The Pepper 9-Lipoxygenase Gene CaLOX1 Functions in Defense and Cell Death Responses to Microbial Pathogens\textsuperscript{1[C][W][OA]}

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Lipoxygenases (LOXs) are crucial for lipid peroxidation processes during plant defense responses to pathogen infection. A pepper (Capsicum annuum) 9-LOX gene, CaLOX1, which encodes a 9-specific lipoxygenase, was isolated from pepper leaves. Recombinant CaLOX1 protein expressed in Escherichia coli catalyzed the hydroperoxidation of linoleic acid, with a $K_m$ value of 113. 9 $\mu$m. Expression of CaLOX1 was differentially induced in pepper leaves not only during Xanthomonas campestris pv vesicatoria (Xcv) infection but also after exposure to abiotic elicitors. Transient expression of CaLOX1 in pepper leaves induced the cell death phenotype and defense responses. CaLOX1-silenced pepper plants were more susceptible to Xcv and Colletotrichum coccodes infection, which was accompanied by reduced expression of defense-related genes, lowered lipid peroxidation, as well as decreased reactive oxygen species and lowered salicylic acid accumulation. Infection with Xcv, especially in an incompatible interaction, rapidly stimulated LOX activity in unsilenced, but not CaLOX1-silenced, pepper leaves. Furthermore, overexpression of CaLOX1 in Arabidopsis (Arabidopsis thaliana) conferred enhanced resistance to Pseudomonas syringae pv tomato, Hyaloperonospora arabidopsidis, and Alternaria brassicicola. In contrast, mutation of the Arabidopsis CaLOX1 ortholog AtLOX1 significantly increased susceptibility to these three pathogens. Together, these results suggest that CaLOX1 and AtLOX1 positively regulate defense and cell death responses to microbial pathogens.

To effectively combat invasion by microbial pathogens, plants activate distinct defense responses that are specifically effective. Despite the presence of plant immune systems, many pathogens can evade or suppress host defense mechanisms. Lipoxygenase (LOX) pathways are crucial for lipid peroxidation processes during plant defense responses to pathogen infection (Casey and Hughes, 2004). Plant LOXs are key enzymes involved in the generation of fatty acid derivatives in oxylipin metabolism.

LOXs comprise a family of non-heme-iron-containing fatty acid dioxygenases, which are ubiquitous in plants and animals (Brash, 1999). LOXs catalyze the conversion of polyunsaturated fatty acids such as linoleic acid into hydroperoxides that are in turn converted to oxylipins. These primary products, which may cause oxidative damage to plant membranes during the hypersensitive response (HR; Slusarenko, 1996), are enzymatically metabolized into traumatin, jasmonic acid (JA), and methyl jasmonate (MeJA). These latter compounds are involved in diverse physiological functions in plant growth and development, senescence, and stress responses. Plant LOXs can be classified as 9-LOXs or 13-LOXs according to the position at which oxygen is incorporated into linoleic acid or linolenic acid, the most important substrates for LOX catalysis in plants (Feussner and Wasternack, 2002). LOX enzymatic activity initiates the different biosynthetic pathways that result in the accumulation of distinct oxylipins. The most understood functional aspects of oxylipin pathways have come mainly from studies of JA produced through the action of 13-LOXs but not 9-LOXs. The metabolism of 13-LOX has been described in tobacco (Nicotiana tabacum) leaves infected by an avirulent strain of Pseudomonas syringae pv phaseolicola (Kenton et al., 1999). During bacterial infection, JA accumulates in tobacco leaves prior to cell death (Kenton et al., 1999). The level of LOX activity and gene expression also increases in tobacco plants during infection with Phytophthora parasitica var nicotianae (Christophe et al., 1996; Rancé et al., 1998). However, the defense-related functions of 9-LOXs are not fully understood. Both 9-LOXs and oxidative processes are proposed to be involved in the HR of tobacco induced by the avirulent pathogen Pseudomonas syringae pv syringae (Montillet et al., 2005).
production of free fatty acid hydroperoxides via the 9-LOX pathway in tobacco is crucial for hypersensitive cell death induced by cryptogein, a purified protein from *Phytophthora cryptogea* (Rusterucci et al., 1999). The function of LOXs in defense against pathogens is likely to be related to the synthesis of fatty acid hydroperoxides and of volatile products with signaling functions (Rusterucci et al., 1999) and antimicrobial activity (Croft et al., 1993; Weber et al., 1999). Gao et al. (2007) recently suggested that oxylipin metabolism mediated by a specific 9-LOX, ZmLOX3, may be involved in fungal pathogenesis in maize (*Zea mays*). ZmLOX3 loss-of-function mutants are susceptible to *Aspergillus flavus* and *Aspergillus nidulans* infection (Gao et al., 2009).

LOX activity may initiate the synthesis of signal molecules or induce structural and metabolic changes in the cell, ultimately leading to cell death that has been termed the HR (Maccarrone et al., 2001). Plant cell death occurs during various phases of development, senescence, and responses to abiotic and biotic stresses, and in particular, in response to pathogen invasion (Morel and Dangl, 1997). Activation of LOXs in plants may be involved in cell death induced by pathogens (Buonaurio and Servili, 1999; Rusterucci et al., 1999). The induction of HR-like cell death by the activation of the 9-LOX-encoding gene *GhLOX1* was shown in cotton (*Gossypium hirsutum*) plants during *Xanthomonas campestris* pv *malvacearum* infection (Marmey et al., 2007). LOX activity increases in parallel with the induction of HR symptoms in tobacco; however, in compatible interactions, LOX activity is delayed and reaches much lower levels (Montillet et al., 2002). In cotton, high LOX activity supports cell death during *X. campestris* pv *malvacearum* infection (Sayegh-Alhammad et al., 2008). The HR, an important defense reaction of plants to pathogen infection, is accompanied by lipid peroxidation processes. In particular, 9-LOX-dependent lipid peroxidation operates during cryptogein-induced HR in tobacco leaves (Rusterucci et al., 1999). In potato (*Solanum tuberosum*), lipid peroxidation occurs as a controlled and directed process that is facilitated by the action of a specific 9-LOX during the HR (Göbel et al., 2003; Montillet et al., 2005). *GhLOX1* is associated with salicylic acid (SA) accumulation during the HR of cotton to *X. campestris* pv *malvacearum* (Marmey et al., 2007).

The bacterial plant pathogen *Xanthomonas campestris* pv *vesicatoria* (*Xcv*) is the causative agent of bacterial spot disease on pepper (*Capsicum annuum*) and tomato (*Solanum lycopersicum*) plants. To identify genes involved in the HR-based innate immune response in pepper, we have isolated and functionally characterized defense-related genes encoding PR1 (for pathogenesis-related protein 1; Kim and Hwang, 2000; Hong and Hwang, 2005), chinatine (Hong et al., 2000), chinin-binding protein (Lee et al., 2001), thionin (Lee et al., 2000), SAR 8.2 (Lee and Hwang, 2003), peroxidase (Choi et al., 2007), and menthone reductase (Choi et al., 2008) from pepper leaves infected with the *Xcv* avirulent strain Bv5-4a. In this study, we used a cDNA macroarray method (Jung and Hwang, 2000) to isolate a novel pepper gene, *CaLOX1*, which encodes a 9-LOX and is specifically induced by avirulent *Xcv* infection of pepper leaves. The purified *CaLOX1* protein was expressed in *Escherichia coli* and investigated for LOX activity. Virus-induced gene silencing (VIGS) is a widely used, powerful technique for reverse genetics. VIGS vectors derived from the *Tobacco rattle virus* (TRV) are the most popular for VIGS. Recently, a VIGS method was established for the functional characterization of defense-related genes in pepper (Baulcombe, 1999; Burch-Smith et al., 2006; Choi et al., 2007; Chung et al., 2007). Here, we analyzed the effect of *CaLOX1* loss of function during pathogen infection using TRV-based VIGS of the *CaLOX1* gene. Arabidopsis (*Arabidopsis thaliana*) plants that constitutively overexpressed *CaLOX1* were also examined to determine the gain-of-function phenotype of *CaLOX1* in plant defense. We further functionally characterized the Arabidopsis mutants *lox1-1* and *lox1-2*, which have T-DNA insertions in *AtLOX1*, a putative *CaLOX1* ortholog. Analysis of the function of *CaLOX1* in pepper and Arabidopsis plants provided insight into the role of *CaLOX1* expression in defense responses and the hypersensitive cell death of plants following pathogen invasion.

**RESULTS**

**Isolation, Sequence Analysis, and LOX Activity of *CaLOX1***

We isolated pathogen-induced pepper genes using a pepper cDNA library prepared from pepper leaves inoculated with the *Xcv* avirulent strain Bv5-4a (Jung and Hwang, 2000). Among the *Xcv*-induced pepper cDNA clones, the pepper LOX gene *CaLOX1* was identified. *CaLOX1* contains 76 bp at the 5’ untranslated region and 184 bp at the 3’ untranslated region. Translation of the unique open reading frame present in *CaLOX1* indicated that it codes for an 861-amino acid protein with an estimated molecular mass of 97.9 kD and an estimated pI of 5.42 (Supplemental Fig. S1). Sequence alignment of the predicted amino acid sequence of *CaLOX1* showed that it shares identities greater than 75% identity with other plant LOXs from potato, tobacco, tomato, and Arabidopsis (Supplemental Fig. S2). In particular, *CaLOX1* showed the greatest match (92% identity) to potato LOX (Kolomiets et al., 1996). Analysis of the deduced amino acid sequence of *CaLOX1* identified two conserved LOX domains for plant LOX-related proteins: the PLAT LH (for polycystein-1, LOX, α-toxin) or LH2 (for lipoxygenase homology 2) domain and the LOX domain. The deduced *CaLOX1* protein contains conserved His residues (positions 547, 556, and 715) that are also observed in other plant LOXs and that have been implicated in iron binding and enzyme catalytic activity (Prigge et al., 1996).
The enzyme activity of CaLOX1 was tested by expressing full-length CaLOX1 in *E. coli* as a fusion protein with an N-terminal His tag. Purified recombinant CaLOX1 expressed in *E. coli* was detected on Coomassie Brilliant Blue-stained SDS-PAGE gels as an apparent single polypeptide (Fig. 1A). To assay enzyme activity, affinity-purified recombinant CaLOX1 was incubated with linoleic acid or arachidonic acid as a substrate at various concentrations (Fig. 1B). We first determined the effects of pH and substrate concentration on CaLOX1 activity. The optimal pH for purified CaLOX1 activity ranged from 6 to 6.5 (Supplemental Fig. S3A). Enzyme activity with arachidonic acid as a substrate was lower, whereas linoleic acid was a good substrate (Supplemental Fig. S3B). Optimal conditions for CaLOX1 enzyme activity were pH 6.0 to 6.5 and 0.1 to 1.5 mM with linoleic acid as a substrate at 25°C (Supplemental Fig. S3C).

We then calculated the kinetic parameters for the substrates linoleic acid and arachidonic acid under standard reaction conditions using a Michaelis-Menten plot analysis program (Fig. 1C). The *K_m* values of CaLOX1 for linoleic acid and arachidonic acid were 113.9 and 20.3 μM, respectively, and the *k_cat* values for the two substrates were 8.7 and 1.3 min⁻¹, respectively. The *K_m*, *V_max*, and *k_cat* values for linoleic acid were much higher than those for arachidonic acid, suggesting that linoleic acid has a significantly higher affinity.

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**Figure 1.** Purification, enzyme activity, and positional specificity of CaLOX1. A, SDS-PAGE of the recombinant His-tagged CaLOX1 fusion protein. Total protein extracts and purified protein were analyzed by 10% SDS-PAGE and stained with Coomassie Brilliant Blue. Lane M, Molecular markers (kDa); lane 1, uninduced soluble fraction of *E. coli* BL21 cell extracts expressing His-tagged CaLOX1 protein; lane 2, soluble fraction of *E. coli* BL21 cell extracts expressing His-tagged CaLOX1 protein after isopropyl β-D-thiogalactopyranoside induction; lane 3, Purified His-tagged CaLOX1 fusion protein. B, LOX activity of CaLOX1 as determined with various concentrations of linoleic acid as a substrate. Values are presented as means ± SD. C, Kinetic parameters of purified CaLOX1. Kinetic parameters were calculated using a Michaelis-Menten plot analysis program. Values represent the mean ± SD of three independent replicates. *K_cat* is defined to equal *V_max/E_l*. *E_l* is total enzyme concentration in molar.

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**Table 1.** Kinetic parameters of purified CaLOX1.

| Substrate          | *K_m*, μM | *V_max*, μmol min⁻¹ | *K_cat*, min⁻¹ | *K_cat/K_m*, min⁻¹ |
|--------------------|-----------|---------------------|----------------|-------------------|
| Linoleic acid      | 113.9±19.6| 17.8±1.5            | 8.7±0.7        | 0.076±0.006       |
| Arachidonic acid   | 20.3±7.3  | 2.6±0.2             | 1.3±0.1        | 0.062±0.005       |

Kinetic parameters were calculated using a Michaelis-Menten plot analysis program. Values represent the mean ± SD of three independent replicates.
for purified CaLOX1 than does arachidonic acid. Estimates of $k_{cat}/K_m$ of the purified CaLOX1 for linoleic acid and arachidonic acid were 0.076 and 0.062 $\mu M^{-1} min^{-1}$, respectively, suggesting that the catalytic efficiencies of both substrates for CaLOX1 are different. This indicates that linoleic acid is an in planta substrate suitable for CaLOX1.

To determine the positional specificity of CaLOX1, we further identified the reaction products from the CaLOX1 enzyme assay following the HPLC analysis. As shown in Figure 1D, 9-hydroperoxylinolenic (9-HPOT) acid, but not 13-HPOT acid, was mainly produced by the reaction of purified CaLOX1 with linoleic acid as a substrate, indicating that CaLOX1 is indeed a 9-specific LOX.

Expression of CaLOX1 in Response to Biotic and Abiotic Stresses

To determine the organ-specific expression of CaLOX1 in pepper plants, we performed RNA gel-blot analysis of expression of CaLOX1 in leaf, root, stem, flower, green fruit, and red fruit (Fig. 2A). The CaBPR1 gene was used as a comparable control (Kim and Hwang, 2000). CaLOX1 transcripts were slightly detected in green fruit but not in other pepper organs.

LOXs and their metabolites have been demonstrated to be involved in plant defense responses to diverse pathogens and stresses (Siedow, 1991; Porta and Rocha-Sosa, 2002; Liavonchanka and Feussner, 2006).

Expression profiles of CaLOX1 in response to the virulent Ds1 and the avirulent Bv5-4a strains of Xcv were examined in infected pepper leaves during both compatible and incompatible interactions (Fig. 2A). Northern analysis of total RNA extracted from infected leaves revealed that the CaLOX1 transcript accumulated in pepper leaves infected with virulent or avirulent Xcv strains. Expression of CaLOX1 was induced 1 h after infection by the virulent strain Ds1, peaked at 20 h, and decreased thereafter. In contrast, induction of CaLOX1 by the avirulent strain Bv5-4a was rapid, peaking at 5 h and strongly maintained 5 to 25 h after inoculation. Expression of CaLOX1 was also significantly increased by mock inoculation. These data indicated that the induction of CaLOX1 transcript in mock-inoculated or virulent Xcv-inoculated leaves may be due, in part, to the wounding effect.

Ethylene, SA, abscisic acid (ABA), methyl viologen, and MeJA are well-known modulators of defense responses in plants. Pepper plants at the six-leaf stage were used to study the CaLOX1 function in stress-induced responses. Treatment with ethylene, SA, NaCl, and methyl viologen significantly induced CaLOX1 expression in pepper leaves (Fig. 2B). However, CaLOX1 transcripts were not or were weakly detected in leaves treated with ABA, MeJA, cold, and wounding. In particular, treatments with ethylene and methyl viologen were very effective in triggering CaLOX1 expression.

Figure 2. RNA gel-blot analysis of pepper CaLOX1 and CaBPR1 expression genes in pepper leaves. The blots were hybridized with biotin probes for the CaLOX1 3’ untranslated region and full-length CaBPR1 cDNA. A, Expression of CaLOX1 and CaBPR1 in various organs of pepper plants and in pepper leaves at different time points after inoculation with the virulent strain Ds1 and the avirulent strain Bv5-4a of Xcv. B, Expression of CaLOX1 in pepper leaves at various time points after treatment with ethylene (10 mL L$^{-1}$), SA (5 mM), MeJA (100 mM), ABA (100 mM), NaCl (200 mM), methyl viologen (100 mM), cold (4°C), and wounding. H, Healthy leaves.
Induction of the Cell Death Response by Transient Expression of CaLOX1 in Pepper Leaves

To determine whether the CaLOX1 gene is involved in cell death, we transiently expressed CaLOX1 in pepper leaves infiltrated with Agrobacterium tumefaciens carrying 35S:00 (empty vector) or 35S:CaLOX1 construct. The empty vector control did not induce cell death or a necrotic response (Fig. 3A), whereas a necrotic phenotype was distinctly observed in pepper leaves infiltrated with different concentrations of Agrobacterium (35S:CaLOX1). Cell death lesions in plants can be visualized by fluorescence under UV light, indicating the cell death-related production of phenolic compounds (Dixon and Paiva, 1995). UV illumination of pepper leaves revealed that such compounds were synthesized and accumulated in regions surrounding the Agrobacterium (35S:CaLOX1)-infiltrated area (Fig. 3A). We confirmed the Agrobacterium-mediated transient expression of CaLOX1 in pepper leaves using an immunoblotting assay. Expression of c-Myc-tagged CaLOX1 was specifically detected in leaf tissues 24, 48, and 72 h after inoculation; however, the protein expressing 35S:c-Myc was not detected in pepper leaves (Fig. 3B). Cell death and hydrogen peroxide (H₂O₂) production in leaves transiently expressing CaLOX1 was visualized by trypan blue and 3,3'–diaminobenzidine (DAB) staining, respectively, but was not visualized at all, or only weakly, in leaves transiently expressing the empty vector (Fig. 3C). We employed an ion leakage test to analyze the severity of cell necrosis caused by plasma membrane damage. Pepper leaves that transiently expressed CaLOX1 exhibited more ion leakage 30 h after agroinfiltration than did empty vector control leaves (Fig. 3D). Changes in defense-related gene expression in leaves transiently expressing CaLOX1 and empty vector control were examined using real-time reverse transcrip-

Figure 3. Transient expression of CaLOX1 and cell death in pepper leaves infiltrated with Agrobacterium GV3101 carrying the 35S:00 or 35S:CaLOX1 construct. A, Visible and UV light-illuminated phenotypes of the transient expression in leaves 3 d after agroinfiltration with different bacterial concentrations (OD 600). B, Immunoblotting assay of the Agrobacterium-mediated transient expression of CaLOX1 in pepper leaves. C, Staining with trypan blue and DAB of transient expression in leaf tissues 1 d after agroinfiltration. Bars = 0.2 mm. D, Ion leakage from transiently expressing leaves at different time points after agroinfiltration. E and F, Induction of CaLOX1, CaBPR1, and CaDEF1 as measured by real-time RT-PCR. The data of the relative transcript levels are normalized to the expression of the 18S rRNA gene as an internal control. Values are presented as means ± so. Asterisks indicate significant differences between the 35S:CaLOX1 and 35S:00 constructs (Student’s t test, P < 0.05). H, Healthy leaves; hai, hours after infiltration.
tion (RT)-PCR. Expression of the CaLOX1, CaBPR1 (for basic pathogenesis-related gene), and CaDEF1 (for defense) genes was rapidly and strongly induced by transient expression of CaLOX1 (Fig. 3, E and F). The transcript levels of CaBPR1 and CaDEF1 increased continuously during the transient expression of CaLOX1, indicating that the CaLOX1-mediated expression of defense-related genes is required for hypersensitive cell death in pepper leaves.

Enhanced Susceptibility of CaLOX1-Silenced Pepper to Bacterial and Fungal Infection

To investigate the CaLOX1 loss-of-function phenotype in pepper, we used the VIGS technique to knock down the expression level of CaLOX1 with TRV vectors containing 621 bp of CaLOX1 cDNA (TRV: CaLOX1) or lacking the CaLOX1 insert (TRV:00). Three to 4 weeks after VIGS, empty vector control (TRV:00) and silenced (TRV:CaLOX1) plants were challenged with the virulent strain Ds1 (compatible) and the avirulent strain Bv5-4a (incompatible) of Xcv. Five and 15 h after inoculation with Xcv (10^8 colony-forming units [cfu] mL^{-1}), CaLOX1 transcript levels were analyzed by RT-PCR in empty vector and silenced plants. CaLOX1 expression was compromised in CaLOX1-silenced plants compared with empty vector control plants. We further evaluated whether CaLOX1 silencing affects the resistance response to Xcv infection. Expression levels of pepper defense-related genes in empty vector control and silenced plants were analyzed by RT-PCR. As shown in Figure 4A, avirulent Xcv infection strongly induced the CaBPR1 gene in empty vector control plants; however, CaBPR1 expression was weakly or not at all induced in CaLOX1-silenced plants infected with avirulent Xcv. The expression of CaPR4 (putative antifungal protein), CaPR5 (osmotin-like protein), CaSAR82A (SAR8.2), CaDEF1, and CaPOA1 (ascorbate peroxidase) in CaLOX1-silenced leaves was similar to that in empty vector control leaves during both virulent and avirulent Xcv infections. CaLOX1-silenced plants exhibited enhanced susceptibility to the Xcv virulent strain Ds1 and the avirulent strain Bv5-4a (Fig. 4, B and C). The Ds1 and Bv5-4a strains (5 × 10^4 cfu mL^{-1}) grew significantly less in empty vector control leaves than in CaLOX1-silenced leaves during infection, especially 4 d after inoculation. Five days after inoculation, more symptoms of susceptibility were observed in CaLOX1-silenced plants. We also stained Xcv-infected empty vector control and silenced leaves with trypan blue and DAB. Strongly polymerized DAB (indicative of H_2O_2 formation) and HR were detected in empty vector control leaves 24 h after inoculation with Bv5-4a, whereas HR and H_2O_2 accumulation were distinctly reduced in CaLOX1-silenced leaves. In compatible interactions with Ds1, both empty vector control and CaLOX1-silenced leaves did not exhibit H_2O_2 formation or hypersensitive cell death. In addition, we also measured H_2O_2 by xylanol orange assay, in which H_2O_2 is reduced by ferrous ions in an acidic solution that forms a ferric product-xylanol orange complex (Fig. 4D). Low levels of H_2O_2 were detected during the Xcv infection in CaLOX1-silenced leaves compared with empty vector control leaves. As shown in Figure 4E, cell death, which is assessed by monitoring cellular ion leakage as a measure of membrane damage, significantly declined in CaLOX1-silenced leaves during the Xcv infection. SA data obtained by HPLC analysis show that lower free and total SA (free SA plus Glc-conjugated SA) levels accumulated in CaLOX1-silenced leaves infected by the virulent (compatible) Xcv strain compared with empty vector control leaves (Fig. 4F). However, no significant differences in SA levels were detected between empty vector control and CaLOX1-silenced plants during infection, except the 18-h early stage of infection by the virulent Xcv (Supplemental Fig. S4A). Collectively, these results indicate that silencing of the CaLOX1 gene compromises the basal defense response of pepper to Xcv infection.

We then challenged control and CaLOX1-silenced pepper plants with the fungal pathogen Colletotrichum coccodes. Leaves of CaLOX1-silenced plants infected with C. coccodes exhibited more chlorotic lesions than did empty vector control plants (Fig. 5). High levels of expression of CaLOX1 were induced in C. coccodes-infected empty vector control plants at the six-leaf stage, whereas silencing of CaLOX1 compromised CaLOX1 expression in pepper leaves. Pepper defense-related genes, such as CaBPR1, CaPR10, CaSAR82A, and CaDEF1, were weakly induced in CaLOX1-silenced plants compared with empty vector control plants. To evaluate disease development in the inoculated plants, disease severity was rated based on a 0 to 3 scale, where 0 = no visible symptoms, 1 = small circular or irregular gray-brown spots on leaves, 2 = dark-brown lesions with mild chlorosis, and 3 = enlarged dark-brown lesions with severe chlorosis (Fig. 5B). The disease severity of CaLOX1-silenced plants infected with C. coccodes was greater than that of empty vector control plants (Fig. 5B). Seven days after inoculation, more susceptible chlorotic symptoms were observed in leaves of CaLOX1-silenced plants than in leaves of empty vector control plants. Together, these results indicate that the expression of CaLOX1 is involved in the resistance of pepper plants to Xcv and C. coccodes.

LOX Activity in Pepper Leaves

To determine whether CaLOX1 activity is induced by pathogen infection, we tested for LOX activity in protein extracts of pepper leaves infected with the
Figure 4. Enhanced susceptibility of CaLOX1-silenced plants to infection by the virulent strain Ds1 (compatible [C]) and the avirulent strain Bv5-4a (incompatible [I]) of Xcv (10^6 cfu mL\(^{-1}\)). A, RT-PCR analysis of the expression of CaLOX1 and pepper defense-related genes in empty vector control (TRV:00) and CaLOX1-silenced plants (TRV:CaLOX1). The experiments were performed three times with 28 PCR cycles. H, Healthy leaves; CaBPR1, basic pathogenesis-related protein 1; CaPR4, putative antifungal protein; CaPR5, osmotin-like protein; CaSAR8.2, SAR8.2; CaDEF1, defensin 1; CaPOA1, ascorbate peroxidase 1; 18S rRNA, control. B, Disease symptoms on leaves of empty vector control and silenced plants inoculated with Xcv Ds1 and Bv5-4a. Five days after inoculation, empty vector control plants showed only mild symptoms, whereas CaLOX1-silenced plants showed extensive lesions (left). Infected leaves were stained with trypan blue and DAB 1 d after Xcv inoculation (right). Bars = 0.2 mm. C, Bacterial growth in leaves of the empty vector control (TRV:00) and CaLOX1-silenced plants 0, 2, and 4 d after inoculation (5 \times 10^4 cfu mL\(^{-1}\)). D, Quantification of H\(_2\)O\(_2\) in leaves of empty vector control plants and CaLOX1-silenced plants 15 h after inoculation with 10^7 cfu mL\(^{-1}\) Xcv using the xylenol orange assay. E, Ion leakage from the leaf tissues at different time points after inoculation with Xcv. F, Levels of SA in the empty vector control and CaLOX1-silenced leaves infected by Xcv. FW, Fresh weight. Values are presented as means ± se. Asterisks indicate significant differences from wild-type plants (Student’s t test, \(P < 0.05\)). [See online article for color version of this figure.]
virulent Ds1 and avirulent Bv5-4a strains of Xcv (Fig. 6). Profiles of LOX activity in extracts from healthy and infected leaves are shown in Figure 6A. Infection with Xcv Bv5-4a induced significantly higher LOX activity in pepper leaves than did infection with Xcv Ds1; however, LOX activity remained at low levels in healthy leaves. We then examined LOX activity in unsilenced (TRV:00) and silenced (TRV:CaLOX1) pepper plants during Xcv infection (Fig. 6B). Empty vector control plants (TRV:00) displayed higher levels of LOX activity during infection with avirulent Xcv compared with those infected with the virulent strain. However, LOX activity was not significantly enhanced in CaLOX1-silenced plants by Xcv infection, indicating that silencing of CaLOX1 compromised LOX activity. These data suggest that pepper plants defend themselves against pathogen infection by activating the LOX pathway.

Figure 5. Enhanced susceptibility of CaLOX1-silenced pepper plants to C. coccodes infection. A, Expression of defense-related genes in leaves of empty vector control and silenced plants. H, Healthy leaves. The experiments were performed three times, and 28 PCR cycles were carried out. B, Disease symptoms and disease severity in leaves of empty vector control and silenced pepper plants 5 d after inoculation. Asterisks indicate significant differences between the means as determined by Student’s t test (P < 0.05). M, Means of disease severities. [See online article for color version of this figure.]

Figure 6. Enhanced activity of LOX in pepper leaves infected with Xcv. A, LOX activity in pepper leaves inoculated with the virulent (compatible) Ds1 and avirulent (incompatible) Bv5-4a strains of Xcv. B, LOX activity in leaves of unsilenced (TRV:00) and silenced (TRV:CaLOX1) pepper plants infected with the Ds1 and Bv5-4a strains. Linoleic acid (0.1 mM) was used as a substrate. All experiments were performed three times with similar results. C, Quantification of lipid peroxidation (MDA concentration) in leaves of empty vector control (TRV:00) and silenced (TRV:CaLOX1) pepper plants infected with Xcv. FW, Fresh weight. Values are presented as means ± SD. Different letters indicate significant differences as determined by the LSD test (P < 0.05). Asterisks indicate significant differences between wild-type and inoculated plants (Student’s t test, P < 0.05).
Induction of lipid peroxidation was also assessed by determining the accumulation of thiobarbituric acid-reactive substances in the pepper leaves infected by *Xcv* (Fig. 6C). The lipid peroxidation was significantly induced in pepper leaves by *Xcv* infection. Lower levels of malondialdehyde (MDA), which is a decomposition product formed by peroxidation of polyunsaturated fatty acids in the membranes, were detected in *CaLOX1*-silenced leaves than in empty vector control plants (TRV:00) during the *Xcv* infection. This indicates that the *CaLOX1* gene functions in lipid peroxidation of leaf tissues of pepper.

Enhanced Resistance of *CaLOX1*-OX Transgenic Arabidopsis to Bacterial Infection

To determine the gain-of-function phenotype of the pepper *CaLOX1* gene in planta, transgenic Arabidopsis lines that express a cauliflower mosaic virus 35S promoter-driven *CaLOX1* construct were generated using an *Agrobacterium*-mediated gene transfer system (Clough and Bent, 1998). At least 10 independent transgenic lines expressed *CaLOX1*, whereas *CaLOX1* transcripts were not observed in wild-type (ecotype Columbia [Col-0]) plants (Fig. 7A). Three *CaLOX1*-OX transgenic lines, 12, 15, and 16, were selected for further study. The *CaLOX1* transgene was constitutively expressed in the representative Arabidopsis transgenic line 12 during *Pseudomonas syringae* pv *tomato* (*Pst*) infection (Fig. 7B). We investigated time course expression of some defense-related genes such as *AtPDF1.2* and *AtPDF1.2*, which serve as markers in the SA and JA pathways, during infection with *Pst* DC3000 and *Pst* DC3000 (*avrRpm1*) in *CaLOX1*-OX transgenic plants. Induction of *AtPDF1.2*, but not *AtLOX1* and *AtPDF1.2*, greatly and rapidly increased in the transgenic plants during *Pst* infection compared with wild-type plants. The *CaLOX1*-OX transgenic plants, in which LOX is activated, may respond differentially to pathogen infection. We examined their responses to *Pst* DC3000 and to *Pst* DC3000 (*avrRpm1*). *Pst* DC3000 is an important bacterial pathogen and a valuable model organism to study plant-pathogen interactions (Quirino and Bent, 2003). Arabidopsis plants were inoculated with 10⁶ cfu mL⁻¹ *Pst*. As shown in Figure 7C, wild-type plants inoculated with *Pst* DC3000 showed disease symptoms, whereas a limited necrotic response was observed in the infected areas of leaves of transgenic lines 3 d after inoculation. Wild-type plants inoculated with *Pst* DC3000 (*avrRpm1*) showed a typical HR. Similarly, *CaLOX1* transgenic lines also showed enhanced resistance to *Pst* DC3000 (*avrRpm1*) compared with wild-type plants. We further quantified the expression of several defense-related genes in *CaLOX1*-OX Arabidopsis transgenic plants. *AtLOX1*, 9-position specific LOX, was strongly expressed in Arabidopsis leaves during *Pst* infection, especially in the incompatible interaction with *CaLOX1*-OX Arabidopsis, which is consistent with the recent finding of Bannenberg et al. (2009). *AtPR1*, which serves as a marker for induction of the SA pathway, was rapidly and strongly induced in *CaLOX1*-OX transgenic plants; however, *AtPDF1.2*, a marker gene for the JA pathway, was not at all expressed during *Pst* infection. To characterize the observed necrotizing process at the microscopic level, 5-week-old wild-type and transgenic plant leaves were infiltrated with *Pst* DC3000 and *Pst* DC3000 (*avrRpm1*). Cell death and H₂O₂ production were detected by staining with trypan blue (dark blue) and DAB (dark brown), respectively (Fig. 7D). A significant macroscopic HR was observed in transgenic plants 24 h after inoculation with *Pst* DC3000 but not in either wild-type or transgenic plants infected by *Pst* DC3000 (*avrRpm1*) (data not shown). In plants inoculated with *Pst* DC3000 (*avrRpm1*), the HR was more strongly induced in transgenic plants than in wild-type plants (Fig. 7D). The visual observation of DAB staining was quantified by measurements of H₂O₂ levels using xylenol orange assay (Fig. 7E). Levels of H₂O₂ significantly increased in *CaLOX1*-OX transgenic leaves during the virulent *Pst* DC3000 infection. *CaLOX1*-OX transgenic plants generated H₂O₂ and underwent significant cell death when inoculated with either the virulent or avirulent strain at 10⁶ cfu mL⁻¹. Treatment with MgCl₂ solution, as a negative control, did not lead to H₂O₂ production or to cell death in wild-type or transgenic lines. In this study, all three transgenic lines gave similar results.

The *CaLOX1* function was further studied by monitoring bacterial growth in *CaLOX1*-OX transgenic lines. We tested the growth of virulent *Pst* DC3000 and avirulent *Pst* DC3000 (*avrRpm1*) in wild-type and transgenic plants 0 and 3 d after inoculation with 10⁶ cfu mL⁻¹ (Fig. 7F). Bacterial growth immediately after inoculation (day 0) indicated that an equal quantity of bacterial inoculum was infiltrated into leaves of both wild-type and transgenic plants. Higher growth of *Pst* DC3000 and *Pst* DC3000 (*avrRpm1*) was observed in wild-type plants 3 d after inoculation compared with transgenic plants (Fig. 7F). To determine whether *CaLOX1* overexpression affects the SA pathway, we also quantified SA levels in wild-type and *CaLOX1*-OX transgenic leaves. Free and total SA (free SA plus Glic-conjugated SA) levels accumulated faster and greater in *CaLOX1*-OX transgenic leaves than in wild-type leaves during *Pst* DC3000 infection (Fig. 7G). These support the idea that the elevated SA levels may be due to the induction of *AtPR1* expression conferred by ectopic *CaLOX1* overexpression. In contrast, there were no significant differences in JA levels between wild-type and *CaLOX1*-OX transgenic Arabidopsis plants infected with *Pst* DC3000 and *Pst* DC3000 (*avrRpm1*) (Supplemental Fig. S4B). These results suggest that *CaLOX1* overexpression confers defense responses to *Pst* infection via the SA-dependent signaling pathway. The *Pst* DC3000- and *Pst* DC3000 (*avrRpm1*)-dependent HR in *CaLOX1*-OX transgenic plants was examined using an electrolyte leakage assay (Fig. 7H). Cell death associated with the HR causes the release of electrolytes, which is measured
as a change in the conductance of a bath solution (Orlandi et al., 1992). Transgenic plants infected by \textit{Pst} DC3000 and \textit{Pst} DC3000 (avrRpm1) exhibited significantly higher levels of ion leakage compared with wild-type plants. These data indicate that ectopic expression of the \textit{CaLOX1} transgene in \textit{Arabidopsis} enhanced basal resistance to bacterial pathogen infection, which was accompanied by a HR response.

Enhanced Susceptibility of \textit{AtLOX1}\_::T-DNA Plants to Bacterial Infection

To investigate the loss-of-function phenotype of the \textit{CaLOX1} ortholog \textit{AtLOX1} in \textit{Arabidopsis}, we isolated two putative \textit{AtLOX1} insertion lines, SALK\_000058 and SALK\_059431, from the Salk Institute T-DNA insertion library database (http://signal.salk.edu/cgi-bin/tdnaexpress) by a BLAST search. The predicted
amino acid sequence of AtLOX1 was 68% identical to that of CaLOX1 (Supplemental Fig. S2), and they both contain the LOX-specific PLAT_LH and LOX domains. Homozygous T-DNA insertion lox1 mutants were obtained by screening each ortholog line by PCR with specific primer pairs. Figure 8A shows a schematic of lox1 genomic structures indicating the positions of the T-DNA insertions. The loss-of-function mutants were named lox1-1 and lox1-2. RT-PCR analysis revealed that AtLOX1 transcripts were not detected in lox1-1 and lox1-2 plants inoculated with Pst DC3000 (avrRpm1) (Fig. 8B). Three days after inoculation with the virulent strain Pst DC3000, severe chlorotic symptoms developed in lox1 plants, whereas typical susceptible symptoms did not occur in wild-type plants (Fig. 8C). Dead cells or cells with damaged membranes were stained with DAB and trypan blue, which indicated that H₂O₂ formation and cell death did not occur in wild-type and lox1 leaves. Significantly, Pst DC3000 grew well in lox1-1 and lox1-2 mutants (Fig. 8D). The growth of Pst DC3000 was 5-fold greater in lox1-1 and lox1-2 mutants than in wild-type plants. Electrolyte leakage of the two mutant lines, lox1-1 and lox1-2, was significantly lower than that of wild-type plants during virulent Pst infection (Fig. 8E). Together, these data indicate that the AtLox1 mutants exhibited enhanced susceptibility to virulent Pst infection.

**LOX Activity in CaLOX1-OX and Arabidopsis lox1 Mutant Plants**

We speculated whether the enhanced resistance of CaLOX1-OX plants is due to increased LOX activity; hence, we analyzed LOX activity in wild-type and CaLOX1-OX Arabidopsis (Fig. 9). The levels of LOX activity were higher in the CaLOX1-OX transgenic lines 12, 15, and 16 relative to that of wild-type plants (Fig. 9A), suggesting that CaLOX1 is enzymatically active in heterologous transgenic Arabidopsis. We also measured LOX activity in the Arabidopsis mutant lox1-1 (Fig. 9B) and found that it was similar to that in wild-type plants. However, the enhanced LOX activity in lox1-1 leaves was lower than that in wild-type leaves 15 h after infection with Pst DC3000 and Pst DC3000 (avrRpm1). These data suggest that mutation of Arabidopsis AtLOX1 may compromise the level of LOX activity.

To further substantiate the effect of CaLOX1 over-expression on LOX activity in plants, we analyzed wild-type and CaLOX1-OX Arabidopsis plants during infection with Pst DC3000 and Pst DC3000 (avrRpm1) (Fig. 9C). In CaLOX1-OX plants, the level of LOX activity was somewhat higher in healthy leaves than in wild-type plants. As expected, infection with virulent Pst DC3000 or avirulent Pst DC3000 (avrRpm1) also induced significantly higher LOX activities in CaLOX1-OX Arabidopsis compared with wild-type plants. Importantly, either virulent or avirulent Pst DC3000 infection drastically stimulated lipid peroxidation in wild-type and CaLOX1-OX Arabidopsis leaves (Fig. 9D). However, there were no significant differences in MDA levels between wild-type and CaLOX1-OX plants during Pst infection, except for a high level of lipid peroxidation in CaLOX1-OX leaves 12 h after inoculation with avirulent Pst DC3000 (avrRpm1).

**Distinct Responses of Arabidopsis CaLOX1-OX and lox1 Plants to Hyaloperonospora arabidopsidis Infection**

To determine whether CaLOX1-OX transgenic and lox1 plants are resistant to the biotrophic oomycete H. arabidopsidis, we inoculated over 100 seedlings of each
line with a suspension of an asexual inoculum of *H. arabidopsidis* isolate Noco2 (5 × 10^3 conidiosporangia mL^-1), which is virulent to Arabidopsis Col-0. As shown in Figure 10A, *H. arabidopsidis* isolate Noco2 was highly virulent to wild-type cotyledons, which exhibited high levels of mycelial growth, sporulation, and sporangiophores. In contrast, *CaLOX1*-OX transgenic plants were significantly resistant to *H. arabidopsidis* isolate Noco2, with less sporangiophore formation in cotyledons compared with wild-type plants. Three and 5 d after inoculation, H_2O_2 production and restricted hyphal growth were observed in transgenic plants, as observed by DAB and trypan blue staining. We also tested *lox1* mutants for resistance to *H. arabidopsidis* and found that they were more susceptible to *H. arabidopsidis* isolate Noco2 than were wild-type plants. DAB staining of infection sites was not observed in these cotyledons. Hyphal growth, and early and heavy asexual sporulation, were distinctly enhanced in *lox1* mutants relative to wild-type and *CaLOX1*-OX transgenic plants.

Effects of the gain or loss of function of *CaLOX1* and *AtLOX1* on plant resistance to *H. arabidopsidis* infection were evaluated by measuring asexual sporulation on Arabidopsis cotyledons (Fig. 10B). These levels varied for each line, ranging from heavy to low. The percentage of sporangiophore formation and the average number of sporangiophores were lower in *CaLOX1*-OX transgenic plants than in wild-type plants. In contrast, *lox1* mutants exhibited higher asexual sporulation than did wild-type plants. Together, these results indicate that the *CaLOX1* and *AtLOX1* genes play a crucial role in basal resistance to the biotrophic oomycete *H. arabidopsidis*.

Distinct Responses of Arabidopsis *CaLOX1*-OX and *lox1* Plants to *Alternaria brassicicola* Infection

Previous studies reported that LOX expression is regulated by JA during infection with fungal pathogens (Melan et al., 1993). We examined whether *CaLOX1* is involved in plant resistance to the fungal pathogen *A. brassicicola*. Because wild-type (Col-0) plants are known to be incompatible with *A. brassicicola* (Moreno et al., 2005), we used the Arabidopsis ecotype Col-0 as a comparable control for resistance to *A. brassicicola*. The HR was induced in wild-type and *CaLOX1*-OX transgenic plants by *A. brassicicola* infection, which results in the development of brown necrotic lesions on leaves (Narusaka et al., 2003). The restricted hyphal growth, cell death, and production of H_2O_2 during infection were similar in wild-type and

Figure 9. Altered LOX activity in Arabidopsis leaves infected with *Pst* DC3000 and *Pst* DC3000 (avrRpm1). A, LOX activity in leaf extracts from wild-type (Col-0 [WT]), *CaLOX1*-OX, and *lox1*-1 mutant plants. B, LOX activity in Arabidopsis wild-type and *lox1*-1 leaves infected with *Pst*. C, LOX activity in Arabidopsis wild-type and *CaLOX1*-OX leaves. Linoleic acid (0.1 mM) was used as a substrate. All experiments were performed three times with similar results. D, Quantification of lipid peroxidation (MDA concentration) in Arabidopsis wild-type and *CaLOX1*-OX leaves infected with *Pst*. FW, Fresh weight. Values are presented as means ± sd. Different letters indicate significant differences as determined by the LSD test (*P* < 0.05) in three independent experiments. Asterisks indicate significant differences between wild-type and inoculated plants (Student’s *t* test, *P* < 0.05).
CaLOX1-OX transgenic plants. In contrast, infection of lox1 mutants by A. brassicicola resulted in spreading lesions and abundant sporulation (Fig. 11, A and C). As shown in Figure 11B, restricted fungal spread and high levels of H$_2$O$_2$ were detected in wild-type and CaLOX1-OX plants by trypan blue and DAB staining. However, in lox1 mutants inoculated with A. brassicicola, the infection hyphae spread profusely from the site of inoculation. The number of spores produced at the infection site was determined using the spore count assay (Fig. 11C). Spores were abundantly produced on leaves of lox1 mutants compared with wild-type and CaLOX1-OX plants. The lox1 mutants were clearly more susceptible to A. brassicicola infection than were wild-type and CaLOX1-OX transgenic plants. Together, these results indicated that CaLOX1 is necessary for plant defense responses to the fungal pathogen A. brassicicola.

**DISCUSSION**

Here, we report the cloning of the pepper 9-LOX gene CaLOX1, which is involved in plant defense and cell death responses to microbial pathogens. Activation of CaLOX1 was crucial for cell death and defense responses in pepper leaves, which were accompanied by reactive oxygen species (ROS) accumulation, lipid peroxidation, SA accumulation, and defense-related gene expression. The strong activity of CaLOX1 also was shown to induce plant defense and cell death in response to pathogen infection. Our molecular genetic study using CaLOX1-silenced pepper plants, CaLOX1-OX Arabidopsis, and Arabidopsis lox1 mutants revealed that the enhanced expression of CaLOX1 and defense-related genes such as the SA-responsive CaBPR1 is required for defense and cell death in plants. In contrast to the CaLOX1 overexpression phenotype, CaLOX1-silenced and lox1 mutants exhibited enhanced susceptibility to pathogen invasion. Overexpression of CaLOX1 in Arabidopsis conferred broad-spectrum resistance to infection by H. arabidopsidis and A. brassicicola as well as Pst. The data presented here suggest that CaLOX1 functions as a positive regulator of broad-spectrum resistance and cell death responses during pathogen infection.
Isolation, Enzyme Activity, and Position Specificity of the Pepper 9-LOX Gene and Protein

LOX genes have been widely used to study the role of JA and oxylipins in defense responses during abiotic and biotic stresses, but their biological functions are not fully understood (Porta and Rocha-Sosa, 2002). Our results here provide evidence of a role for the pepper 9-LOX gene, CaLOX1, in regulating disease resistance. Sequence alignment analysis revealed that CaLOX1 shares high homology with LOXs from potato, tobacco, tomato, and Arabidopsis. The His residues that act as ligands to the active site iron are highly conserved in LOXs (Prigge et al., 1996). The pepper CaLOX1 protein contains conserved His residues involved in catalysis, as observed in other LOXs (Hornung et al., 1999). The presence of these conserved His residues predicted that CaLOX1 was a LOX.

Linolenic and linoleic acids are the most common plant substrates for LOXs (Siedow, 1991), and linoleic acid is particularly important as a substrate (Lorenzi et al., 2006). A comparison of the substrate specificity of the six Arabidopsis LOXs revealed that the 9-LOXs AtLOX1 and AtLOX5 oxygenate linoleic acid and linolenic acid rather than arachidonic acid (Bannenberg et al., 2009). The catalytic efficiency of CaLOX1 ($k_{cat}/K_m$) was higher with linoleic acid than with arachidonic acid, which suggests that linoleic acid is an efficient substrate for the formation of the enzyme-substrate complex. The LOX pathway has several branches and produces many signaling molecules that respond to a variety of pathogens or stress factors. That is, LOXs are the entry point to pathways that provide a variety of oxylipin molecules involved in signaling for stress responses. LOX enzymes oxygenate polyunsaturated fatty acids in a position-specific manner. The biosynthesis of plant oxylipins is initiated by primary fatty acid oxygenases, including the 9- and 13-LOXs (Bannenberg et al., 2009). Specifically, only 13-hydroperoxylinolenic acid produced by 13-LOX leads to the biosynthesis of JA (Feussner and Wasternack, 2002). However, JA is not synthesized through 9-hydroperoxylinolenic acid by 9-LOX. The positional specificity of the LOX enzyme is a crucial factor to determine whether the LOX is involved in the biosynthesis of JA. HPLC analysis of the reaction products for the positional specificity of CaLOX1 revealed that 9-hydroperoxylinolenic acid is mainly produced by the reaction of purified CaLOX1 with linoleic acid as a substrate, which suggests that CaLOX1 is a 9-LOX.

CaLOX1 Is Required for the Cell Death Response in Pepper

CaLOX1 was highly expressed during the HR of pepper leaves during the incompatible interaction...
with *Xcv* Bv5-4a. This suggests an essential role for LOX in establishing the HR. HR cell death may be due, in part, to lipid peroxidation initiated by LOX activity. This hypothesis is supported by the findings of Falloul et al. (2002) and Montillet et al. (2002) that lipid peroxidation and LOX activity are stimulated in the HR of plants to pathogenic bacteria. Importantly, LOX activity also increased rapidly and to a higher level in HR cell death during *Xcv* infection. The accumulation of LOXs is specifically associated with leaf cell death in Arabidopsis (Montillet et al., 2002). LOX activation may be associated with the HR and H$_2$O$_2$ oxidative stress to induce programmed cell death (Maccarrone et al., 2001).

**CaLOX1**-dependent cell death was observed in parallel with enhanced CaLOX1-transient expression in pepper plants infiltrated with *Agrobacterium* (35S: *CaLOX1*). Intriguingly, CaLOX1 transient expression strongly induced the SA-responsive gene CaBPR1 and the JA-responsive gene CaDEF1 in pepper leaves. Simultaneously, UV-visible fluorescence and ROS accumulation were also detected in leaves transiently expressing CaLOX1, suggesting an accumulation of ROS and phenolic compounds associated with cell death. These results raise the possibility that CaLOX1 acts as a trigger of cell death responses in pepper plants during pathogen infection. Metabolic products of the LOX pathway are likely to be required for the induction of cell death responses that limit pathogen growth at the infection site. For example, 9- and 13-HPO cause cell death in lentil (*Lens culinaris*) root protoplasts (Maccarrone et al., 2000). Signal transduction pathways that are dependent on SA or JA may be activated during the cell death response in pepper transiently expressing CaLOX1, leading to biosynthesis or the release of potential antimicrobial effector molecules (Morel and Dangl, 1997). HR cell death is closely related to the generation of lipid peroxides and ROS (Porta and Rocha-Sosa, 2002). Accumulation of hydrogen peroxides in the cell death region in CaLOX1-transiently expressing pepper leaves is consistent with the findings of Maccarrone et al. (2000) that early production of H$_2$O$_2$ during the HR in lentil root protoplasts induces cell death as well as an increase in LOX activity.

**Silencing of CaLOX1 Suppresses Defense Responses to Microbial Pathogens in Pepper**

To investigate the loss of function of CaLOX1 in pepper plants, we used the VIGS technique with the TRV vector system, a fast and highly effective tool for gene down-regulation (Liu et al., 2002; Hein et al., 2005; Sarowar et al., 2007). CaLOX1-silenced pepper plants showed not only enhanced susceptibility to the virulent fungus *C. coccodes* and the bacterial pathogen *Xcv* Ds1 but also a delayed HR to the avirulent *Xcv* Bv5-4a. These observations suggest that CaLOX1 is required for basal defense and HR-mediated resistance to *C. coccodes* and *Xcv* infection. Interestingly, reduced induction of CaBPR1 (a SA marker gene) in the silenced plants was intimately associated with the inhibition of SA accumulation during the virulent *Xcv* infection. The suppression of CaLOX1 in CaLOX1-silenced plants also seems to be crucial for the inhibition of HR-associated cell death. Importantly, levels of CaLOX1 transcripts in unsilenced and CaLOX1-silenced plants paralleled LOX activities. CaLOX1 induction and LOX activity were low in CaLOX1-silenced plants. These results are consistent with the earlier demonstration that LOX transcript accumulation patterns and LOX activities are coincident in potato (Kolomiet et al., 2000). In our study, VIGS of the CaLOX1 gene also inhibited localized cell death in pepper leaves infected with pathogens, which was accompanied by the suppression of H$_2$O$_2$ accumulation, lipid peroxidation, SA accumulation, and cell death-related genes. These results suggest that lipid peroxidation and SA and SA accumulation in pepper are crucial for the execution of hypersensitive cell death and defense responses induced by CaLOX1 expression.

**Expression of CaLOX1 and the CaLOX1 Ortholog AtLOX1 Confers Basal Resistance to a Broad Range of Pathogens in Arabidopsis**

**CaLOX1** overexpression in Arabidopsis plants conferred enhanced resistance to *Pst* DC3000, *Pst* DC3000 (*avrRpm1*), *H. arabidopsidis*, and *A. brassicicola*. Infection with *Pst* DC3000 (*avrRpm1*) induced a HR and H$_2$O$_2$ accumulation in CaLOX1-OX Arabidopsis. During Pst DC3000 infection, CaLOX1-OX plants exhibited small HR-like necrotic lesions at the infection site and inhibition of pathogen growth. Infection of CaLOX1-OX leaves by pathogens resulted in oxidative stress, which led to the irreversible damage of cellular membranes and the appearance of necrotic disease symptoms. Moreover, CaLOX1-OX Arabidopsis plants exhibited an increase in LOX activity compared with wild-type plants. Upon infection of CaLOX1-OX plants with *Pst* DC3000 and *Pst* DC3000 (*avrRpm1*), LOX activity was significantly enhanced, suggesting that CaLOX1 is critically involved in the defense response in heterologous Arabidopsis. In contrast, plants homozygous for T-DNA insertions of the Arabidopsis CaLOX1 ortholog (the *lox1-1* and *lox1-2* mutants) were susceptible to infection by *Pst* DC3000, *H. arabidopsidis*, and *A. brassicicola*. As expected, we observed decreased LOX activity in the Arabidopsis *lox1* mutant relative to wild-type plants during *Pst* infection. Interestingly, this suggests that AtLOX1 may function in the Arabidopsis defense response to *Pst* infection in a manner similar to that in CaLOX1-OX Arabidopsis lines. CaLOX1 overexpression in transgenic Arabidopsis plants enhanced AtPR1 expression during *Pst* DC3000 and *Pst* DC3000 (*avrRpm1*) infection. The increased level of SA-responsive AtPR1 expression in CaLOX1-OX Arabidopsis plants supports the notion that CaLOX1 may be involved in SA-mediated signal-
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ferred by LOX plants. Analyses of genes are highly conserved for disease resistance in LOX resistance to pathogen attack suggests that these Xanthomonas campestris inoculation with /C176 on soil, seeds were vernalized at 4°C for 2 d to overcome dormancy.

Production of Recombinant CaLOX1 in Escherichia coli

The CaLOX1 gene was cloned into the vector pET28a to generate pET28a::CaLOX1, which encodes CaLOX1 with a His tag fused to the N terminus. The CaLOX1 gene was amplified by PCR with the forward primer 5'-GAATTCTATATCGACACACTGTTGGGTA-3' and the reverse primer 5'-CATGTACTCGGAAAGATTG-3' with a BamHI site and the reverse primer 5'-GAATTCTATATCGACACACTGTTGGGTA-3' with an EcoRI site, based on the CaLOX1 full sequence. For CaLOX1 expression, pET28a::CaLOX1 was transformed into E. coli BL21 (DE3). Transformant bacteria were incubated in a flask containing 50 mL of Luria-Bertani medium supplemented with kanamycin (50 μg mL⁻¹) and cultured at 37°C, until optical density at 600 nm (OD600) reached approximately 0.5. CaLOX1 protein expression was induced in the bacterial culture for 10 h at 18°C by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) to 0.5 mM. pET28a::CaLOX1 was purified with 1.5 mg of nickel-nitrilotriacetic agarose resin (Invitrogen) in a 10-mL purification column, from which the target protein was eluted with an imidazole solution (250 mM). Purified CaLOX1 was subjected to 10% SDS-PAGE and stained with Coomassie Brilliant Blue to confirm purity.

CalloX1 Enzyme Assay

Protein extracts were prepared from healthy and bacteria-infected pepper and Arabidopsis leaves, which were homogenized with 1 mL of 0.1 mM sodium phosphate buffer (pH 6.5) using a pestle and mortar. The homogenate was centrifuged at 15,000 g for 30 min at 4°C, and the resulting supernatant was used as the enzyme source. Protein concentrations of crude fractions were determined with a dye-binding protein assay kit (Bio-Rad) following the manufacturer’s instructions for bovine serum albumin as a standard.

LOX activity in the soluble fractions was spectrophotometrically monitored by measuring the increase of the conjugated diene hydroperoxide at 234 nm at 25°C for 10 min (Richard et al., 1992; Rance et al., 1998). The assay mixture included 0.1 mM sodium phosphate buffer (pH 6.5), 0.1 mM linoleic acid (Sigma), and 5 to 10 μL of resuspended enzyme in a total volume of 1.2 mL. The enzyme reaction was initiated by adding purified CaLOX1 from E. coli or protein extracts from plants, and the change in absorbance was recorded. LOX activity is expressed in nkat mg⁻¹ protein using a molar extinction coefficient of 25,000 M⁻¹ cm⁻¹.

Kinetic parameters of hydroperoxidation of linoleic acid and arachidonic acid for purified CaLOX1 were analyzed, and the linear parts of the kinetic progress curves were evaluated as described (Seo et al., 2001). Various concentrations (0–200 μM) of substrates were tested to determine the kinetic parameters. k₀, Vₗₗₗ, and kₗₗₗ for each substrate were calculated using Prism 5.0 (GraphPad Software). Kinetic parameters were determined by analyzing Michaelis-Menten plots from three independent experiments.

Positional Specificity of CaLOX1

Positional specificity of CaLOX1 protein was analyzed by silica gel HPLC using a Zorbax SIL column (4.6 × 150 mm; Agilent). Purified recombinant CaLOX1 protein was incubated for 5 min with 0.1 mM sodium phosphate buffer (pH 6.5) containing 0.1 mM linoleic acid (Sigma) at 25°C. The reaction was stopped by the addition of 0.1 M HCl. Reaction products were extracted in 10:1 hexane:ethanol. After shaking for 1 min, the organic layer was applied to the HPLC column using a solvent system of hexane:ethanol:acetic acid (70:30:1, v/v/v) and a flow rate of 1 mL min⁻¹. A flow rate of 1 mL min⁻¹. 9-HPOT and 13-HPOT standards were purchased from Cayman Chemical (Mizuno et al., 2003).

Pathogen Inoculation and Disease Rating

Pepper plants were inoculated with the virulent strain DS1 and avirulent strain Bv5-4a of Xcv (Jung and Hwang, 2000). Xcv was grown at 28°C on yeast nutrient broth (5 g of yeast extract, 8 g of nutrient broth, and 1 L of water). Pepper plants at the six-leaf stage were inoculated by infiltrating bacterial suspension (5 × 10⁶ cfu mL⁻¹; Chung et al., 2007; Kim et al., 2007). Inoculated plants were incubated for 18 h in a moist chamber at 28°C and then in a growth room as described previously (Jung and Hwang, 2000). Infected leaves were sampled at various time points after inoculation.

Pseudomonas syringae pv tomato strain DC3000 and Pst DC3000 (avrRpm1) were used. The bacteria were grown at 28°C in yeast nutrient broth containing rifampicin (50 mg mL⁻¹). To study bacterial growth, bacterial suspensions (10⁷ cfu mL⁻¹) were infiltrated into Arabidopsis leaves using a syringe. At appropriate time points, three independent leaves infiltrated with Pst were harvested.

Colletotrichum coccodes isolate 2-25 was used in this study. The fungus was grown on oatmeal agar plates for 5 to 7 d at 28°C in the dark. Conidia concentration was adjusted to 5 × 10⁶ mL⁻¹ with sterile tap water using a hemacytometer. Pepper plants at the six-leaf stage were sprayed with the

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MATERIALS AND METHODS

Plant Material and Growth Conditions

Pepper (Capsicum annum ‘Nockwang’) plants were raised in a plastic tray (55 × 35 × 15 cm) after seed germination. At the two-leaf stage, plants were transplanted and grown in pots containing soil mix (peat moss:vermiculite: perlite, 3:1:1, v/v/v) at 28°C with a 14-h-light/10-h-dark regime at a light intensity of 70 μmol photons m⁻² s⁻¹. 35S:CaLOX1, lox1-1, lox1-2, and wild-type plants in the Arabidopsis (Arabidopsis thaliana) Col-0 background were used in this study. Arabidopsis plants were grown in pots containing peat moss, perlite, and vermiculite (2:1:2, v/v/v) at 24°C with a 14-h-light/10-h-dark regime at a light intensity of 130 μmol photons m⁻² s⁻¹. Prior to sowing on soil, seeds were vernalized at 4°C for 2 d to overcome dormancy.

cDNA Library Screening and DNA Sequencing

A pepper cDNA library was constructed from pepper leaves 18 h after inoculation with Xanthomonas campestris pv vesicatoria. The cDNAs were cloned into the vector λ ZAP II using the λ ZAPII-cDNA library synthesis kit (Stratagene). Pathogen-induced cDNAs were identified using cDNA probes from uninoculated pepper leaves or leaves inoculated with the Xcv avirulent strain Bv5-4a (Jung and Hwang, 2000). DNA sequencing was performed with an ABI 310 DNA sequencer (PE Biosystems) using PRISM BigDYE Terminator sequencing ready reaction kits. Nucleotide sequences were assembled and analyzed using DNA Strider 1.2.1. Database searches were run with the BLASTx program (http://www.ncbi.nlm.nih.gov/BLAST/) and the ExPasy Proteomics Server (http://www.expasy.org). The CaLOX1 cDNA was found among sequenced clones.

Production of Recombinant CaLOX1 in Escherichia coli

The CaLOX1 gene was cloned into the vector pET2a to generate pET2a::CaLOX1, which encodes CaLOX1 with a His tag fused to the N terminus. The CaLOX1 gene was amplified by PCR with the forward primer 5'-GCATC-

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conidial suspension in 0.05% Tween 20, incubated in a moist chamber in the dark for 48 h at 28°C, and then raised in the growth room.

_Hyaloperonospora arabidopsidis_ isolate Noco2 was maintained on Arabidopsis Col-0 plants by weekly subculturing. To produce large quantities of inoculum, 7- to 10-d-old seedlings were inoculated with _H. arabidopsidis_ and the spores produced on cotyledons were collected in water. Seven-day-old seedlings were sprayed with a suspension of assexual inoculum (5 × 10^7 conidia mL^-1_) and the inoculated seedlings were covered with a transparent dome to maintain high humidity (80%–100%) and grown for 7 d at 17°C (Lee et al., 2008). Seven days after inoculation, sporangiophore production was monitored using a dissection microscope. Asexual sporulation of _H. arabidopsidis_ was visually assessed by counting the number of sporangiophores on both sides of the cotyledons. Disease ratings consisted of five classes based on the number of sporangiophores: zero to five, six to 10, 11 to 15, 16 to 20, and over 20 sporangiophores per cotyledon.

*Alternaria brassicicola* was cultured on potato dextrose agar medium at 24°C for 10 d. Spore concentrations were determined using a hemacytometer and adjusted to 5 × 10^5 conidia mL^-1_. A. brassicicola was inoculated by placing 10 μL droplets of suspension on leaves of 5-week-old plants. For mock treatment, 10 μL droplets of water were placed onto the leaves. Inoculated plants were kept at 100% relative humidity at 24°C. Four days after inoculation, lesion diameters and the numbers of newly formed spores were measured (Ob et al., 2005).

**Stress Treatment**

SA (5 μM), methyl viologen (10 μM), ABA (100 μM), and MeJA (100 μM) were applied onto pepper leaves at the six-leaf stage. Plants treated with MeJA were tightly sealed in a plastic bag. For ethylene treatment, the plants were sealed in a flask and aliquots of pure ethylene gas were injected into the flask to 10 μL mL^-1_. For salt treatment, pepper plants were removed from the soil and soaked in 200 mM NaCl. For cold treatment, pepper plants were exposed to low temperature (4°C). To induce drought stress, plants were exposed to dehydration by withholding water. For water deficit experiments, leaves were pricked with a needle. The treated pepper plants were sampled at various time points after treatment, frozen in liquid nitrogen, and stored at −70°C until used for RNA isolation.

**RNA Gel-Blot and RT-PCR Analysis**

Total RNA was isolated from leaf tissues of pepper and Arabidopsis plants using TRIzol (Invitrogen) according to the manufacturer’s instructions. For RNA gel-blot analysis, total RNA (10 μg) was separated on 1.2% formaldehyde agarose gels and transferred onto a nylon membrane (Hybond+; Amersham Pharmacia Biosciences), followed by UV cross-linking. Probes for RNA gel-blotting were prepared by PCR amplification of cDNA inserts of the expected size. Independent kanamycin-resistant transgenic plants were identified by PCR amplification of an cDNA insert of the expected size.

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**Identification of T-DNA Insertion Lines**

Homozygous insertion mutant seeds were obtained from the Arabidopsis Biological Resource Center (http://www.arabidopsis.org/; Ohio State University. The lco51-1 (SALK_059431) and lco51-2 (SALK_00058) mutants were isolated from the SALK T-DNA lines. The seeds were planted on Murashige and Skoog agar plates containing kanamycin (50 μg mL^-1_), and kanamycin-resistant plants were transferred to soil. Mutant plants were confirmed by PCR using T-DNA and gene-specific primer sets, as described on the T-DNA Express homepage (http://signal.salk.edu/Tdnaexpress.html). Two sets of PCR were carried out using the three primers LP (left gene-specific primer), RP (right gene-specific primer), and LB (left border primer of the T-DNA insertion) for each SALK line: lco51-1 LP primer, 5'-AGCTCCTTGAGACCTCATTCCACGAGC-3'; lco51-1 RP primer, 5'-GGAGCACCCCTCAGCAGACGAG-3'; lco51-1 LB primer, 5'-TTCGTGTCATAGCTGAGAAG-3'.

**Measurement of Ion Leakage**

Leaves of 5-week-old Arabidopsis plants were infiltrated with 10^6 cfu mL^-1_ _Pst D3000_ and _Pst DC3000_ (avrRpm1) to determine ion leakage. Leaf discs (1 cm in diameter) were removed immediately following infiltration, washed in 30 mL of double distilled water for 30 min, and transferred into 20 mL of double distilled water. Conductance was then measured with a conductivity meter (model sensLONT; Hach; Mackey et al., 2003).
Quantification of Lipid Peroxidation

Lipid peroxidation was estimated by determining the MDA contents in pepper and Arabidopsis plants as described previously (Heather and Packer, 1968). Leaf samples (200 mg) were homogenized in 0.5% TCA. The homogenates were centrifuged at 10,000g for 15 min at 4°C. Extracts was mixed with 0.5% thiobarbituric acid prepared in 20% TCA and incubated at 95°C for 30 min. The reaction was stopped on ice, and samples were centrifuged at 12,000g for 15 min. The absorbance of the resultant supernatant was measured at 532 and 600 nm. The nonspecific Abs was subtracted from the Absg. The MDA concentration was determined using the extinction coefficient 155 mM⁻¹ cm⁻¹.

Staining with DAB, Trypan Blue, and Aniline Blue

To monitor plant cell death and fungal growth, control leaves or leaves inoculated with pathogens were stained with 1 mg mL⁻¹ DAB (Sigma), lactophenol-trypsin blue (10 mL of lactic acid, 10 mL of glycerol, 10 g of phenol, and 10 mg of trypan blue, dissolved in 10 mL of distilled water), and 0.01% aniline blue in 150 mM K2HPO₄, pH 9.5. After DAB staining overnight, the leaves were cleared by boiling for 10 min in ethanol and destained overnight in ethanol. For trypan blue staining, infected plants were boiled for 5 min in the staining solution and destained overnight in chloral hydrate (2.5 g mL⁻¹). For aniline blue staining, infected plants were cleared of chlorophyll using alcoholic lactophenol and rinsed in 50% ethanol and water. The samples were then stained for 30 min with 0.01% aniline blue (Rigano et al., 2007) and mounted in 70% glycerol for microscopic observation.

Quantification of H₂O₂ by Xylenol Orange Assay

H₂O₂ production in plants was spectrophotometrically measured using xylenol orange assay, which forms a complex with the Fe³⁺ produced by the hydroperoxide-based oxidation of Fe²⁺ (Bindschedler et al., 2001). One milliliter of assay reagent [25 mM FeSO₄ and 25 mM (NH₄)₂SO₄, dissolved in 2.5 M H₂SO₄] was added to 100 mL of 125 μM xylene orange and 100 mM sorbitol. To measure the H₂O₂ content, leaf discs excised using a cork borer were withdrawn and centrifuged at 5,000g for 10 min, and 100 μL of the supernatant was added to 1 mL of xylene orange reagent. After 30 min of incubation, the peroxide-mediated oxidation of Fe²⁺ to Fe³⁺ was determined by measuring the Abs₆₅₀ of the Fe³⁺-xylene orange complex.

Measurement of SA

SA and SA glycoside were extracted and quantified according to the method described by Verberne et al. (2002). Leaf tissue samples (0.5 g) were frozen in liquid nitrogen, ground to a fine power, and sequentially extracted with 90% and 100% methanol. As an internal standard for SA, 3-hydroxybenzoic acid (Sigma) was added at a mass ratio of 50 mg g⁻¹ leaf fresh weight. SA was determined by fluorescence (excitation 305 nm, emission 405 nm) after separation on a c₂₅ reverse-phase HPLC column (Waters).

Extraction and Measurement of JA

For analysis of JA, leaf samples were extracted with ice-cold 100% methanol and centrifuged at 10,000g for 10 min. Supernatants were decanted and pellet reextracted with 100% methanol. Dihydrojasmonic acid was added as an internal standard for quantification of JA. Extraction of JA was continued by rotating the samples at 28°C for 2 h. Extracted samples was adjusted to 70% methanol with ice-cold water, and pH was lowered to 3 with HCl. The samples were passed through a Sep-Pack C18 cartridge (Waters), which has been preswashed with 70% methanol. Cartridges were washed with 70% methanol, and eluates were combined. Samples were partitioned with CHCl₃, dried over anhydrous MgSO₄, and methylated with methylation reagent followed by hexanecetyl-butylic methyl ether (11, v/v). The supernatants were decanted and stored at −20°C. JA was analyzed by gas chromatography-mass spectrometry (Agilent 6890) with a DB-5MS column (30 m × 0.25 mm, 0.25 μm; J&W Scientific). The temperature gradient was 60°C for 1 min, 60°C to 190°C at 15°C min⁻¹, 190°C to 220°C at 5°C min⁻¹, 220°C to 290°C at 25°C min⁻¹, and 290°C for 1 min.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers FJ377808 (CaLOX1), AF053343 (CaPR1), AF442388 (CaDEF1), AF442387 (CaPOA1), AF313766 (CaSAR82), AF244122 (CaPR4), AF244121 (CaPR10), At1g59020 (AtLOXI), and At1g62250 (AtUBQ5).

Supplemental Data

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

Supplemental Figure S1. Nucleotide and derived amino acid sequences of CaLOX1 cDNA encoding pepper LOX.

Supplemental Figure S2. Alignment of the deduced amino acid sequence of CaLOX1 with other plant LOXs.

Supplemental Figure S3. LOX activity of recombinant His tag-CaLOX1 expressed in E. coli BL21 (DE3).

Supplemental Figure S4. Levels of JA in pepper and Arabidopsis.

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