Running Head: LSD1 interacts with catalases to regulate cell death

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LESION SIMULATING DISEASE1 Interacts with Catalases to Regulate Hypersensitive Cell Death in Arabidopsis

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ABSTRACT

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 (ROS). 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 co-immunoprecipitation 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 CAT 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 wild type, suggesting that the interaction between LSD1 and catalases is involved in the regulation of the ROS generated in the peroxisome. Genetic studies revealed that LSD1 interacted with CAT 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.

Key words: Catalase, LSD1, peroxisome, programmed cell death, salicylic acid
Programmed cell death (PCD) is endogenously programmed, whose commence 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 accumulations of specific signaling molecules, including ion fluxes, reactive oxygen species (ROS), salicylic acid (SA) and reactive nitrogen intermediates (RNI) (Heath, 2000; Mur et al., 2008; Coll et al., 2011). The HR not only induces the local response but also systemic acquired resistance (SAR) (Vlot et al., 2009).

In Arabidopsis, 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-photoperiod 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 (EDS1), PHYTOALEXIN DEFICIENT4 (PAD4) and NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1), key regulators of specific pathogen resistance, are necessary for LSD1-regulated hypersensitive cell death (Rusterucci et al.,
LSD1 has also been shown to be involved in light acclimation to conditions that promote photooxidative stress, regulation of lysigenous aerenchyma formation and regulation of low temperature cell death (Mateo et al., 2004; Muehlenbock et al., 2007; Muhlenbock et al., 2008; Huang et al., 2010).

Despite extensive efforts in the past decades, little is known about the biochemical activity of LSD1 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 intracellular NB-LRR receptor (Coll et al., 2010). Recently, a LITAF domain protein AtGILP was identified to interact with LSD1 to negatively regulate hypersensitive cell death (He et al., 2011). These findings provide important clues to the understanding the LSD1 function in the regulation of PCD.

The LSD1-regulated cell death has long been associated with the 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 up-regulation of COPPER-ZINC SUPEROXIDE DISMUTASE (CuZnSOD) to limit the spread of cell death (Kliebenstein et al., 1999). Moreover, the lsd1 mutant showed 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, CAT is a highly conserved enzyme catalyzing the conversion of hydrogen peroxide to water and oxygen, and plays a key role in the removal the excessive amount of hydrogen peroxide (Mhamdi et al., 2010). The Arabidopsis genome contains three catalase genes (CAT1, CAT2 and CAT3) (Frugoli et al., 1996). These CAT 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 CAT and their activities imply a complex regulatory mechanism of three functional 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 CATALASE3 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 1520-bp native LSD1 promoter in the lsd1 null mutant background (Columbia-0 [Col-0] background) (Rusterucci et al., 2001). Immunoblotting analysis showed that whereas no LSD1 was detectable in the lsd1 mutant using an anti-LSD1 antibody (Huang et al., 2010), LSD1-FLAG fusion protein was expressed in the LSD1:LSD1-FLAG transgenic plants (Fig. 1A and Supplemental Fig. S1A and S1B). 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 PCR (RT-PCR) showed that the increased expression of FMO1 in 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 LSD1-containing protein complex followed by mass spectrometry-based proteomic analysis. Total soluble proteins prepared from two-week-old seedlings of LSD1-FLAG or Col-0 in LD 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, 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 CAT3 protein were identified, representing total protein coverage of 26% (Fig. 1D and Supplemental Fig. S2A). In a second experiment, 8 tryptic peptides were obtained from CAT3 protein, 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 AtGILP (Kaminaka et al., 2006; Coll et al., 2010; He et al., 2011). 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 condition or other technical difficulties. Hereafter, we report our detailed analysis on the regulatory role of the LSD1-CAT interactions in the 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 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% - 84% identical, considering conservative substitutions (Frugoli et al., 1996). Similar to that of 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., 2011) (see 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 the 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-CAT interaction in yeast cells (Fig. 2C). These results indicate that LSD1 interacts with all three CAT proteins in a zinc finger-dependent manner.

**LSD1 Interacts with Catalases In Planta**

Because LSD1 physically interacts with catalases protein in vitro, it is expected that LSD1 and catalase proteins may present in a 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) (see also Supplemental Fig. S3). We therefore tested possible interaction of LSD1 with CAT2 and CAT3 in planta. To this end, we first constructed the CAT2:FLAG-CAT2 and CAT3:FLAG-CAT3 transgenes, and transformed 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 (the first nucleotide of the CAT3 open reading frame is referred to as 1), resulting in the conversion of Gln150 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 (see Supplemental Fig. S3B).

We performed a co-immunoprecipitation (Co-IP) assay. In the extracts prepared from the LSD1:LSD1-FLAG transgenic seedlings, all three CAT proteins were efficiently
precipitated by the anti-FLAG antibody-coupled agarose beads, as revealed by a polyclonal anti-CAT antibody (Fig. 3A). Note that, the anti-CAT antibody (Agrisera, Cat# AS09501) used in this experiment was able to recognize all three CAT proteins (Supplemental Fig. S3A and S3C). 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 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 wild type and the lsd1 mutant (Supplemental Fig. S5A and S5B). This result indicates that the interaction between LSD1 and catalase does not affect the subcellular localization of CAT2 and CAT3.

The lsd1 Mutant Is Hypersensitivity to the Catalase Inhibitor 3-AT

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 H₂O₂ 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. S6A and S6B). 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 long-day condition. In the absence of 3-AT, no difference was observed between wild type and the 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. 4A and 4B). When treated with a higher concentration of 3-AT (5 μM), approximately 90% lsd1 and 80% Col-0 were chlorotic (Fig. 4A and 4B). The results suggest that LSD1-regulated PCD is tightly coupled with the 3-AT-sensitive catalase activity.

**LSD1 Genetically Interacts with CAT Genes in a Light-dependent RCD Process**

To define genetic interactions between LSD1 and CAT, 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 wild type (Fig. 5A). However, the lsd1, lsd1 cat2-1 and lsd1 cat3-2 double mutants, and lsd1 cat2-1 cat3-2 triple mutants, 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 to cat2-1, cat3-2, cat2-1 cat3-2 and wild type in mature plants (Supplemental Fig. S7).

The RCD of lsd1 can be induced by long-photoperiod conditions (Dietrich et al., 1994). In SD (8 h of light/16 h of dark), no obvious 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 area displayed lesions in lsd1 cat2-1, lsd1 cat3-2, and lsd1 cat2-1 cat3-2, respectively (Fig. 5B). Compared to 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 lesions penetrance and the severity of the mutant
phenotype in the light-dependent RCD process.

We also generated stable transgenic lines of 35S:FLAG-CAT2 and 35S:FLAG-CAT3 in lsd1 mutant background to explore the function of the overexpression of CAT in lsd1. However, despite the substantially increased expression level and increased catalase enzyme activity (Supplemental Fig. S9A and S9B), all these transgenic plants showed a similar phenotype as the lsd1 mutant in LD (Supplemental Fig. S9C), indicating that overexpression of CAT does not reduce the RCD phenotype of lsd1 in LD.

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

It has been shown that induction of spreading cell death in lsd1 was triggered by inoculation with a low concentration bacterial suspension of an avirulent Pseudomonas syringae, which had no visible effect on 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 grown in SD conditions were infected with the avirulent bacterium Pst DC3000 (avrRpm1) at a relatively low concentration [5 × 10⁶ colony-forming unit (CFU) per mL]. Ion leakage of these samples was measured over times, a readout which corresponded to plant cell death (Mackey et al., 2003). Compared to lsd1, cat mutants (including cat2-1, cat3-2 and cat2-1 cat3-2) and Col-0 showed only a slight increase in conductivity (Fig. 6). However, there was increased conductivity for lsd1 cat2-1, lsd1 cat3-2 double mutants and lsd1 cat2-1 cat3-2 triple mutant (Fig. 6). The results indicate that 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 proved to be SA dependent. SA not only triggers but also
mediates RCD phenotype in *lsd1* (Aviv et al., 2002). As a key plant defense hormone, SA can also modulate the catalase activity (Durner and Klessig, 1996; Vlot et al., 2009). Lesion formation in *cat2* was accompanied with accumulation of high levels of SA in LD, and exogenous SA induced the cell death of *cat2* in SD (Chaouch et al., 2010). The *sid2-2* (*SA induction-deficient*) mutant carries a mutation to *ICS1* which encodes an isochorismate synthase of the SA biogenesis pathway (Wildermuth et al., 2001). SA level after pathogen infection in *sid2* mutants is only 5%-10% of the wild type (Abreu and Munne-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 apparent RCD phenotype was observed in *lsd1* grown in LD conditions (Fig. 7A and 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 and Supplemental Fig. S7). This result indicates that 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 colony-forming unit (CFU) per mL], 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 happened in Col-0 and *sid2-2* (Fig. 7B). The *lsd1 sid2-2* double mutant showed delayed and reduced ion leakage compared to *lsd1* (Fig. 7B). Notably, the enhanced ion leakage in *lsd1 cat2-1, lsd1 cat3-2* and *lsd1 cat2-1 cat3-2* plants were partially suppressed by the *sid2-2* mutation (Fig. 7B). Compared to *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 also required for HR cell death regulated by LSD1 and catalases.

DISCUSSION

The Arabidopsis 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 response (Zamocky et al., 2008). In this study, we revealed that LSD1 physically and genetically interacted with catalases in the light-dependent RCD and HR cell death processes in Arabidopsis. We also presented 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 the interaction with other proteins (Kaminaka et al., 2006; Coll et al., 2010; He et al., 2011). In the present study, the LSD1-like zinc finger was also found to be essential for the interaction between LSD1 and catalase, further demonstrating the importance of 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 hydrogen peroxide (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., 2011). Thus, LSD1 may act as a house-keeping gent 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 mis-localization 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 the 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 catalase activity in leaves, leads to the appearance of lesion in a day-length 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). 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 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 H₂O₂, 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 photosystem I 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 photosystem II (Mateo et al., 2004). Consistently, our data suggest that the cell death regulated by LSD1 and catalases is also associated with H$_2$O$_2$ generated in peroxisomes and SA produced in chloroplasts. In summary, results presented in this study indicate that *LSD1* genetically interacts with *CAT* 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
Columbia (Col-0) accession of wild type *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 (MS) medium (half-strength MS salts, 1% sucrose and 0.8% agar). Plants were grown under 16 h light/8 h dark cycle (LD) or 8 h light/16 h dark cycle (SD) or continuous white light (light intensity of 60 μmol m⁻² s⁻¹) at 22°C.

Seeds of *lsd1* (Col-0 background) (Rusterucci 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 ABRC (Arabidopsis Biological Resource Center). The *cat3-2* (Col-0 background) mutant was identified in this study (see 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 pairs: LSD1FP and LSD1BG), which included a 1520-bp promoter and a 1919-bp coding region. The fragment was ligated to pBlueScript SK (−) (Stratagene), which fused with a single copy of FLAG tag in C-terminal. Then the XhoI/XbaI fragment containing C-terminal FLAG-tagged *LSD1* genomic DNA driven by the endogenous promoter was cloned into the XhoI/SpeI digested binary hygromycin-selected vector pER8 (Zuo et al., 2000).

To generate *CAT2*:FLAG-CAT2, a *CAT2* endogenous 2002-bp promoter was PCR amplified (primer pairs: CAT2PF and CAT2PB), in-framed fused to FLAG tag to yield SK-CAT2pro:FLAG. A *CAT2* genomic DNA was amplified by PCR (primer pairs: CAT2GF
and CAT2GB), this PCR fragment was ligated to SK-CAT2pro:FLAG using PstI/SpeI sites, and then CAT2::FLAG-CAT2 was cloned to the XhoI/SpeI 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 and CAT3PB and CAT3GF and CAT3GB, respectively.

To construct 35S::FLAG-CAT2, FLAG-CAT2 DNA fragments were amplified from CAT2::FLAG-CAT2 plasmid (primer pairs: KpnIFLAG and CAT2GB), and then ligated into pMW101 at the KpnI/SpeI site. 35S::FLAG-CAT3 was made by a similar approach with the primers KpnIFLAG and CAT3GB.

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

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

Reverse Transcription-PCR (RT-PCR) and Quantitative RT-PCR (RT-qPCR)
Total RNA was prepared by the RNAprep pure Plant RNA Purification Kit (Tiangen Biotech, Beijing) according to the manufacturer’s instructions. RT-PCR and RT-qPCR 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 and 100 × proteinase inhibitor (Sigma-Aldrich, Cat #: P9599). Extracts were centrifuged at 13,000 rpm at 4°C twice to collect soluble fraction. Approximately 400 mg proteins were incubated with Protein A agarose beads (Sigma-Aldrich, Cat #: P2545) for 30 min using a
rotator, and then the samples were centrifuged at 1200 rpm at 4°C. The supernatant was incubated with high affinity anti-FLAG agarose beads (Sigma-Aldrich, Cat #: A2220) 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 in SDS-PAGE gels. Differentially expressed bands were excised and the trypsin-digested samples subsequently identified by CapLC Q-TOF MS/MS.

Co-immunoprecipitated proteins were analyzed by immunoblot with anti-FLAG (Abmart, Cat #: M20008), anti-CAT (Agrisere, Cat #: AS09501) or anti-LSD1 (Huang et al., 2010) antibodies.

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 EcoRI/XhoI sites of pGADT7 (Clotech). The LSD1 full-length CDS was amplified by PCR (primer pairs: LSD1F1 and LSD1B1). The PCR product was ligated to pBlueScript SK (−) (Stratagene) and subcloned to the EcoRI/BamHI sites of pGBK7 (Clotech). The LSD1-NTD and LSD1-CTD were cloned with the same procedure and different primers. The LSD1-NTD’s primers were LSD1F1 and LSD1NTDB, the LSD1-CTD’s primers were LSD1CTDF and LSD1B1. The LSD1-ZF1 fragment was amplified from SK-LSD1 with primers LSD1ZF1F and LSD1ZF1B, then the ZF1 truncated version of LSD1-ZF1 was subcloned to the EcoRI/BamHI sites of pGBK7. The 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 (Clotech, http://www.clotech.com/).
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 *Pst DC3000 (avrRpm1)* was injected at the concentration of $5 \times 10^6$ colony-forming unit (CFU) per mL 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 measured over time with a Orion 3-Star Plus Conductivity Meters (Thermo Scientific) as described (Torres et al., 2005).

Enzyme Analysis

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

Whole Mounting Immunofluorescence Labeling

The anti-CAT2 and anti-CAT3 monoclonal antibodies were produced by AbMart (Shanghai, China) with the specific peptides. Immunofluorescence labeling for CAT2 in the roots from 6-d-old seedlings were performed according to the method of (Boudonck et al., 1998) with minor modifications. Briefly, the roots were placed on glutaraldehyde-activated APTES ($\gamma$-aminopropyltriethoxysilane)-coated slides. Then the samples were fixed for 1 h in 4% (w/v) formaldehyde (freshly prepared by dissolving solid paraformaldehyde in 1 x MTSB/2% Triton X-100) (1 x MTSB: 50 mM Pipes, 5 mM EGTA, 5 mM MgSO$_4$, 89 mM KOH, pH 6.9). 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 and 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% DMSO was pipetted onto the slides and they were 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 (BSA) 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, Cat #: A11029, 1:1000) 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, Cat #: A11029, 1:1000). The fluorescence signal was visualized using a confocal laser scanning microscope (Olympus Fluo View™, FV1000).

**Accession Numbers**

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession number: LSD1 (At4g20380), CAT1 (At1g20630), CAT2 (At4g35090), CAT3 (At1g20620) and SID2 (At1g74710), FMO (AT1g19250), 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 salicylic acid is required for RCD regulated by LSD1 and catalase.

**Supplemental Figure S8** The lsd1 and cat 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.

**ACKNOWLEDGMENTS**

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LITERATURE CITED


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demonstrate that redox state is a key modulator of daylength-dependent gene expression, and define photoperiod as a crucial factor in the regulation of H₂O₂-induced cell death. Plant J **52**: 640-657


Vlot AC, Dempsey DA, Klessig DF (2009) Salicylic acid, a multifaced hormone to combat disease. Annu Rev Phytopathol **47**: 177-206


**FIGURE LEGENDS**

**Figure 1** Identification of LSD1-Interacting Proteins in Arabidopsis.

(A) The *LSD1:LSD1-FLAG* transgene rescuing the phenotype of *lsd1* mutant grown in long-day (LD). Twenty-five-day-old (top panel) and thirty-day-old (middle panel) Col-0, *lsd1* and *LSD1:LSD1-FLAG* transgenic seedlings. Bar, 1 cm. Bottom: Immunoblotting analysis of LSD1-FLAG fusion proteins in extracts prepared from 2-week-old seedlings using α-FLAG antibody. Equal loading was verified by using α-tubulin antibody.

(B) RT-PCR analysis of the expression of *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 light/8 h dark). *Actin7* (*At5g09810*) was used as internal control.

(C) Isolation of LSD-interacting proteins. LSD1-interacting proteins were isolated from the protein extracts prepared from two-week-old seedlings with the indicated genotypes. The samples were separated by SDS-PAGE and stained with silver. Differential expressed bands (indicated with red arrow) were excised and identified by mass spectrometry.

(D) Analysis of the protein bands isolated in panel C by CapLC Q-TOF mass spectrometry. The identified CAT3 peptides were shown in bold.

**Figure 2** LSD1 Interacts with Catalases in Yeast Cells.

(A) Interactions between LSD1 and catalase proteins in 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 the selective SD medium minus Trp and Leu (SD-TL), then grown on SD medium minus Ade, His, Trp, and Leu (SD-AHTL) supplemented with 40 μg/mL X-α-Gal for β-galactosidase activity.

(B) Schematic diagram of LSD1 constructs. LSD1: full length (189 amino acid residues), NTD: N-terminal of LSD1 contains three LSD1-like zinc fingers (residues 1-121), CTD:
C-terminal of LSD1 (residues 122-189), ZF1: deletion of zinc finger 1 (residues 12-33); ZF2: deletion of zinc finger 2 (residues 53-74); ZF3: 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. See panel A for technical details.

**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 with α-FLAG agarose beads, followed by immunoblot detection with α-CAT or α-FLAG antibodies.

(B) Co-immunoprecipitation of LSD1, CAT2 and CAT3. Proteins were extracted from Col-0, CAT2:FLAG-CAT2 and CAT3:FLAG-CAT3 transgenic plants, and then immunoprecipitated with α-FLAG agarose beads. The sample was analyzed by immunoblotting with α-LSD1 or α-FLAG antibodies.

**Figure 4** The *lsd1* Mutant Is Hypersensitive to 3-AT.

(A) Two-week-old seedlings of Col-0 and *lsd1* germinated and grown in 1/2 MS medium containing various concentrations of 3-AT in LD. Bar, 1 cm.

(B) Quantitative analysis of the number of plants showing chlorosis with the indicated genotypes. The means of three replicates (biological repeats) ± SD are shown. ** indicates significant difference (*P* < 0.01, student’s *t*-test).

**Figure 5** LSD1 Genetically Interacts with CAT2 and CAT3.

(A) Twenty-five-day-old plants grown in LD. Bar, 1cm.

(B) Quantitative analysis of lesion areas in leaves with the indicated genotypes (percentage of rosette leaf areas). n.d., not detected. ** indicates significant difference (*P* < 0.01, student’s *t*-test).
**Figure 6** Quantification of Cell Death by Ion Leakage in *Pst* DC3000 (*avrRpm1*)-Infected Plants.

Seven-week-old plants in SD were treated with $5 \times 10^6$ colony-forming unit (CFU) per mL *Pst* DC3000 (*avrRpm1*). Ion leakage measurements of leaf discs were started 5 HPI. Conductivity ($\mu$S cm$^{-1}$) was detected at the time points indicated. The means of three replicates (biological repeats) ± SD are shown. HPI: hours post inoculation.

**Figure 7** Accumulation of Salicylic Acid Is Required for Cell Death Regulated by LSD1 and Catalases.

(A) Twenty-eight-day-old plants grown in LD. Bar, 1cm.

(B) Quantification of cell death by ion leakage measurements after the infection of *Pst* DC3000 (*avrRpm1*) in SD. Approximately six-week-old plants in SD were treated with $5 \times 10^6$ colony-forming unit (CFU) per mL *Pst* DC3000 (*avrRpm1*). Conductivity ($\mu$S cm$^{-1}$) was detected at the time points indicated. The means of three replicates (biological repeats) ± SD are shown. HPI: hours post inoculation.
A

LSD1: LSD1-FLAG

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ACT7

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