.]

grown in SD conditions were infected with the avirulent bacterium *Pst* DC3000 (*avrRpm1*) at a relatively low concentration (5×10^6 colony-forming units [CFU] mL^{-1}). Ion leakage of these samples was measured over time, a readout for measuring plant cell death (Mackey et al., 2003). Compared with *lsd1*, *cat* mutants (including *cat2-1*, *cat3-2*, and *cat2-1 cat3-2*) and Col-0 showed only slight increases in conductivity (Fig. 6). However, there was increased conductivity for *lsd1 cat2-1* and *lsd1 cat3-2* double mutants and the *lsd1 cat2-1 cat3-2* triple mutant (Fig. 6). These results indicate that the reduction of catalase activity enhanced pathogen-induced cell death in the *lsd1* mutant.

Accumulation of SA Is Required for PCD Regulated by LSD1 and Catalase

Genetic and pharmacological experiments showed that SA plays an important role in HR cell death. RCD in *lsd1* has been shown to be SA dependent. SA not only triggers but also mediates the RCD phenotype in *lsd1* (Aviv et al., 2002). As a key plant defense hormone, SA can also modulate catalase activity (Durner and Klessig, 1996; Vlot et al., 2009). Lesion formation in *cat2* was accompanied by the accumulation of high levels of SA in LD conditions, and exogenous SA induced the cell death of *cat2* in SD conditions (Chaouch et al., 2010). The *SA induction-deficient2-2* (*sid2-2*) mutant carries a mutation to *ICS1*, which encodes an isochorismate synthase of the SA biogenesis pathway (Wildermuth et al., 2001). The SA level after pathogen infection in *sid2* mutants is only 5% to 10% of the wild-type level (Abreu and Munné-Bosch, 2009).

To address whether SA is required for the interactions between LSD1 and catalases during light-dependent RCD and HR cell death, we introduced a *sid2-2* mutation into *lsd1*, *cat* (*cat2-1*, *cat3-2*, and *cat2-1 cat3-2*), and *lsd1 cat* (*lsd1 cat2-1*, *lsd1 cat3-2*, and *lsd1 cat2-1 cat3-2*) backgrounds. As expected, the double mutant *lsd1 sid2-2* did not undergo the RCD, whereas an apparent RCD phenotype was observed in *lsd1* grown in LD conditions (Fig. 7A; Supplemental Fig. S7). The enhanced RCD in *lsd1 cat2-1*, *lsd1 cat3-2*, and *lsd1 cat2-1 cat3-2* was also restricted in *sid2-2 lsd1 cat2-1*, *sid2-2 lsd1 cat3-2*, and *sid2-2 lsd1 cat2-1 cat3-2* grown in LD conditions (Fig. 7A; Supplemental Fig. S7). This result indicates that the accumulation of SA was required for RCD regulated by LSD1 and catalases.

We next investigated HR cell death in these multiple mutants. Wild-type and various mutant plants were injected with the avirulent bacterium *Pst* DC3000 (*avrRpm1*) at a relatively low concentration (5×10^6 CFU mL^{-1}), and then ion leakage of the samples was measured. There was an obvious increase in ion leakage in *lsd1* (Fig. 7B), consistent with previous observations (Torres et al., 2005). In contrast, ion leakage measurements after pathogen inoculation showed that almost no cell death occurred in Col-0 and *sid2-2* (Fig. 7B).

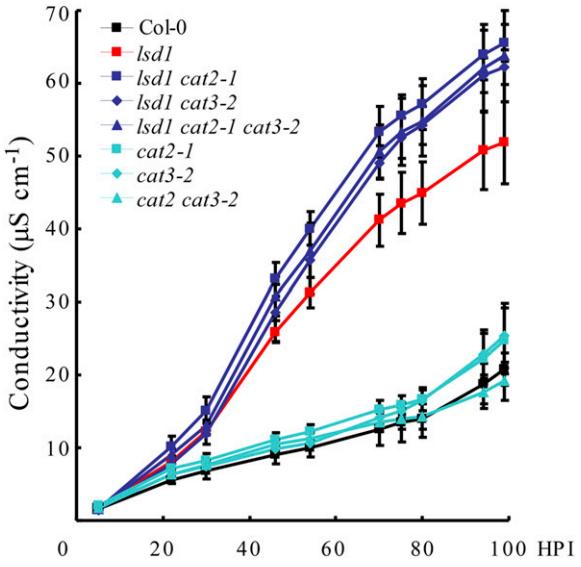

Figure 6. Quantification of cell death by ion leakage in *Pst* DC3000 (*avrRpm1*)-infected plants. Seven-week-old plants in SD conditions were treated with 5×10^6 CFU mL^{-1} *Pst* DC3000 (*avrRpm1*). Ion leakage measurements of leaf discs were started 5 h post inoculation (HPI). Conductivity (micro Siemens [μS] cm^{-1}) was detected at the time points indicated. Means of three replicates ± SD are shown. [See online article for color version of this figure.]

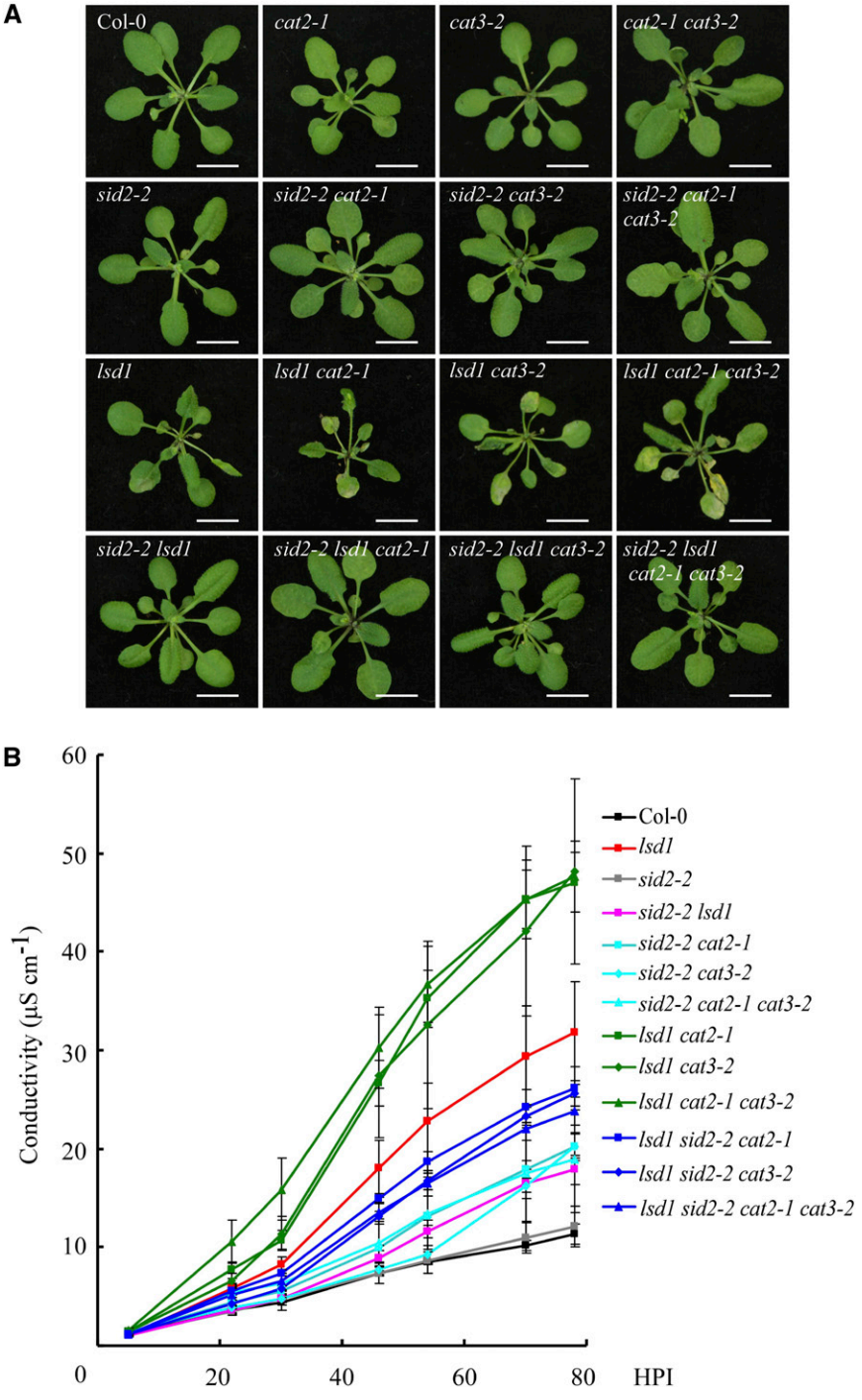

Figure 7. Accumulation of SA is required for cell death regulated by LSD1 and catalases. A, Twenty-eight-day-old plants grown in LD conditions. Bars = 1 cm. B, Quantification of cell death by ion leakage measurements after the infection with *Pst* DC3000 (*avrRpm1*) in SD conditions. Approximately 6-week-old plants in SD conditions were treated with 5×10^6 CFU mL^{-1} *Pst* DC3000 (*avrRpm1*). Conductivity (micro Siemens [μS] cm^{-1}) was detected at the time points indicated. Means of three replicates ± SD are shown. HPI, Hours post inoculation. [See online article for color version of this figure.]

The *lsd1 sid2-2* double mutant showed delayed and reduced ion leakage compared with *lsd1* (Fig. 7B). Notably, the enhanced ion leakage in *lsd1 cat2-1*, *lsd1 cat3-2*, and *lsd1 cat2-1 cat3-2* plants was partially suppressed by the *sid2-2* mutation (Fig. 7B). Compared with the *sid2-2* single mutant, the *sid2-2 cat2-1*, *sid2-2 cat3-2*, and *sid2-2 cat2-1 cat3-2* plants also exhibited increased ion leakage (Fig. 7B). Taken together, these results indicate that SA accumulation was required for HR cell death regulated by LSD1 and catalases.

DISCUSSION

The Arabidopsis gene *LSD1* encodes a negative regulator of PCD, and this gene does not exist in bacteria, yeast, and animals (Dietrich et al., 1997; Epple et al., 2003). Catalase is a vital enzyme to metabolize H_2O_2, which is an important signaling molecule regulating plant growth and stress responses (Zamocky et al., 2008). In this study, we reveal that LSD1 physically and genetically interacted with catalases in the light-dependent RCD and HR cell death processes in

Arabidopsis. We also present genetic evidence demonstrating that SA accumulation was required for the process regulated by LSD1 and catalases.

Although LSD1 has long been identified as a key regulator of PCD, the precise mechanism of how it regulates this process remains unknown. Our data together with previous reports indicate that the protein interactions mediated by LSD1 play important roles in the regulation of PCD. LSD1 contains three LSD1-like zinc fingers required for interaction with other proteins (Kaminaka et al., 2006; Coll et al., 2010; He et al., 2011a). In this study, the LSD1-like zinc finger was also found to be essential for the interaction between LSD1 and catalases, further demonstrating the importance of the LSD-like zinc finger in its interaction with other proteins. It should be noted that all LSD1-interacting proteins display distinctive biochemical activities and are localized in different cellular organelles. Catalase is a type of peroxisomal enzyme catalyzing the degradation of H_2O_2 (Mullen et al., 1997; Chaouch et al., 2010), and bZIP10 is a transcription factor shuttling between the cytoplasm and nucleus (Kaminaka et al., 2006). Another LSD1-interacting protein, GILP, is localized in the plasma membrane with unknown biochemical activity (He et al., 2011b). Thus, LSD1 may act as a housekeeping gene to interact with proteins with diverse functions and subcellular localization patterns to regulate PCD-associated signaling pathways. In this regard, we speculate that LSD1 may function as a molecular chaperone to monitor or sense the damaged activity, structure, or mislocalization of a target protein induced by the cellular ROS level and/or the cellular redox status. This notion is also supported by genetic evidence presented in previous studies (Jabs et al., 1996; Dietrich et al., 1997; Coll et al., 2011) and this study.

LSD1 regulates oxidative stress-induced cell death, and catalase is the crucial enzyme for eliminating oxidative stress (Jabs et al., 1996; Mhamdi et al., 2010). In Arabidopsis, the knockout of *CAT2*, which contributes to the majority of catalase activity in leaves, leads to the appearance of lesions in a daylength- and photorespiratory-dependent manner (Queval et al., 2007; Chaouch et al., 2010). At the same time, the accumulation of SA also participated in the resistance to biotic stress in *cat2* (Queval et al., 2007). A previous study indicated that *lsd1* showed a similar phenotype to catalase-deficient plants (Mateo et al., 2004). The reduced catalase activity in *lsd1* observed in this study is consistent with a previous report (Mateo et al., 2004). Our biochemical and genetic data indicate that the interaction between LSD1 and catalase may affect catalase activity by an unknown mechanism, especially under stress conditions.

The block of SA accumulation rescues the RCD phenotype in *lsd1* and the enhanced RCD in *lsd1 cat2-1*, *lsd1 cat3-2*, and *lsd1 cat2-1 cat3-2* in LD conditions. These results support the perspective that SA is required for the optimal photosynthetic performance of plants as well as being involved in the initiation of cell death (Mur et al., 1997; Mateo et al., 2006). Additionally, *lsd1* is hypersensitive to 3-AT, the inhibitor of catalase, suggesting that the cell death in *lsd1* was related to peroxisomal H_2O_2, which was mainly controlled by catalase. Previous studies showed that paraquat, which efficiently induces cell death in wild-type plants by inducing ROS generation in PSI of chloroplasts (Babbs et al., 1989), was incapable of triggering RCD in *lsd1* (Jabs et al., 1996). Instead, the initiation and propagation of RCD in *lsd1* has been proposed to be related to PSII (Mateo et al., 2004). Consistently, our data suggest that the cell death regulated by LSD1 and catalases is also associated with H_2O_2 generated in peroxisomes and SA produced in chloroplasts. In summary, the results presented in this study indicate that *LSD1* genetically interacts with catalase genes, and their encoded proteins are in a same protein complex, thereby playing an important role in regulating PCD.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The Col-0 accession of wild-type Arabidopsis (*Arabidopsis thaliana*) was used in this study. Seeds were surface sterilized with 10% bleach for 10 min and washed three times with sterile water. Sterilized seeds were then plated on Murashige and Skoog medium (one-half-strength Murashige and Skoog salts, 1% Suc, and 0.8% agar). Plants were grown under 16-h-light/8-h-dark (LD) or 8-h-light/16-h-dark (SD) conditions or under continuous white light (light intensity of 60 μmol m^{-2} s^{-1}) at 22°C.

Seeds of *lsd1* (Col-0 background; Rustérucci et al., 2001) were kindly provided by Dr. Jeff Dangl. Seeds of *cat2-1* (SALK_076998; Col-0 background; Bueso et al., 2007) and *sid2-2* (CS16438; Col-0 background; Wildermuth et al., 2001) were obtained from the Arabidopsis Biological Resource Center. The *cat3-2* (Col-0 background) mutant was identified in this study (Supplemental Fig. S4).

Plasmid Construction and Generation of Transgenic Plants

To make *LSD1:LSD1-FLAG*, a DNA fragment of LSD1 was amplified by PCR (primer pair LSD1FP and LSD1BG), which included a 1,520-bp promoter and a 1,919-bp coding region. The fragment was ligated to pBluescript SK– (Stratagene), which fused with a single copy of the FLAG tag at the C terminus. Then, the *Xho*I/*Xba*I fragment containing the C-terminal FLAG-tagged *LSD1* genomic DNA driven by the endogenous promoter was cloned into the *Xho*I/*Spe*I-digested binary hygromycin-selected vector pER8 (Zuo et al., 2000).

To generate *CAT2:FLAG-CAT2*, a *CAT2* endogenous 2,002-bp promoter was PCR amplified (primer pair CAT2PF and CAT2PB) and in-frame fused to the FLAG tag to yield SK-*CAT2*pro:FLAG. *CAT2* genomic DNA was amplified by PCR (primer pair CAT2GF and CAT2GB); this PCR fragment was ligated to SK-*CAT2*pro:FLAG using *Pst*I/*Spe*I sites, and then *CAT2:FLAG-CAT2* was cloned to the *Xho*I/*Spe*I sites of pER8 (Zuo et al., 2000). The *CAT3:FLAG-CAT3* construct was generated in a similar way. The primer pairs of *CAT3* promoter and genomic DNA were CAT3PF/CAT3PB and CAT3GF/CAT3GB, respectively.

To construct *35S:FLAG-CAT2*, *FLAG-CAT2* DNA fragments were amplified from *CAT2:FLAG-CAT2* plasmid (primer pair *Kpn*IFLAG and CAT2GB) and then ligated into pMW101 at the *Kpn*I/*Spe*I sites. *35S:FLAG-CAT3* was made by a similar approach with the primers *Kpn*IFLAG and CAT3GB.

These constructs were transformed into *Agrobacterium tumefaciens* strain GV3101, which was then used for the transformation of Arabidopsis by the floral dip method (Clough and Bent, 1998).

All primer sequences used in the plasmid constructs are presented in Supplemental Table S1.

RT-PCR and Quantitative RT-PCR

Total RNA was prepared by the RNAprep pure Plant RNA Purification Kit (Tiangen Biotech) according to the manufacturer's instructions. RT-PCR and quantitative RT-PCR analyses were carried out as described by Mu et al. (2008). All primer pairs used in PCR analyses are listed in Supplemental Table S1.

Affinity Purification and Mass Spectrometry

Two-week-old Arabidopsis seedlings were ground in liquid nitrogen and extracted in 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 100 mM NaCl, 10% glycerol, 0.5% IGEPAL CA-630 (Sigma-Aldrich), and 100× proteinase inhibitor (Sigma-Aldrich). Extracts were centrifuged at 13,000 rpm at 4°C twice to collect soluble fraction. Approximately 400 mg of protein was incubated with protein A agarose beads (Sigma-Aldrich) for 30 min using a rotator, and then the samples were centrifuged at 1,200 rpm at 4°C. The supernatant was incubated with high-affinity anti-FLAG agarose beads (Sigma-Aldrich) for 4 h at 4°C and washed several times. Bound proteins were eluted by boiling the beads in 6× SDS sample buffer and fractionated on SDS-PAGE gels. Differentially expressed bands were excised, and the trypsin-digested samples were subsequently identified by CapLC Q-TOF tandem mass spectrometry.

Coimmunoprecipitated proteins were analyzed by immunoblot with anti-FLAG (Abmart), anti-CAT (Agrisera), or anti-LSD1 (Huang et al., 2010) antibody.

Immunoblotting was performed as described previously (Ren et al., 2009).

Yeast Two-Hybrid Assay

To make the yeast two-hybrid assay constructs, the full-length cDNA fragments of *CAT1*, *CAT2*, and *CAT3* were PCR amplified with primers CAT1F1 and CAT1B1, CAT2F1 and CAT2B1, and CAT3F1 and CAT3B1, respectively. The PCR products were ligated to pBluescript SK– (Stratagene) and subcloned to the *Eco*RI/*Xho*I sites of pGADT7 (Clontech). The *LSD1* full-length coding sequence was amplified by PCR (primer pair LSD1F1 and LSD1B1). The PCR product was ligated to pBluescript SK– (Stratagene) and subcloned to the *Eco*RI/*Bam*HI sites of pGBKT7 (Clontech). *LSD1-NTD* and *LSD1-CTD* were cloned with the same procedure and different primers. The *LSD1-NTD* primers were LSD1F1 and LSD1NTDB, and the *LSD1-CTD* primers were LSD1CTDF and LSD1B1. The *LSD1-ZF1* fragment was amplified from *SK-LSD1* with primers LSD1ZF1F and LSD1ZF1B, and then the ZF1 truncated version of *LSD1-ZF1* was subcloned to the *Eco*RI/*Bam*HI sites of pGBKT7. *LSD1-ZF2* and *LSD1-ZF3* were cloned in the same way but with different primers.

Yeast two-hybrid assay was performed using Yeastmaker Yeast Transformation System 2 according to the user manual.

Lesion Detection, Cell Death Induction, and Cell Death Quantification

Percentage lesion areas under LD conditions were calculated using IQmaterials software (Chaouch et al., 2010). Plants were grown in SD conditions and used for the following experiment. The avirulent bacterium *Pseudomonas syringae* pv. *tomato* DC3000 (*avrRpm1*) was injected at the concentration of 5×10^6 CFU mL^{-1} in 10 mM $MgCl_2$ into fully expanded leaves. After 3 h, 5.0-mm leaf discs were collected and washed with distilled water for 1 h. Twelve leaf discs were placed in a tube with 15 mL of distilled water, and conductivity was measured over time with Orion 3-Star Plus Conductivity Meters (Thermo Scientific) as described (Torres et al., 2005).

Enzyme Analysis

Analysis of the catalase zymogram was carried out as described (Zimmermann et al., 2006). Catalase activity was measured spectrophotometrically by the decrease of A_{240} (Aebi, 1984; Weydert and Cullen, 2010).

Whole-Mount Immunofluorescence Labeling

The anti-CAT2 and anti-CAT3 monoclonal antibodies were produced by Abmart with specific peptides. Immunofluorescence labeling for CAT2 in the roots from 6-d-old seedlings was performed according to the method of Boudonck et al. (1998) with minor modifications. Briefly, the roots were placed on glutaraldehyde-activated γ-aminopropyltriethoxysilane-coated slides. Then, the samples were fixed for 1 h in 4% (w/v) formaldehyde (freshly prepared by dissolving solid paraformaldehyde in 1× MTSB [50 mM PIPES, 5 mM EGTA, 5 mM $MgSO_4$, and 89 mM KOH, pH 6.9]/2% Triton X-100). The fixed samples were washed for 5 min in distilled water, and then the samples were rinsed in 20%, 40%, 60%, 80%, and 100% methanol for 20 min individually, followed by rinsing in 100%, 80%, 60%, 40%, and 20% methanol for 20 min individually. After washing in distilled water for 5 min, the samples were digested by 1% (w/v) driselase/0.5% cellulase/0.075% macerozyme in distilled water (pH 5.2) for 20 min. The roots were washed three times in 1× MTSB. Then, 3% IGEPAL CA-630 plus 10% dimethyl sulfoxide was pipetted onto the slides and incubated for 30 min at room temperature. After washing four times with 1× MTSB/0.01% Triton X-100, the samples were blocked in 3% bovine serum albumin for 2 h. The slides were incubated overnight at 4°C with a 1:500 dilution of anti-CAT2 antibody. Then, the slides were rinsed three times in 1× MTSB/0.01% Triton X-100, and the Alexa Fluor 488-conjugated secondary antibody (Invitrogen) was pipetted onto the slides for incubation of 2 h. After washing the slide three times in 1× MTSB/0.01% Triton X-100 and once in distilled water, the fluorescence signal was visualized using a confocal laser scanning microscope (Olympus Fluo View; FV1000).

Immunofluorescence labeling for CAT3 in hypocotyls from 5-d-old seedlings grown in darkness was performed according to the method of Sauer et al. (2006) for hypocotyls with primary antibody anti-CAT3 (1:500 dilution) and Alexa Fluor 488-conjugated secondary antibody (Invitrogen). The fluorescence signal was visualized using a confocal laser scanning microscope (Olympus Fluo View; FV1000).

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: LSD1 (At4g20380), CAT1 (At1g20630), CAT2 (At4g35090), CAT3 (At1g20620), SID2 (At1g74710), FMO (AT1g19250), and ACT7 (At5g09810).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Characterization of *LSD1*:*LSD1-FLAG* transgenic plants and anti-LSD1 antibody.

Supplemental Figure S2. Mass spectrometric analysis of LSD1-interacting peptides.

Supplemental Figure S3. Analysis of catalase proteins and their enzymatic activities.

Supplemental Figure S4. Characterization of the *cat2-1* and *cat3-2* mutants.

Supplemental Figure S5. Subcellular localization of catalases.

Supplemental Figure S6. Reduced catalase activity in *lsd1*.

Supplemental Figure S7. Accumulation of SA is required for RCD regulated by LSD1 and catalase.

Supplemental Figure S8. The *lsd1* and *catalase* multiple mutant phenotype.

Supplemental Figure S9. Overexpression of *CAT2* or *CAT3* incapable of rescuing the *lsd1* mutant phenotype.

Supplemental Table S1. Primers used in this study.

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