The *Arabidopsis* LSD1 gene plays an important role in the regulation of low temperature-dependent cell death

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Summary

- In higher plants, the crosstalk between cold stress responses and reactive oxygen species (ROS) signaling is not well understood.
- Two chilling-sensitive mutants, *chs4-1* and *chs4-3*, were characterized genetically and molecularly.
- The *CHS4* gene, identified by map-based cloning, was found to be identical to *LESION SIMULATING DISEASE RESISTANCE 1* (*LSD1*). We therefore renamed these two alleles *lsd1-3* and *lsd1-4*, respectively. These two mutants exhibited an extensive cell death phenotype under cold stress conditions. Consistently, *lsd1-3* plants exposed to cold showed up-regulation of the *PR1* and *PR2* genes, and increased accumulation of salicylic acid. These results indicate that low temperature is another trigger of cell death in *lsd1* mutants. Furthermore, *lsd1-3* plants accumulated higher concentrations of H₂O₂ and total glutathione under cold conditions than wild-type plants. Genetic analysis revealed that *PAD4* and *EDS1*, two key signaling regulators mediating resistance responses, are required for the chilling-sensitive phenotype of *lsd1-3*.
- These findings reveal a role of LSD1 in regulating cell death triggered by cold stress and a link between cold stress responses and ROS-associated signaling.

Introduction

Temperature is one of the major environmental factors that influence plant growth and development as well as distribution. In order to survive, plants respond and adapt to stress through various biochemical and physiological processes. When exposed to low temperatures, plant cytosolic Ca²⁺ concentrations increase transiently, followed by altered expression of diverse cold-regulated (*COR*) genes (Gilmour *et al.*, 1992; Nordin *et al.*, 1993; Yamaguchi-Shinozaki & Shinozaki, 1994). Some of these genes are regulated by C-repeat/dehydration responsive element-binding factor (CBF) transcription factors, which are considered to be the central components responsible for cold tolerance (Shinwari *et al.*, 1998).

Several lines of evidence suggest that plant responses to cold stress are directly linked to reactive oxygen species (ROS) signaling (Lee *et al.*, 2002; Davletova *et al.*, 2005a; Vogel *et al.*, 2005; Einset *et al.*, 2007a,b, 2008). ROS are key signal transduction molecules during responses to environmental stresses and developmental stimuli (Mittler *et al.*, 2004). During environmental stresses, ROS activate stress-response pathways and induce defense mechanisms. On the other hand, excess ROS produced under stress and the failure to maintain ROS balance cause oxidative damage to cells, leading to growth defects or initiation of cell death (Torres & Dangl, 2005). Cold stress increases transcript abundance and protein concentrations of ROS-scavenging enzymes, as well as ROS accumulation (O’Kane *et al.*, 1996). Several genes have been implicated in both cold stress and ROS signaling. For example, overexpression of *ZAT12*, a C2H2 zinc finger-type transcription factor gene, induces cold-inducible genes and confers increased cold tolerance in plants when overexpressed. Furthermore, *ZAT12* plays a central role and regulates a number of genes involved in plant responses to oxidative stress (Davletova *et al.*, 2005b). Glycine betaine (GB) is a chemical that improves tolerance to stress caused by chilling (Park *et al.*, 2004). A membrane trafficking protein RabA4c and a ferric reductase FRO2 play roles in GB’s effect on ROS accumulation.
during chilling conditions (Einset et al., 2007a, 2008). An ethylene response factor, JERF3, enhances superoxide dismutase activity, and reduces ROS content when over-expressed, thus enhancing tolerance to freezing and other abiotic stresses (Wu et al., 2008).

The Arabidopsis lesion simulating disease resistance 1 (ldl1) mutant shows abnormal regulation of ROS-dependent cell death, characteristics of a runaway cell death (RCD) phenotype when exposed to long photoperiod or infected with an avirulent pathogen (Dietrich et al., 1994). LSD1 encodes a C2H2 zinc finger protein with homology to GATA-type transcription factors (Dietrich et al., 1997). Cell death conditioned by ldl1 requires EDS1 and PAD4, two key regulators that are needed for specific pathogen resistance (Rusterucci et al., 2001). LSD1 is thought to limit the spread of cell death via up-regulation of CuZn-superoxide dismutase that scavenges superoxide (Kliebenstein et al., 2001). For the complementation assay, a genomic LSD1 fragment encompassing the native promoter (3616 bp) was amplified with primer LSD1-2 and LSD1-3. For generation of double mutants, single mutants were crossed and the double mutants were identified by PCR genotyping in the F2 population.

Plants were grown at 22 or 4°C, respectively, under long-day (LD, 16 h light : 8 h dark) or short-day photoperiods (SD, 10 h light : 14 h dark) at 100 μmol m⁻² s⁻¹, with 50–70% relative humidity in soil or on MS medium (Sigma) containing 2% sucrose and 0.8% agar.

Genetic mapping and cloning of the CHS4 Gene
For genetic mapping of the chs4-1 mutation, the mutant was crossed to the Ler plant. F1 plants from the cross were self-fertilized, and the resulting F2 seeds were collected. A total of 850 chs4-1 mutant plants were not selected from the segregating F2 population based on the chilling-sensitive phenotype of the mutant. Genomic DNA from these F2 plants was extracted and used for PCR-based mapping with simple sequence length polymorphism (SSLP) and cleaved amplified polymorphic sequence (CAPS) markers. Initial mapping placed the mutation to the markers F28A21 and F1N20 on the chromosome IV. New mapping markers were developed based on insertion/deletions identified from Cereon Arabidopsis polymorphism and Ler sequence collection (http://www.arabidopsis.org). Genomic DNA corresponding to candidate genes was PCR amplified from the mutants and sequenced to identify the mutation.

Plasmid construction and plant transformation
For the complementation assay, a genomic LSD1 fragment encompassing the LSD1 native promoter (3616 bp) was amplified with primer LSD1-2 and LSD1-1pF by PCR using Probest High fidelity DNA polymerase (Takara, Japan), and cloned into the binary vector pCAMBIA1300 (CAMBIA, Canberra, Australia) with the GFP tag to generate the LSD1::LSD1-GFP.

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To construct CHS4::GUS fusion, a 2138 bp genomic fragment upstream of the LSD1 ATG start codon was amplified with primer LSD1-1pF and LSD1-1pR, and fused with the β-glucuronidase (GUS) reporter gene in the vector pZPGUS2 (Diener et al., 2000).

The Agrobacterium tumefaciens strain GV3101 carrying different constructs was used to transform wild-type or chs4-1 plants via floral dip transformation (Clough & Bent, 1998).

Ion leakage assay
The electrolyte leakage test was performed as described previously (Lee et al., 2002). Three-week-old plants grown in soil under normal conditions were treated at 4°C for different lengths of time. The percentage of electrolyte leakage

Materials and Methods
Plant material and growth conditions
Arabidopsis thaliana plants in accession Columbia (Col), Wassilewskija (Ws) and Landsberg erecta (Ler) were used in this study. ldl1-1 and edd1-1 are in the Ws background, and pad4-1 and PRI::GUS transgenic plant are in the Col background. pad1-1c was generated by introgressing ldl1-1 into the Col background (Rusterucci et al., 2001). The chs4-1 and chs4-3 (Schneider et al., 1995) in the Col background were obtained from ABRC (stock numbers CS8001 and CS8003). The mutation in chs4-1 was identified by derived cleaved amplified polymorphic sequence (dCAPS) primers LSD1-2 and LSD1-3. For generation of double mutants, single mutants were crossed and the double mutants were identified by PCR genotyping in the F2 population.
was calculated as the ratio of the percentage of the conductivity before autoclaving to the percentage after autoclaving.

**Measurement of total glutathione**

To compare total glutathione concentration and the redox state of glutathione in wild-type and *lsd1-3* plants, reduced glutathione [GSH] and oxidized glutathione [GSSG] in wild-type and *lsd1-3* plants were determined as described previously (Rao & Ormrod, 1995). Three-week-old plants grown in soil were treated at 4°C for 10 d.

**Salicylic acid measurement**

Free salicylic acid (SA) and total SA were extracted and measured from 3-wk-old plants grown at 22°C or treated at 4°C for 10 d as described previously (Li et al., 1999) with some modifications. The last extract residue was dissolved in acetonitrile, and analyzed by high-performance liquid chromatography (HPLC) using 5% acetate (pH 3.2) as the mobile phase.

**Histochemical staining assay**

Trypan blue staining and 3,3′-diaminobenzidine (DAB) staining were performed as described previously (Bowling et al., 1997; Thordal-Christensen et al., 1997). Histochemical detection of GUS activity was performed as described previously (Yang et al., 2006).

**Quantitative real-time PCR**

Total RNA was isolated from 10-d-old seedlings using TRIzol (Invitrogen), followed by treatment with RNase-free DNase I (Takara) at 37°C for 20 min to degrade genomic DNA. Two micrograms of RNA were subjected to first-strand cDNA synthesis using M-MLV reverse transcriptase (Promega), and an oligo (dT21) primer. The primers used for real-time PCR are listed in the Supporting Information, Table S1. Real-time PCR was performed in 20 μl reactions containing 2 μl fivefold-diluted cDNA, 0.4 μM of each primer, and 10 μl SYBR Green PCR Master Mix (Takara). Analysis was performed using the ABI PRISM 7500 real-time PCR system (Applied Biosystems, Foster City, CA). Primer efficiencies were measured and relative expression level was calculated using the comparative C_\text{r}^- method (User Bulletin 2 for ABI PRISM 7700 sequence detection system). All experiments were repeated at least three times with similar results.

**Preparation of anti-LSD1 antibodies and western blot analysis**

An *LSD1* cDNA fragment was cloned into pGEX-4T1 vector (Pharmacia, USA), and purified GST-LSD1 recombinant protein was used to immunize rabbits to generate the antiserum. The anti-LSD1 antibody was then affinity-purified using purified MBP-LSD1 recombinant protein as a ligand. The purified antibody was extensively characterized using protein extracts prepared from wild-type and *lsd1* mutant plants.

**Results**

The chilling sensitive mutant *chs4* is allelic to *lsd1*

*Arabidopsis* chilling-sensitive 4 (*chs4*) mutants, *chs4-1* and *chs4-3*, were previously isolated from plants grown at low temperatures (Schneider et al., 1995). In general, chilling sensitivity has been referred to as a variety of types of physiological damage, including wilting, necrosis, chlorosis, and/or leakage of ions from cell membranes (Lyons, 1973). These two *chs4* mutants had no obvious growth defects when grown at 20–22°C (Fig. 1a). However, when *chs4* mutants were treated at 4°C, the old leaves became yellowish and wilted, and exhibited necrosis (Fig. 1a). Nevertheless, the *chs4* plants could flower and set seeds normally if they were transferred to normal temperatures after cold treatment for no longer than 3 wk. Prolonged treatment of *chs4-1* plants with low temperatures caused growth arrest and an eventual plant death (Fig. 1a).

The *chs4-1* mutation was shown to be a recessive mutation in a single nuclear gene (Schneider et al., 1995). A map-based cloning approach was used to identify the *CHS4* gene. The *chs4-1* mutation was mapped initially between markers F28A21 and F1N20 on chromosome IV. Further mapping narrowed *CHS4* to an interval of c. 300kb spanning BACs F18F4 and T7J7 (Fig. 1b). Sequencing analysis detected a single nucleotide substitution of G-to-A in the last exon–intron junction site of *LSD1*. This
mutation presumably leads to abnormal splicing, resulting in a predicted protein with 29 additional amino acid residues. This prediction was verified by reverse transcription PCR and sequencing analyses (Figs 1c, S1). In chs4-3, a single C-to-T mutation was found within the seventh exon of LSD1, resulting in a conversion of Pro to Leu at residue 167 (P167L). No differences in LSD1 mRNA levels were detected between wild-type and chs4-3 plants (Fig. 1c).

Immunoblot analyses revealed reduced levels of the mutated proteins in chs4 mutant plants (Fig. 1d), suggesting that these two mutants might be weak alleles of lsd1. The defective phenotypes of chs4-1 and chs4-3 are likely the result of a mutation-induced deactivation of LSD1 protein.

To verify if the chs4 mutant phenotype is caused by mutations in LSD1, a wild-type genomic fragment of LSD1 under the control of its own promoter was transformed into chs4-1 plants. All 15 T1 transgenic plants analyzed showed wild-type-like morphology (Fig. 1e). In addition, F1 plants from a cross of chs4-1 and lsd1 (Dietrich et al., 1997) had the lsd1 phenotype (Fig. S2), demonstrating that these two mutants are indeed alleles. For conciseness, we refer to lsd1, lsd1c (Rusterucci et al., 2001), chs4-1, and chs4-3 as lsd1-1, lsd1-1c, lsd1-3, and lsd1-4, respectively, hereafter.

Expression pattern of LSD1

To further elucidate the physiological function of LSD1, we monitored the LSD1 expression pattern by GUS staining of transgenic plants expressing GUS driven by a 2.1 kb fragment of the LSD1 promoter. GUS expression was detected in cotyledons, roots, rosette leaves, stems, inflorescences, and flowers, but was not detected in siliques (Fig. 2a–f). Western blot analyses showed similar patterns of LSD1 protein.
accumulation (Fig. 2g), indicating that LSD1 may function in most tissues and during most developmental stages.

We then used immunoblot analysis to determine whether LSD1 expression was responsive to different stimuli. During a 12 h treatment period, LSD1 was highly induced by methyl violgen (MV), an oxidative inducer. Treatment with benzothiadiazole (BTH), a biologically active analog of SA, slightly increased the LSD1 protein level, whereas LSD1 expression was not altered in response to cold treatment (Fig. 2h). However, LSD1 protein accumulated to higher levels after cold treatment at 4°C for >3 d (data not shown). Consistently, more LSD1 transcripts were detected in plants treated with cold (4°C), methyl violgen (MV; 5 µM), or benzothiadiazole (BTH; 0.5 mM) at 0, 3, 6, and 12 h, respectively. Protein was analyzed by immunoblotting with antibodies against LSD1. Loading consistency in (g) and (h) was determined by a nonspecific band (c. 32 kD) recognized by the anti-LSD1 antibody.

Cell death occurs in lsd1-3 plants under cold stress conditions

Visible necrosis was observed on leaves of lsd1-3 and lsd1-4 plants under cold stress conditions (Figs 1a, 3a). Trypan blue staining verified that extensive cell death occurred in cold-treated lsd1-3 and lsd1-4 plants, but not in wild-type plants (Fig. 3a). Cold-treated lsd1-3 plants stained with DAB showed the accumulation of higher levels of hydrogen peroxide (H₂O₂) than wild-type plants (Fig. 7c). Furthermore, PR1 and PR2 were highly expressed in lsd1-3 plants under cold conditions as revealed by northern blot analysis (Fig. 3b). lsd1-3 plants carrying a PR1::GUS construct showed a strong increase in GUS activity after cold treatment as compared with wild type PR1::GUS transgenic plants (Fig. 3c). Therefore, lsd1-3 plants may constitutively activate defense responses at cold stress.

Salicylic acid is an essential signaling molecule in plant defense responses (Kunkel & Brooks, 2002). Therefore, endogenous SA levels were measured to examine the role of SA in the lsd1-3 phenotype. The endogenous levels of both free and total SA in lsd1-3 plants were five- and threefold higher than in wild-type plants at 22°C. Cold treatment enhanced accumulation of free and total SA in both wild-type and lsd1-3 plants. However, compared with wild-type plants, the lsd1-3 mutant showed c. nine- and 10-fold increases in concentrations of free and total SA, respectively (Fig. 3d). This result indicates that lsd1-3 plants accumulated more SA under cold conditions, leading to hypersensitive response-like cell death phenotype.

The cell death phenotype of lsd1 is dependent on temperature

Runaway cell death of the null mutant lsd1-1 can be induced by LD conditions (16 h light : 8 h dark) (Dietrich et al., 1994). However, lsd1-3 plants were morphologically indistinguishable from wild-type plants grown either in LD or SD (10 h light : 14 h dark) conditions at 22°C (Figs 1a,
Previous studies have demonstrated that temperature-dependent cell death in several mutants is compromised by high temperatures (Yang & Hua, 2004; Noutoshi et al., 2005; Yang et al., 2006, 2007; Zhou et al., 2008; Gao et al., 2009; Gou et al., 2009). Next we asked whether high temperatures had any effect on the lsd1-conferred phenotype. The RCD phenotype of lsd1-1c triggered by LD was partially suppressed by high temperatures (28°C) (Fig. 4d). Consistently, induction of the PR1 and PR2 genes in lsd1-1c grown at 22°C was abolished when grown at 28°C (Fig. 4e). These results suggest that temperature is an important factor to modulate cell death conferred by the LSD1 mutation.

**lsd1** mutants have defective membrane integrity and cold-regulated gene expression

To determine the extent of chilling injury on membrane integrity in lsd1 plants, lsd1-3 leaves excised from plants treated at 4°C for varying lengths of time were subjected to electrolyte leakage assays. Electrolyte leakage did not change significantly in wild-type plants during a 21 d cold treatment. However, lsd1-3 plants showed a dramatic increase in electrolyte leakage from 6 d after cold treatment, and the electrolyte leakage displayed a time-dependent increase during 6–21 d of cold treatment (Fig. 5a). This indicates that the membrane integrity of lsd1-3 plants was damaged by cold stress.

Next, we examined whether the expression of cold-regulated genes contributes to the lsd1-conferred phenotype. Quantitative real-time PCR analysis showed that cold induced a higher expression of CBF1, CBF2, and CBF3 and their targets RD29A and COR47, which was consistent with previous studies (Jaglo-Ottosen et al., 1998; Miura et al., 2007). CBF1, CBF2, and CBF3 were rapidly induced in lsd1-3 and wild-type plants following cold treatment. Compared with wild-type plants, cold induction of CBF1, CBF2, and CBF3 genes was decreased in lsd1-3 plants after 3 and 6 h of cold treatment (Fig. 5b). RD29A and COR47 were induced at low levels in lsd1-3 plants to varying extents after 12 and 24 h of cold treatment (Fig. 5b). Therefore, LSD1 might modulate plant responses to cold stress, at least partially by altering the expression of cold-regulated genes.

The total glutathione concentration is increased in lsd1-3 plants under cold stress conditions

Glutathione plays important roles in many processes, including the G1-to-S transition in the cell cycle (Vernoux et al., 2000), transcriptional and translation regulation (Kan et al., 1988; Baena-Gonzalez et al., 2001), cell death (Henmi et al., 2001), and disease resistance (May et al., 1996). Glutathione is also implicated in chilling tolerance (Kocso et al., 2001). To determine the role of glutathione in lsd1-3 plants, cellular redox changes were analyzed.
by comparing total glutathione ([GSH] + [GSSG]) and the ratio of reduced glutathione : total glutathione ([GSH] : ([GSH] + [GSSG])) of \textit{lsd1-3} and wild-type plants under cold stress conditions. Comparable amounts of total glutathione were detected in wild type and \textit{lsd1-3} plants at 22°C and under short-day (SD) conditions for 50 d and then transferred to 22°C and long-day (LD) conditions for 3 d. (b) 3,3¢-diaminobenzidine (DAB) staining of leaves from plants in (a). (c) Wild-type and \textit{lsd1-1c} plants were grown at 22°C and under short-day (SD) conditions for 21 d or cold-treated at 4°C for 21 d. (d) Wild-type and \textit{lsd1-1c} plants were grown at 22°C and in SD conditions for 28 d and then placed at 28°C and in LD conditions for 10 d. (e) Analysis of \textit{PR1} and \textit{PR2} expression in wild-type Col (black bars) and \textit{lsd1-1c} (gray bars) plants by real-time PCR. Four-week-old plants grown at 22°C and under SD conditions were transferred to 22°C and LD conditions for 10 d, 28°C and LD conditions for 10 d, or 4°C and SD conditions for 10 d.

**Fig. 4** Phenotypes of \textit{Arabidopsis lsd1} mutants in response to different light periods and temperatures. (a) Wild-type, \textit{lsd1-1}, and \textit{lsd1-3} plants were grown at 22°C and under short-day (SD) conditions for 50 d and then transferred to 22°C and long-day (LD) conditions for 3 d. (b) 3,3¢-diaminobenzidine (DAB) staining of leaves from plants in (a). (c) Wild-type and \textit{lsd1-1c} plants were grown at 22°C and under short-day (SD) conditions for 21 d or cold-treated at 4°C for 21 d. (d) Wild-type and \textit{lsd1-1c} plants were grown at 22°C and in SD conditions for 28 d and then placed at 28°C and in LD conditions for 10 d. (e) Analysis of \textit{PR1} and \textit{PR2} expression in wild-type Col (black bars) and \textit{lsd1-1c} (gray bars) plants by real-time PCR. Four-week-old plants grown at 22°C and under SD conditions were transferred to 22°C and LD conditions for 10 d, 28°C and LD conditions for 10 d, or 4°C and SD conditions for 10 d.

ROS-associated gene expression of \textit{lsd1-3} plants during cold stress

As noted previously, greater H₂O₂ accumulation in \textit{lsd1-3} than in wild-type plants was observed under cold stress (Fig. 7c). Excess H₂O₂ was shown to induce expression of genes involved in oxidative stress (Iba, 2002; Mittler et al., 2004; Rizhsky et al., 2004). Therefore, the transcript abundance of genes encoding ROS-producing enzyme NADPH oxidase (RbohD), and ROS-scavenging enzymes, such as ascorbate peroxidase (APX1), catalase (CAT1), and glutathione reductase (GR1), in \textit{lsd1-3} plants was examined. Under normal growth conditions, RbohD expression was at a lower level in \textit{lsd1-3} plants than in wild-type plants (Fig. 6b). No notable differences in expression of APX1, CAT1, and GR1 were detected between wild-type and \textit{lsd1-3} plants at 22°C (Fig. 6b). The transcript abundances of RbohD, APX1, CAT1, and GR1 were substantially higher in \textit{lsd1-3} plants than in wild-type plants upon exposure to cold for 10 d. ZAT12 transcripts were more abundant in \textit{lsd1-3} plants than in wild-type plants after 10 d of 4°C treatment (Fig. 6b). Ferritins are considered to be essential for cell protection against oxidative damage (Ravet et al., 2009). \textit{FER1} was also up-regulated in \textit{lsd1-3} plants in cold conditions (Fig. 6b). Moreover, the extent of cold induction of these genes was similar in \textit{lsd1-3} and \textit{lsd1-1c} plants (Fig. S3). Taken together, these results suggest that the \textit{lsd1-3} phenotype in cold conditions is associated with impairment of the ROS detoxification pathway and the oxidative signaling pathway caused by disruption of \textit{LSD1}. 

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pad4 and eds1 mutations suppress the chilling sensitivity of lsd1-3 plants

LSD1-regulation of basal defense and cell death is dependent on EDS1 and PAD4, two key regulators that are required for many R gene-mediated defenses (Rusterucci et al., 2001; Aviv et al., 2002). Previous studies have also demonstrated that temperature-dependent growth defects and cell death in some mutants are compromised by eds1 and pad4 (Yang & Hua, 2004; Yang et al., 2006, 2007; Gao et al., 2009; Gou et al., 2009). To test whether the chilling sensitive phenotype of lsd1-3 requires EDS1 and PAD4, lsd1-3 eds1-1 and lsd1-3 pad4-1 double mutants were generated and analyzed. Neither lsd1-3 eds1-1 nor lsd1-3 pad4-1 showed visible cell death phenotypes in cold conditions (Fig. 7a, b). In addition, H2O2 accumulation in lsd1-3 plants was completely suppressed by the eds1 and pad4 mutations (Fig. 7c). In accordance with the cell death phenotype, the increased expression of PR genes at 4°C was also completely abolished by eds1 and pad4 (Fig. 7d). These data indicate that the chilling sensitivity of lsd1-3 is dependent on EDS1 and PAD4.

Discussion

In this study, we characterized two weak alleles of lsd1, lsd1-3 and lsd1-4, which were previously named chs4-1 and chs4-3, respectively, based on their phenotype at chilling temperatures (Schneider et al., 1995). lsd1 mutant plants display a cell death phenotype within their leaves when exposed to...
4°C. lsd1 mutants also show constitutive defense responses, including elevated PR gene expression, and accumulation of endogenous SA and excess ROS at 4°C. Furthermore, the lsd1 mutant phenotype is completely suppressed by eds1 and pad4. These characteristics indicate that low temperature is another trigger of cell death in lsd1 mutants dependent on PAD4 and EDS1.

LSD1 is a negative regulator of SA-dependent programmed cell death (PCD) and plant defense responses to pathogens (Dietrich et al., 1997; Rusterucci et al., 2001; Aviv et al., 2002). LSD1 is also necessary for acclimation to conditions that promote photooxidative stress, including long-day photoperiods, high-light conditions, and photorefractory conditions (Dietrich et al., 1997; Mateo et al., 2004). Here, lsd1 plants displayed growth inhibition and visible PCD within their leaves under cold stress. The LSD1 mutation caused reduced cold induction of CBFs and their target genes, suggesting that LSD1 has specific effects on expression of cold-regulated genes to modulate plant responses to cold stress. How LSD1 regulates the expression of cold-regulated genes remains elusive. LSD1 protein expression was not regulated by cold during a 12 h time period. However, prolonged cold exposure (at least 3 d) can increase LSD1 protein levels (data not shown), implying that LSD1 may not respond to low temperatures directly. Compared with the wild-type, the lsd1 mutation-activated defense response may have a higher threshold to additional stress signals, which may explain a reduced response of lsd1 mutants to cold treatment, as we have observed.

Consistent with a previous study (Kaminaka et al., 2006), we found that LSD1 was largely localized in the cytoplasm at 22°C, and low temperatures had no effect on the subcellular localization of LSD1 (Fig. S4). LSD1 interacts with a bZIP transcription factor, AtbZIP10, thereby retaining AtbZIP10 in the cytoplasm, which results in modulation of cell death and the basal defense (Kaminaka et al., 2006). In the cold signaling pathway, Hos1 has been shown to translocate from the cytoplasm to the nucleus following cold stress. Once in the nucleus, Hos1 interacts with and polyubiquitinates ICE1, a positive regulator of CBF3, thus negatively regulating the expression of cold-regulated genes that contribute to cold acclimation (Lee et al., 2001; Dong et al., 2006). Therefore, LSD1 might regulate the nuclear localization of ICE1 and/or...
HOS1 proteins by binding them either directly or indirectly through other proteins functioning in this pathway. Further localization studies of the HOS1 and ICE1 proteins in lsd1 mutants under cold stress may help elucidate the LSD1 function in regulation of temperature-dependent plant growth and cell death.

lsd1-3 and lsd1-4 are weak alleles of lsd1-1c, as they do not show obvious defects in response to LD conditions, under which lsd1-1c exhibits an RCD phenotype. Sequencing analysis revealed that the point mutations in lsd1-3 and lsd1-4 did not produce stop codons, and that both mutated proteins could presumably be translated. Indeed, mRNA concentrations of LSD1 were not altered in these two mutants. However, the mutated protein levels were dramatically reduced in lsd1-3 and lsd1-4 plants, which is probably the result of the decreased stability of the mutated proteins. Nevertheless, it appears that the residual mutated proteins in lsd1-3 and lsd1-4 plants are still functional, and that they repress RCD under normal conditions when no excess ROS are accumulated. However, under unfavorable environmental conditions, such as cold stress, the residual activity of the mutated LSD1 proteins was insufficient to inhibit RCD caused by overaccumulated ROS in lsd1-3 and lsd1-4 mutants, leading to their cold-sensitive phenotypes.

Our data show that LSD1 plays an essential role in avoidance of temperature-induced oxidative stress. In lsd1 mutants, excess ROS lead to up-regulation of genes encoding ROS-scavenging enzymes and enhance the concentration of the nonenzymatic antioxidant glutathione, which can contribute to plant protection against adverse situations. However, even the increased amount of these protectants was unable to prevent the damage caused by excess ROS accumulation during cold conditions in lsd1 mutants.

Several environmental factors can initiate cell death. For example, lsd1 plants initiate RCD during long photoperiods and high-light conditions, whereas lsd2, lsd5, and len1 plants develop lesions under short-day conditions but not under long-day conditions (Dietrich et al., 1994; Ishikawa et al., 2003). Furthermore, lesion formation and cell death are compromised by high temperature and/or humidity in transgenic plants overexpressing the tomato disease resistance gene Pto (Li et al., 2002) and in some lesion-mimic mutants, including bon1, bap1, ssi4, sib1, bir1, and cpr30 (Yang & Hua, 2004; Noutoshi et al., 2005; Yang et al., 2006, 2007; Zhou et al., 2008; Gao et al., 2009; Gou et al., 2009). By contrast, cell death in rice spl7 mutants requires high temperatures and UV solar radiation (Yamanouchi et al. 2002). In our study, cell death occurs in lsd1-3 only at low temperatures. Null mutations in EDS1 and PAD4 suppress the chilling-sensitive phenotype of lsd1-3. This finding is in agreement with the observation that pad4 and eds1 block lsd1-conditioned RCD triggered by long photoperiods, high light, photorespiratory conditions, or SA (Jabs et al., 1996; Rusterucci et al., 2001; Mateo et al., 2004). Therefore, a similar mechanism is likely utilized by LSD1-regulated cell death machinery in response to different environmental cues.

In summary, our data indicate that low temperature is another trigger of the cell death caused by the imbalance of ROS in lsd1 mutants, and that the cell death phenotype requires EDS1 and PAD4. Thus, LSD1 plays an important role in ROS responses to repress cell death under various environmental stimuli.

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Supporting Information
Additional supporting information may be found in the online version of this article.

Fig. S1 Comparison of predicted amino acids of LSD1 and mutated forms in lsd1-3 and lsd1-4 mutants.

Fig. S2 Allelism test of chs4-1 and lsd1 mutants.

Fig. S3 Expression of reactive oxygen species (ROS)-associated genes in lsd1 plants by real-time PCR.

Fig. S4 Immunoblot analysis of LSD1 in lsd1-3 plants under cold stress conditions.

Table S1 Gene-specific primers used in this study.

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