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A Novel Zinc Finger Protein Is Encoded by the Arabidopsis *LSD1* Gene and Functions as a Negative Regulator of Plant Cell Death

Robert A. Dietrich,* Michael H. Richberg,*
Renate Schmidt,‡§ Caroline Dean,‡
and Jeffery L. Dangl*†
*Department of Biology
†Curriculum in Genetics and Molecular Biology
University of North Carolina
Chapel Hill, North Carolina 27599
‡Department of Molecular Genetics
John Innes Center
Norwich NR4 7UH
United Kingdom
§Max-Delbrück Laboratory
Carl-von-Linné Weg 10
50829 Köln
Germany

Summary

Arabidopsis *lsd1* mutants are hyperresponsive to cell death initiators and fail to limit the extent of cell death. Superoxide is a necessary and sufficient signal for cell death propagation. Thus, *LSD1* monitors a superoxide-dependent signal and negatively regulates a plant cell death pathway. We isolated *LSD1* via its map position. The predicted LSD1 protein contains three zinc finger domains, defined by CxxCxRxxLMYxxGASxVxC xxC. These domains are present in three additional Arabidopsis genes, suggesting that LSD1 defines a zinc finger protein subclass. *LSD1* is constitutively expressed, consistent with the mutant phenotype. Alternate splicing gives rise to a low abundance mRNA encoding an extra five amino-terminal amino acids. We propose that LSD1 regulates transcription, via either repression of a prodeath pathway or activation of an antideath pathway, in response to signals emanating from cells undergoing pathogen-induced hypersensitive cell death.

Introduction

Controlled induction of cell death occurs both during normal plant development and the rapid, localized response to pathogen infection known as the hypersensitive response (HR; Goodman and Novacky, 1994; Dangl et al., 1996). The HR is a feature of most, but not all, disease resistance reactions. It is not known whether HR is required to halt pathogen growth. Genetic control of disease resistance and HR is commonly determined by specific interactions between alleles of pathogen *avr* (avirulence) gene loci and an allele of a corresponding plant disease resistance (*R*) locus. When these are present in both host and pathogen, the result is disease resistance. If either the plant *R* allele or the pathogen *avr* gene are absent or inactive, disease occurs. The simplest mechanistic interpretation of allele-specific disease resistance is that the *R* gene product recognizes the *avr* gene product directly, triggering the chain of signal transduction events culminating in a halt of pathogen growth. (reviewed by Dangl, 1995; Staskawicz et al., 1995; Bent, 1996). Direct avr–R protein interaction has not been shown in plants, but expression of *avr* genes in plant cells can initiate *R*-dependent HR, and avr–R protein–protein interactions can occur in yeast two-hybrid systems (Gopalan et al., 1996; Scofield et al., 1996; Tang et al., 1996; Van den Ackerveken et al., 1996).

Inducible plant cell responses subsequent to pathogen recognition include calcium influx, K^+–H^+ exchange leading to alkalinization of the extracellular space, and an oxidative burst (reviewed by Hammond-Kosack and Jones, 1996). Production of reactive oxygen intermediates (ROI) occurs with kinetics and magnitude, suggesting a key role in either pathogen elimination, signaling of downstream effector functions, or both (reviewed by Baker and Orlandi, 1995; Low and Merida, 1996). Both H_2O_2 (Levine et al., 1994, 1996) and superoxide have been implicated in initiating HR (Jabs et al., 1996). ROI production may be mediated by a plasma membrane NADPH oxidase analogous to that used by mammalian neutrophils, although other models exist (Bolwell et al., 1995). Cell death during the HR may be caused by ROI toxicity, or it may be a consequence of signals derived from ROI. Morphological descriptions of cell death during infection suggest, in at least some cases, parallels with animal apoptosis (Mittler et al., 1995; Kosslak et al., 1996; Levine et al., 1996; Ryerson and Heath, 1996; Wang et al., 1996; reviewed by Dangl et al., 1996). HR is correlated with the onset of systemic acquired resistance (SAR) to secondary infection in distal tissue, a process requiring salicylic acid (SA) accumulation in at least tobacco and Arabidopsis (reviewed by Ryals et al., 1996). SA accumulates following the oxidative burst in and around infection sites, and SA is an inhibitor of a variety of enzymes, suggesting that SA or a radical derived from it poisons the infected cell, causing its death (Enyedi et al., 1992; Malamy et al., 1992; Chen et al., 1994; Durner and Klessig, 1995; Rueffler et al., 1995). The sum of these signals culminates in transcriptional activation of a variety of plant genes, HR, and production of both local and systemic signals that protect the plant from further infection. It is unclear whether these effector functions are controlled by linear, interdigitating, or bifurcating signal pathways.

We and others isolated Arabidopsis mutants that exhibit constitutive initiation of HR-like cell death in the absence of pathogen (Dietrich et al., 1994; Greenberg et al., 1994). These resemble a variety of mutants in crop species broadly categorized as "lesion mimic mutations" (Walbot et al., 1983; Johal et al., 1994). Our series of nonallelic mutations expressed histochemical and molecular markers associated with disease resistance responses and also exhibited heightened resistance to virulent bacterial and oomycete pathogens when lesions were present. Thus, these cell death phenotypes can trigger pathogen nonspecific resistance resembling SAR. We proposed that these mutants subdivided the lesion mimic class into a "lesions simulating disease resistance" or *lsd* phenotype (Dietrich et al., 1994).

Greenberg and Ausubel (1993) additionally isolated a mutant that, though expressing accelerated cell death, was more susceptible to pathogen. It is thus possible to identify genetically at least two types of cell death, those that feed into a pathway culminating in establishment of a disease resistant state, and those that do not.

The *lsd1* mutant is exceptional. In conditions permissive for wild-type plant growth and in the absence of detectable microscopic lesions, the *lsd1* mutant is hyperresponsive to challenge by a variety of stimuli including pathogens and low doses of chemicals that trigger SAR (Dietrich et al., 1994). *lsd1* plants are resistant to virulent pathogens in conditions where no spontaneous cell death lesions were observed. Following initiation of cell death in a local spot on a leaf, lesions propagate throughout the leaf and kill it 2–4 days later. Propagation of locally initiated cell death is confined to the inoculated leaf. Thus, *LSD1* functions to negatively regulate both the initial response to pathogens and the subsequent spread of cell death. Production of superoxide is a necessary and sufficient trigger for this phenotype, and superoxide production precedes onset of cell death by 8–16 hr following initiation by three different triggers (Jabs et al., 1996). Therefore, *LSD1* responds to either superoxide or a signal derived from it to down-regulate or dampen the cell death response, resulting in the typical locally bounded HR. Here, we report the isolation of the *LSD1* gene and show that it is the first member of a new subclass of zinc finger proteins in Arabidopsis.

Results

Genetic and Physical Mapping of *lsd1*

The *lsd1* mutation segregates as a monogenic recessive (Dietrich et al., 1994). We first establish linkage to the *AG* (*agamous*) CAPS marker on chromosome 4 in F2 progeny of a cross between *lsd1* (Ws-0 background) and Col-0 by using the codominant amplified polymorphic sequences (CAPS) mapping procedure (Konieczny and Ausubel, 1993). The closely linked g13838 probe (3 recombinants in 1632 meioses) was used to identify YAC (yeast artificial chromosome) clones (Schmidt et al., 1995, 1996), and we constructed a physical contig (Figure 1A). We derived new CAPS markers 1H1L-1.6 and 5F7R-1.5 from YAC ends, and they mapped closest to *lsd1* (1 and 3 recombinants, respectively, from 2054 meioses). We hybridized these two CAPS markers to filters containing bacterial artificial chromosome (BAC) clone arrays (Choi et al., 1995; distributed by the Arabidopsis Biological Resource Center, Ohio State University) and isolated five BAC clones (Figure 1B). Because 5F7R-1.5 and 1H1L-1.6 genetically flank *lsd1* (Figure 1B), BAC clone 1G5 should contain the gene.

To provide multiple redundancy of genomic cloned DNA encompassing *lsd1*, we connected BACs 6H3 and 8A6 by walking in a genomic phage library. We defined a 1.3 kb EcoRI fragment present only on BACs 8A6 and 1G5. This 8A6-1.3 clone (small box in Figure 1C) was used to isolate three phage clones, two of which are depicted in Figure 1C. Labeled inserts from each detected BAC clones 1G5, 6H3, and 8A6. We converted 8A6-1.3 into a CAPs marker and found that it cosegregated with *lsd1* in 2054 meioses. This map resolution of approximately 0.05 map units suggested that *lsd1* was within 5–15 kb (at 100–300 kb per map unit; Schmidt et al., 1995, 1996) in either direction of 8A6-1.3.

We probed genomic Arabidopsis DNA blots of digested wild-type Ws-0 and *lsd1* to confirm colinearity of the cloned and genomic DNA immediately surrounding 8A6-1.3. Several probes detected a genomic DNA rearrangement in *lsd1* relative to wild-type Ws-0 (data not shown). This rearrangement corresponded to loss of restriction sites and a deletion of ~900 bp (Figure 1D). The *lsd1* mutant comes from an Agrobacterium-mutagenized population of Arabidopsis, and this transformation procedure can generate non-T-DNA-associated mutations (Feldmann, 1991). We subcloned and sequenced wild-type genomic DNA fragments at this position and compared their sequences to several databases, including the Arabidopsis EST database (Rounsley et al., 1996, http://www.tigr.org/tdb/at/at.html). One EST clone (EST 82D11T7) exhibited blocks of identity to our genomic DNA sequence, suggesting the presence of introns in the latter. Because the gene encoding this EST is largely deleted in *lsd1*, it became a candidate *LSD1* gene.

Complementation of *lsd1*

To confirm that the gene encoded within the genomic deletion was *LSD1*, we constructed subclones from genomic phage (Figure 1D) for complementation into the T-DNA binary vector pSGCGF (see Experimental Procedures). Because the method for generation of transgenic Arabidopsis, vacuum infiltration with Agrobacterium carrying binary T-DNA vectors, triggers the *lsd1* phenotype, we devised an alternate complementation strategy. We transformed *lsd1* × Col-0 F1 plants and identified hygromycin-resistant (F2) transgenics. Among these, we identified individuals homozygous for Ws-0 alleles at 5F7R-1.5, 1H1L-1.6, and 8A6-1.3, and thus *lsd1*/*lsd1* homozygous mutants. These individuals contained both mutant and wild-type alleles for the CAPS marker that spans the *lsd1* deletion, because a wild-type allele is present on the transgene. These plants were treated with droplets of 2,6-dichloroisonicotinic acid (INA), an inducer of SAR and the *lsd1* phenotype (Uknes et al., 1993a; Dietrich et al., 1994). If the mutations were complemented, then INA treatment should not lead to spreading cell death. Table 1 shows that transgenic plants carrying either a 7 kb XhoI fragment or a 4.5 kb PstI–XhoI (Figure 1D) all survived this treatment. Selfed F3 progeny from a complemented F2 individual carrying each fragment were also analyzed. All F3 progeny that inherited the transgene were complemented (Table 1), while all of their nontransgenic sibs still exhibited the *lsd1* phenotype (data not shown). In addition, we identified F2 transformants carrying the 7 kb XhoI fragment that were heterozygous at the CAPS markers flanking *lsd1*. Selfed progeny from these should segregate both the transgene and the *lsd1* mutation. Among these progeny, we identified F3 individuals that were homozygous Ws-0 through the *lsd1* interval and carried the transgene. As shown in Table 1, these also were all complemented for protection against INA-induced spreading cell death. We conclude that the 4.5 kb PstI–XhoI fragment carries the *lsd1* gene and sufficient *cis* control elements to ensure its expression.

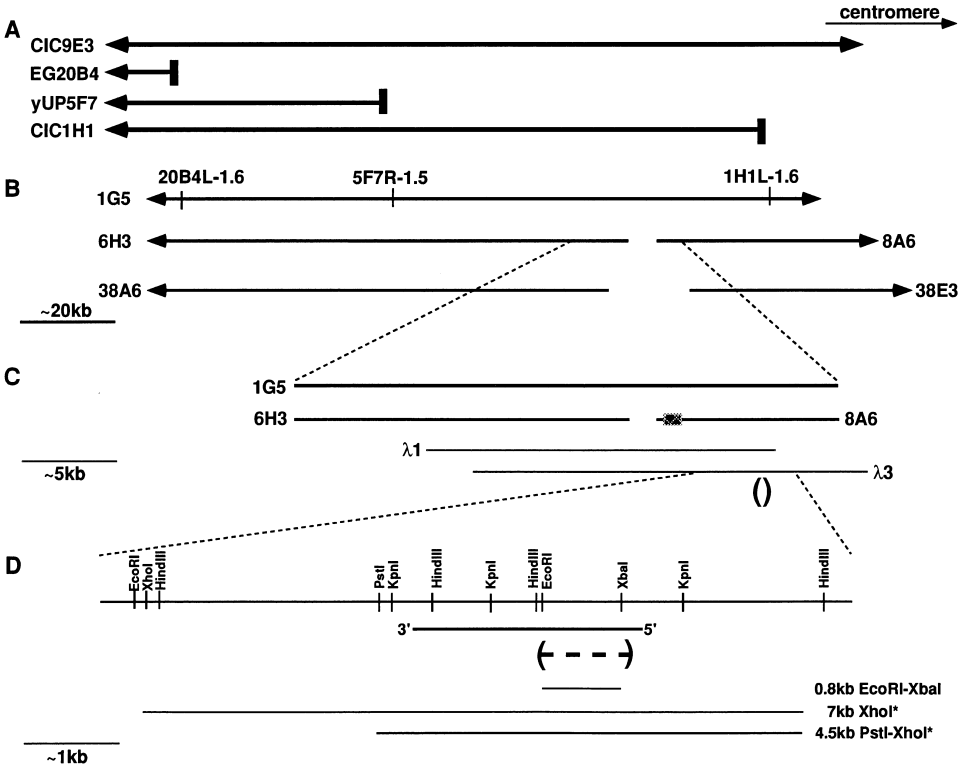

Figure 1. Physical Delineation of the *lsd1* Mutation

(A) YAC clones at *lsd1*. Arrowheads imply YAC clone extension, solid vertical black bars denote YAC ends used to isolate genomic phage clones and subsequently converted into CAPS markers.

(B) The three BAC clones denoted contained the CAPS markers listed above BAC 1G5. Arrowheads imply BAC clone extension. The scale in (A) and (B) is the same.

(C) Genomic phage clones positioned under an expansion of three of the BACs. Diamond-filled bar represents the 8A6-1.3 clone, which cosegregated with *lsd1*, used to isolate these phage. The *lsd1* deletion is noted at bottom by parentheses.

(D) Restriction map around *lsd1*. The extent of the deletion is shown in parentheses, as is the extent of hybridization of the various restriction fragments with *lsd1* cDNAs. Two genomic restriction fragments used in complementation experiments are shown at bottom. The asterisk refers to an XhoI site derived from the λ phage cloning junction.

All transgenic plants complemented for the INA-induced *lsd1* mutant phenotype were also complemented for initiation of spreading cell death after transfer to nonpermissive long day conditions as well (Dietrich et al., 1994; data not shown). Thus, the complementing DNA corrects the mutant phenotype induced by two independent stimuli.

Table 1. Complementation of the *lsd1* mutant

	Number of Plants Complemented/ Number of Transgenics Tested From:	
Construct	Independent F2s	Transgenic F3 Progeny
7 kb XhoI	1/1[a]	20/20[b]
	3/3[c]	21/21[c]
4.5 kb PstI–XhoI	2/2[a]	14/14[b]
35S cDNA	1/1[a]	19/19[b]

[a] Selected for hygromycin resistance and screened for homozygous Ws-0 alleles through the *lsd1* genetic interval as described in text, except where noted in (C). Individual F2s were both drop tested with INA and shifted to LD conditions.

[b] Selfed progeny from a complemented F2 individual (homozygous Ws-0 alleles through the *lsd1* interval) were screened by PCR at F3 for presence of the hygromycin resistance gene and then INA tested.

[c] F2 parents were identified as hygromycin resistant and heterozygous through the *lsd1* interval, then selfed and rescreened as hygromycin resistant and homozygous Ws-0 through the *lsd1* interval at F3 before INA testing.

Identification of Alternately Spliced *LSD1* Transcripts

We sequenced the complementing 4.5 kb PstI–XhoI genomic DNA fragment and 8 independent cDNAs (see Experimental Procedures) and completed the sequence of the full 82D11T7 EST clone. We identified two classes of cDNAs expressing open reading frames of either 184 or 189 amino acids (Figure 2). An alternate splice adds 61 bp to the 5′ region of some cDNAs and also provides an alternate translation start (underlined in Figure 2) that adds 5 amino acids. The cDNA sequences matched the genomic sequence, except at the positions of 7 introns (Figure 2). Nucleotide 1 of the longest cDNA is at position 1891 in the 4.5 kb PstI–XhoI genomic sequence. Thus, 1891 nucleotides of promoter are sufficient for appropriate expression in complementation of the *lsd1* mutation. The cDNA 5′ ends are clustered (data not shown), suggesting that the longest could be full length. We also complemented the *lsd1* mutation by transformation of

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1     cttacgcgtcatgtaaaaaaaaaagaagcgtaaattacgaaaaacagagagataaatccgggcattgagattttggagatagagagagagaaaaatcgaa
101   atctattgtctatctcctcaatttggattggattttctgcatatcatcgctctagatttcgcgggttttggattcgattccttaccctttctccaatcgaa
201   gtttttgggtttgaattggatttgggtttcgttccaaaatcagctctttttgttaatcaggtttcatctgtgtgggtcttgttttgaagcaatttgtgt

301   gtgtttggatgaaagtagcagatatgcaggaccagctggtgtgtcatggttgtaggaatttattgatgtatcctagaggagcatctaatgtgcgttgtgc
            M  K  V  A  D  M  Q  D  Q  L  V  C  H  G  C  R  N  L  L  M  Y  P  R  G  A  S  N  V  R  C    30

401   gttatgtaacactatcaacatggttcctcctcctcctccacctcacgacatggcacacattatatgtggtggttgtagaacaatgcttatgtatacgcgt
 31   A  L  C  N  T  I  N  M  V  P  P  P  P  P  P  H  D  M  A  H  I  I  C  G  G  C  R  T  M  L  M  Y  T  R   64

501   ggggctagtagcgtaagatgctcttgctgtcaaactacgaaccttgtgccagcgcactccaatcaggttgcccatgctccttccagtcaggttgcgcaga
        G  A  S  S  V  R  C  S  C  C  Q  T  T  N  L  V  P  A  H  S  N  Q  V  A  H  A  P  S  S  Q  V  A  Q   97

601   tcaattgtgggcattgtcggacgaccctcatgtatccttacggtgcatcatccgtcaaatgcgctgtttgtcaattcgtaactaacgttaatatgagcaa
        I  N  C  G  H  C  R  T  T  L  M  Y  P  Y  G  A  S  S  V  K  C  A  V  C  Q  F  V  T  N  V  N  M  S   130

701   tggaagggtacctctcccaactaaccggccaaatggaacagcttgtccccctctacatcaacttcaacaccaccctctcagacccaaaccgttgttgta
      N  G  R  V  P  L  P  T  N  R  P  N  G  T  A  C  P  P  S  T  S  T  S  T  P  P  S  Q  T  Q  T  V  V  V  164

801   gaaaaccccatgtccgttgatgaaagcggaaagttggtgagcaatgttgttgttggagtgacaactgacaaaaagtaatcaagaatgagtgagatcttaa
        E  N  P  M  S  V  D  E  S  G  K  L  V  S  N  V  V  V  G  V  T  T  D  K  K  •                    189

901   agatcaaatccaaattcttcctctattcctgcgtttggtttgtgcatattacatacgcggaaaaactgtatgttatatatctcttgactccttttaacc
1001  caagagaaaaagcttatcagaatctcttgttactgcattattggggtttattcaaagttgaagacacaaggtttttgctcgaataatttggcattctttt
1101  gctccatggaacttgaccttctcttctgttagttgacttctaaaactccatcggcccttgtggcattgttaatgtatgtatgaatataatctgatacacc
1201  aaccaatcattaacatttgggtttg
```

Figure 2. *LSD1* cDNA Sequence and Predicted Translation of the LSD1 proteins

Nucleotide position 1 is the 5′ end of the longest cDNA and position 1225 is the 3′ end of the most 3′ cDNA. Bold a55 is the 5′ start of the *lsd1* mutant deletion; the 3′ border is in the fourth intron (data not shown). Amino acid residues are numbered at the right, beginning with the Met of the long splice variant. Bold C residues in the amino acid sequence are the six cysteines of the three C-x-x-C zinc finger domains. Single underlines are the nucleotides and amino acids added in the long alternate splice product. Double underlines are splice junctions. Intron sizes in bp are: intron 1, 88; 2 (short splice), 68; 2 (long splice), 129; 3, 89; 4. 489; 5, 100; 6, 92; 7, 87.

the full insert from EST clone 82D11T7 expressed from the strong and constitutive cauliflower mosaic virus 35S promoter (see Table 1), proving that this cDNA contains the entire *LSD1* coding region. No other significant open reading frames were observed in the 4.5 kb PstI–XhoI genomic clone.

A rare mRNA of approximately 1.2 kb was detected in RNA blots from leaf tissue of wild-type Ws-0 plants probed with EST 82D11T7 (Figure 3). This length is consistent with the size of the longest cDNA, supporting the conclusion that we have identified a nearly full-length transcript. Importantly, this mRNA was completely lacking in mRNA prepared from *lsd1* leaves, furthering the argument that it encodes LSD1. The finding that *lsd1* is an mRNA null allele was corroborated by sequencing across the genomic deletion in the mutant (Figure 2). Expression of *LSD1* mRNA was unaffected by application of INA (Figure 3, top). The expected high level of INA-induced PR-1 mRNA accumulation in leaves of both wild-type and *lsd1* (Figure 3, middle) demonstrates the efficacy of INA treatment.

The *lsd1* phenotype can be observed in all cell types examined after initiation of lesion formation (Dietrich et al., 1994). RNA blot analysis of seedlings, stems, leaves, and flowers demonstrated that the *LSD1* gene is expressed constitutively in each of these Arabidopsis tissues (data not shown). Thus, the requirement for *LSD1* activity in these tissues is consistent with the expression pattern of the gene.

The *LSD1* mRNA Encodes a Novel Zinc Finger Domain

We searched a variety of databases with the predicted translation product of the *LSD1* cDNA sequence. Several striking features emerged. First, there are three zinc finger domains (Figure 4A) that share remarkable internal identity with one another. These are C-x-x-C, or type IV,

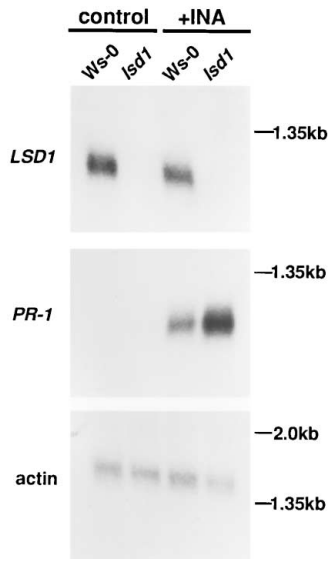

Figure 3. The *lsd1* Mutation Is an mRNA Null Allele

RNA blots (1 mg of poly(A)$^+$ RNA) from leaf tissue of 5-week-old plants kept in short days (permissive for *lsd1* growth) 3 days after spraying with either INA or wettable powder control. Spreading *lsd1* lesions had just started to appear at the time of leaf harvest. Probes were purified inserts from the *LSD1* cDNA as represented by EST 82D11T7 (top), a PR-1 cDNA (Uknes et al., 1993b), and an actin cDNA. The blot was probed successively in the order displayed. Similar results were observed by probing with the 0.8 kb EcoRI–XbaI genomic fragment covering the *lsd1* deletion shown in Figure 1C.

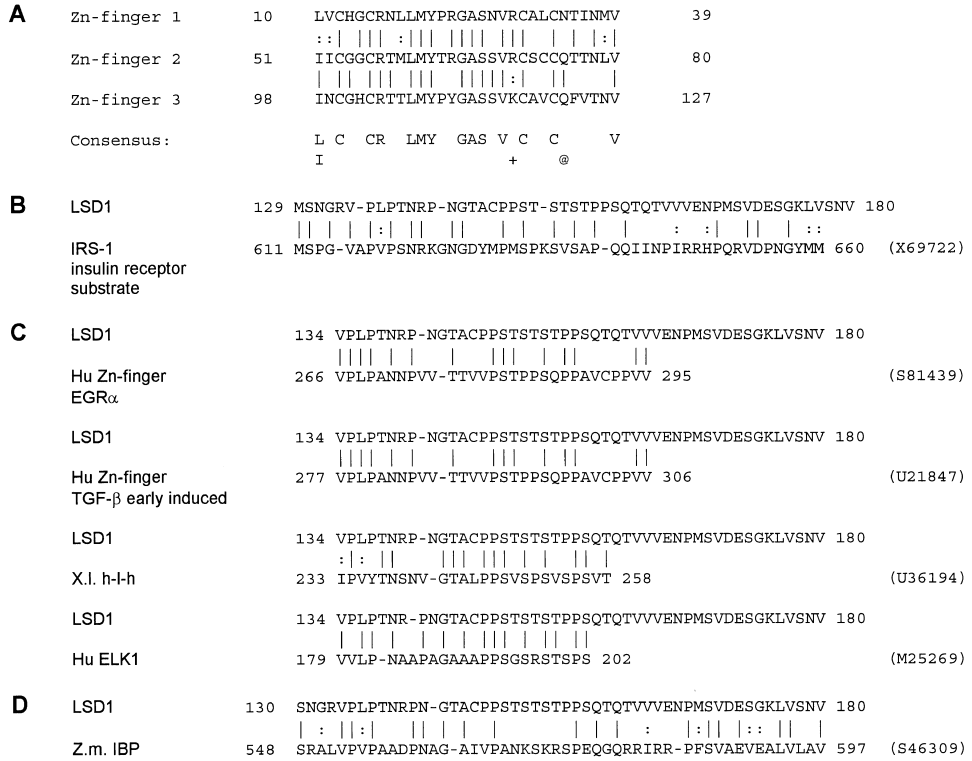

Figure 4. Deduced Structural Features of LSD1

(A) Alignment of the three LSD1 zinc finger domains. Numbers at left and right refer to amino acid residue position in the deduced LSD1 protein from figure 2. Vertical lines indicate pairwise identity; colons indicate conservative substitutions. A consensus sequence is listed below, with conservative substitutions noted. In the second line of consensus, (+) is basic, positively charged; (@) is polar, uncharged, hydrophilic amide.

(B–D) The carboxyl portion of the deduced LSD1 protein is related to known DNA-binding and transcription factors. Accession numbers for each protein are listed at right. (B) shows homology of LSD1 with mammalian insulin receptor substrate proteins. The LSD1 translation product is shown on top, aligned with the mouse insulin receptor substrate. All mammalian insulin receptor substrates are identical in this region. (C) shows homology of LSD1, top line in each comparison, with four known transcription factors: a human early growth response (EGRα) zinc finger protein, a human TGF-β early induced zinc finger protein, a Xenopus laevis H-L-H transcription factor, and the human ELK-1 protein. (D) shows homology of LSD1 with a putative maize transcription initiator binding protein.

zinc fingers, according to the classification of Sánchez-García and Rabbitts (1994), and they are most similar to plant relatives of the GATA-1 transcription factor (Evans and Felsenfeld, 1989; Omichinski et al., 1993). The plant members of this subfamily described to date include the *CO* gene, which controls transition to flowering (Putterill et al., 1995), a set of related DNA-binding proteins (Yanagisawa, 1995; De Paolis et al., 1996), and a gene whose transcription is salt stress–induced (Lippuner et al., 1996). None of these proteins shares with LSD1 the consensus homology within the zinc fingers. The second homology domain is derived from the carboxyl portion of LSD1, from residues 129 to 180 (Figures 4B–4D). This region of LSD1 exhibits homology to three broad classes of regulatory proteins: all mammalian insulin receptor substrates, a set of animal transcription factors, and a maize transcription initiator binding protein.

The conceptual LSD1 translation product also identified three additional Arabidopsis ESTs via their predicted amino acid homology. Importantly, each has at least one C-x-x-C zinc finger and most of the associated consensus residues found in the LSD1 internal homologies. They are ESTs 172A7T7, 132J21T7, and 199N11T7. Thus, it is probable that LSD1 is the first member of a widely distributed zinc finger subfamily in plants, defined by the internal homology within each zinc finger.

Discussion

The HR is a nearly ubiquitous feature of plant disease resistance reactions. Positive regulation of pathogen recognition initiates a stereotypic set of physiological responses. These rapid responses include ion fluxes, oxidative burst, defense gene activation, and the HR. Parts of this cascade are linearly regulated in at least some systems: blocking of Ca^{2+} influx blocks anion channel activity, the oxidative burst, and downstream events, which can include cell death; blocking anion channels effects only ROI production and defense gene activation, but not Ca^{2+} influx (Nürnberger et al., 1994; Levine et al., 1996; May et al., 1996; Jabs et al., 1997). It is probable that intracellular signal transduction pathways culminate in a variety of effector functions that may be stimulus dependent. This is supported by observations that blocking of the oxidative burst prevents both defense gene transcriptional activation and accumulation of potentially antimicrobial phytoalexins in one system (Nürnberger et al., 1994), while the oxidative

burst and phytoalexin biosynthesis are separable in others (Davis et al., 1993; Rustérucci et al., 1996). Similarly, related *R* genes can interfere with the function of one another and can activate separable downstream defense responses (Reuber and Ausubel, 1996; Ritter and Dangl, 1996).

Negative regulation of the HR and plant defense responses in toto is implied by mutants in several plant species that exhibit spontaneous HR-like lesions in the absence of pathogen. Whether the cell death associated with the overall class of lesion mimic mutations is invariably related to control of the normal disease resistance response pathway is debatable (reviewed by Dangl et al., 1996). While *R* gene action is cell autonomous (Bennetzen et al., 1988), parts of the extracellular *R* gene–dependent responses described above are not. Thus, plants must have mechanisms controlling the spread of positively acting defense signals to neighboring cells. Candidates for direct negative control of both cell death and disease resistance responses are the genes defined by the recessive Arabidopsis *lsd1* and *acd2* mutations (Dietrich et al., 1994; Greenberg et al., 1994) and alleles of the recessive barley *ml-o* gene (Wolter et al., 1993). *ml-o* alleles condition resistance to powdery mildew caused by Erysiphe graminis f. sp. hordei and exhibit variable levels of spontaneous cell death. Mutations in each of these three genes are further characterized by derepressed defense responses. Their phenotypes show that at least some perturbations leading to misregulation of cell death control are also loss of negative regulatory functions normally operative in dampening or down-regulating the resistance response.

The null *lsd1* phenotype suggests that the wild-type product is a negative regulator of cell death. In addition, *lsd1* reacts to both virulent pathogens and to chemicals that trigger SAR, with an HR-like response. But it is important to note that *lsd1* expresses wild-type timing of *R* gene–driven HR (Dietrich et al., 1994). Thus, cell-autonomous signals required for *R* gene function are intact in *lsd1*, but the response to cell-nonautonomous signals emanating from cells undergoing HR is perturbed. Collectively, these features of the mutant phenotype suggest that LSD1 limits both the initiation of defense responses and the subsequent extent of the HR. The fact that *lsd1* is hyperresponsive to signals initiating the defense response and HR-like cell death additionally suggests that these pathways are functionally intact in the wild-type cell but require a threshold level of signal for full activation. If so, then LSD1 functions to dampen these responses until the critical signal threshold is reached. Such a function is reminiscent of the "social control" of animal cell death (Raff, 1992) whereby cell death is a default for any cell not receiving and correctly processing cytokine and positional signals from its neighbors.

A model explaining how LSD1 dampens both cell death and disease resistance response functions in the cell should be compatible with the cell-nonautonomous mutant phenotype of cell death propagation once it is initiated, low level constitutive expression of *LSD1* mRNA, and possible LSD1 function predicted from its sequence. In our model, LSD1 acts as a transcription factor (or as a protein that sequesters a transcription factor). As outlined above, the oxidative burst in an infected cell generates a superoxide-dependent signal up-regulating the HR pathway. This signal overcomes the negative regulatory function of the available LSD1 and drives primary responding cells into the HR pathway. Additionally, cells undergoing HR amplify the signal to neighboring cells, probably via a sustained extracellular oxidative burst. The primary signal molecule might diffuse over short ranges (Levine et al., 1994), could act as an autocrine signal, and could lead to the accumulation of a secondary signal molecule in a steep spatial gradient from the infection site. A threshold is reached in this gradient. Above it, the prodeath pathway operates; below it, the prodeath response would be attenuated by LSD1. Such a gradient is formed by SA and SA conjugates (Enyedi et al., 1992), SA biosynthesis can be induced by hydrogen peroxide (Leon et al., 1995), and subeffective doses of SA can amplify pathogen-derived signals (Kauss et al., 1992; Kauss and Jeblick, 1995; Mauch-Mani and Slusarenko, 1996). Thus, an SA gradient could dictate LSD1 activity.

This model does not require that LSD1 activity be up-regulated. The constitutive expression levels could suffice to protect cells below the critical signal threshold for death induction. There is, however, the time lag of 12–16 hr observed between superoxide production initiated in *lsd1* by a variety of triggers and the onset of cell death (Jabs et al., 1996). This provides sufficient time for up-regulation of LSD1 activity before irrevocable commitment to death. If so, then cell death could spread until sufficient active LSD1 accumulates. Alternatively, this time lag could represent a requirement for biosynthesis of prodeath intermediates, and LSD1 normally could operate by interdicting this pathway. LSD1 could positively regulate anti-cell death targets, including genes involved in cell survival, ROI detoxification, or degradation of a key intermediate in the prodeath pathway. Alternatively, LSD1 could act as a transcriptional repressor directly on genes in the prodeath effector pathway. This scenario differs from the first only in that the set of target genes would be different. The availability of extragenic suppressors of *lsd1* will aid in identifying LSD1 targets (Jabs et al., 1996; M. H. R., R. A. D., and J. L. D., unpublished data).

This model explains the runaway cell death phenotype of the *lsd1*. In the absence of LSD1, the threshold normally required before commitment to HR is removed. Thus, minimal up-regulation of the superoxide-dependent signal drives the cell into the HR pathway. Hence, the ability of *lsd1* to respond to virulent pathogens as if resistant derives from the lack of background inhibition of the HR pathway normally operating in the cell. Extracellular superoxide produced during the oxidative burst initiates the same series of events in cells immediately surrounding the site of initiation, and the cell death propagation indicative of the *lsd1* phenotype results. Because the null *lsd1* mutant still requires superoxide for initiation of cell death propagation, it is unlikely that superoxide regulates LSD1 activity directly. This further suggests that a superoxide-dependent signal is the autocrine that propagates the response to neighboring cells.

The predicted homology of LSD1 to a variety of zinc

finger proteins and transcription factors is consistent with these speculations. Zinc finger proteins of the C-x-x-C subclass have known roles in transcriptional activation (Sánchez-García and Rabbitts, 1994), and some plant members of the GATA-1 subfamily have been demonstrated to be DNA-binding proteins (Yanagisawa, 1995; De Paolis et al., 1996). The predicted LSD1 protein sequence does not, however, share homology flanking the C-x-x-C domains with these, or any other, GATA-1 type transcription regulators. This flanking domain is known to mediate selective zinc finger contacts with DNA (Kaptein, 1991; Omichinski et al., 1993). Lack of homology in this domain might imply an LSD1 DNA target different from the canonical GATA site. There is also precedence for a GATA-1 type zinc finger protein acting as a repressor. The Drosophila *PANNIER* gene directly down-regulates genes required for neural precursor cell development (Ramain et al., 1993). Mutant *pannier* alleles with alterations in the cysteine residues of the zinc finger result in dominant loss of function mutations, suggesting that this protein functions in association with another, or perhaps as a homodimer. It will be informative to assess the relative roles for the alternately spliced forms of *LSD1* and to construct *lsd1* alleles with mutations analogous to those *pannier* alleles.

If LSD1 requires activation, then its response to ROI-dependent signals could be broadly analogous to the activation of preexisting cytoplasmic NF-κB. NF-κB is known to be activated by a variety of signals, including indirect activation by ROI (reviewed by Baeuerle and Baltimore, 1996; Baldwin, Jr., 1996). In addition, NF-κB activation can be both pro- or anti-apoptotic, depending on the nature and intensity of the signal and the cellular context (Grimm et al., 1996). In this regard, it should be noted that the A20 zinc finger protein sequesters NF-κB as part of a multimeric complex and thus inhibits its activation (Song et al., 1996). The sequestration function maps to the A20 zinc finger domain. In addition to NF-κB, the activity of at least one zinc finger protein transcription factor, *egr-1*, is sensitive to the cellular redox state (Nose and Obha, 1996).

The proteins with which LSD1 shares homology at its carboxyl terminus (Figure 4) also mediate rapid responses to extracellular stimuli. Some act as transcription factors, yet they utilize a divergent set of DNA-binding domains. The human Egrα and TGF-β early-induced proteins each possess three zinc finger DNA-binding domains, but of a different class than LSD1 (Blok et al., 1995; Subramaniam et al., 1995). In contrast, the Xenopus Hairy/Enhancer of Split homologue and the human Elk-1 protein use bHLH (Dawson et al., 1995) and ETS domains, respectively, for DNA binding (Janknecht and Nordheim, 1992). The mammalian insulin receptor substrate is tyrosine phosphorylated (Keller et al., 1993; Araki et al., 1994), and no role for this molecule in transcription has been described. The last protein sharing this carboxy-terminal homology with LSD1 was described as a transcriptional "initiator binding protein" from maize (Lugert and Werr, 1994). There is no specific function attributed to the domain of homology shared by these proteins and LSD1, and in none of these cases does the domain of homology with LSD1 appear at the carboxyl terminus.

These speculations for LSD1 function are consistent with physiological data discussed above. Genetic experiments have identified other loci encoding positively acting components of disease resistance pathways (see Hammond-Kosack and Jones, 1996), and double mutant analysis suggests that *LSD1* is also involved in regulating flux through such pathways (U. H. Neuenschwander, R. A. D., J. L. D., and J. Ryals, unpublished data; see Dangl et al., 1996; Ryals et al., 1996). Further analysis of the function of LSD1 in the context of emerging signal transduction models for control of plant cell death and disease resistance using both genetic and physiological tools will be rewarding.

Experimental Procedures

Care and Maintenance of Plants

Plants were grown in a chamber with 9 hr of light per day, at 22°C day temperature and 20°C night temperature, essentially as described (Dietrich et al., 1994).

Isolation of DNA and RNA, Probe Preparation, and Cloning

Small-scale genomic DNA preps were from ~1 cm long rosette leaves (Lukowitz et al., 1996). The DNA pellet was resuspended in 50 μL of TE, and 1 μL was used in a 20 μL PCR reaction. Large-scale genomic DNA preps were done based on the protocol of Rogers and Bendich (1985), modified such that CTAB concentration in the 2× CTAB buffer was increased to 3% and the precipitated DNA was resuspended in TEN buffer and digested with 100 μg/mL RNAse, followed by two extractions with chloroform/iso-amyl alcohol and a final precipitation. RNA was isolated by grinding of fresh tissue in liquid nitrogen to a fine powder and extraction in 1 mL of Trizol reagent (Gibco-BRL) per 100 mg of tissue fresh weight, according to the protocol of the manufacturer. Poly(A)$^+$ RNA was isolated using DynaBeads (Dynal). RNA blots were formaldehyde agarose gels and contained either 15 μg of total RNA or 1 μg of poly(A)$^+$ RNA. HyBond filters for DNA or RNA blots (Amersham) were hybridized in 6 × SSC, 5× Denhardt's solution, 0.1% SDS, and 100 μg/mL sheared Herring sperm DNA at 65°C. Washes were in 0.2 × SSC, 0.1% SDS at the same temperature. RNA blots were stripped for rehybridization in 5 mM TRIS/2 mM EDTA, (pH 8.0), 0.1× Denhardt's solution for 1 hr at 65°C.

Isolation of New CAPS Markers and Genetic Mapping of *lsd1*

After establishing linkage to the *AG* CAPS marker (Konieczny and Ausubel, 1993), we subcloned and end-sequenced a 1.6 kb HindIII fragment from the RFLP cosmid marker g3883 (position 73.5 on the Arabidopsis RI map; Lister and Dean, 1993; see http://nasc.nott.ac.uk/RI_data/top_frame.html) and primers designed based on this sequence. This primer set amplified a RAPD marker (size difference in Ws-0 versus Col-0 without restriction digestion), and map data generated using this marker allowed us to place *lsd1* below (telomeric to) it. Probe B9-1.8, isolated as a 1.8 kb SstI–EcoRI fragment from the JGB9 genomic phage clone (RI map position ~75; gift of Dr. George Coupland, Cambridge Laboratories, Norwich, UK) was converted into a CAPS marker. Mapping of this polymorphism placed *lsd1* above (centromeric to) it. Using DNA from F2 individuals, recombinants were identified as homozygous for one of these CAPS markers and heterozygous for the other. F3 progeny from these recombinants were then scored as either homozygous *lsd1*, segregating *lsd1*, or homozygous wild-type for lesion spread. All CAPS markers we developed are described at AApDB.

Map Refinement

YACs were defined (Schmidt et al., 1995; Schmidt et al., 1996, http://genome-www.stanford.edu/Arabidopsis/JIC-contigs.html) and confirmed by DNA blotting to establish a contig, and their ends were isolated by vectorette PCR as described (Matallana et al., 1992). These ends were also used to isolate genomic phage from a Ws-0 genomic library. Insert fragments of 1–3 kb were cloned into pBS

and end-sequenced for derivation of primers identifying new CAPS. PCR conditions (DNA Engine MJ Research) for all CAPS primer pairs except 8A6-1.3 and *lsd1* deletion primers are: 92°C for 3 min; 35 cycles of denature at 92°C for 30 s, anneal at 50°C for 30 s, and extend at 72°C for 2 min 30 s; and 72°C for 3 min. For 8A6-1.3 and the *lsd1* deletion primer pairs, we used 53°C annealing.

Vector Construction for Complementation

The Agrobacterium vacuum infiltration procedure was used to generate transgenic plants (Bechtold et al., 1993). Vectors were derived from pGPTV-Hyg (Becker et al., 1992) as follows: pSGCGF was made by restricting pGPTV-Hyg with HindIII and SacI and replacing this fragment with a HindIII–SacI fragment containing the polylinker from pIC20H (provided by Steve Goff, Novartis, Research Triangle Park, NC). Either the 7 kb XhoI fragment or 4.5 kb PstI–XhoI genomic fragment was cloned into this, the former into the unique vector SalI site, the latter as a SacI–SalI fragment derived from an intermediate cloning step into pBS as a PstI–XhoI fragment. The pHyg35S vector was made by cloning a four enhancer–containing 35S promoter fragment as a HindIII–XbaI fragment into pGPTV-Hyg (provided by Dr. Douglas C. Boyes, Univ. of North Carolina, Chapel Hill). The EST 82D11 cDNA sequence was isolated as a SalI–XbaI fragment from pZL1 (Newman et al., 1994) and cloned into XhoI–XbaI–digested pHyg35S. INA was applied at 0.3 mg/mL powder containing 25% active ingredient, or 4 mM.

Cloning

The genomic Ws-0 library is in λGEM11. The cDNA library is an oligo-dT primed library prepared from poly(A)$^+$ Col-0 mRNA from leaves challenged with P. syringae DC3000 (*avrRpm1*) cloned into λZAPII (Stratagene) according to the instructions of the manufacturer.

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GenBank Accession Numbers

The accession number for the *LSD1* cDNA sequence reported in this paper is UU87833; for the 4.5 kb PstI–XhoI genomic DNA sequence, UU87834.

The accession numbers for EST clones 82D11T7, 172A7T7, 132J21T7, and 199N11T7 are T45220, R6552, T45809, and H76651, respectively.